From: Cynthia Elkins [mailto:CElkins@biologicaldiversity.org]
Sent: Tuesday, March 21, 2023 3:26 PM
To: CityClerk <CityClerk@longbeach.gov>
Subject: CBD Sources, Part 2.1

-EXTERNAL-

Please see the attached references in support of the Center for Biological Diversity's comments to the City Council regarding the Long Beach Unit Annual Plan and Program Plan.

Thank you,
Cynthia Elkins

Cynthia Elkins, Paralegal
Center for Biological Diversity
https://www.biologicaldiversity.org

Bulletin of Environmental Contamination and Toxicology
https://doi.org/10.1007/s00128-021-03176-w

Red Blood Cell Cytotoxicity Associated to Heavy Metals and Hydrocarbons Exposure in Flounder Fish from Two Regions of the Gulf of Mexico

Juan Pablo Ek-Huchim[1] · Flor Árcega-Cabrera[2] · Ana Luisa May-Tec[1] · Monica Améndola-Pimenta[1] · Víctor Ceja-Moreno[1] · Rossanna Rodríguez-Canul[1]

Received: 30 August 2020 / Accepted: 8 March 2021

Abstract

In this study, the genotoxic effect of contaminants was assessed through detection of DNA damage using the micronucleus (MNs) test in erythrocytes from 149 flounder fish collected in two regions of the Gulf of Mexico (GoM). The frequency of microcytes (MCs) was also evaluated in the same group of fish collected from the Perdido Foldbelt (PF) and the Yucatan Platform (YP). The MCs frequency was different among locations of the YP ($p = 0.011$), while MNs frequency varied among locations of PF ($p = 0.024$). MCs and MNs values correlated with heavy metals from fish muscle, fish species and localities. Mean number, prevalence, and intensity of MCs and MNs correlated with Al, PAHs, depth, and locality. MNs frequency showed a species-specific association ($p = 0.004$). MNs and MCs were associated with heavy metals and PAHs from fish muscle and sediments, and the MNs frequency was species dependent.

Keywords Micronuclei · Flounder · Perdido Foldbelt · Yucatan Platform · Gulf of Mexico

Crude oil is one of the most widespread pollutant released into the marine environment causing a wide range of biological effects in native species (Bejarano 2018). In the Gulf of Mexico (GoM) there is always a constant menace of oil spill events because crude oil production activities are very important in the region (Allan et al. 2012; Ward and Tunnell 2017). In 2010, around 4.4×10^6 oil barrels were released into the sea for 87 days in the Deepwater Horizon oil spill blowout (Perez et al. 2017). Offshore fish was used to detect the accumulation of polycyclic aromatic hydrocarbons (PAHs) after the DWH disaster (Murawski et al. 2020).

Flounder fish have been used as bioindicators of habitat disturbance because they are in direct contact with the marine sediments (Bolognesi et al. 2006; Conti and Iacobucci 2008; Holt and Miller 2010). And some components of oil are highly persistent (Gregson et al. 2021). Sediments act as sinkholes for hydrocarbons, heavy metals, and other contaminants. These substances become bioavailable to flatfish and are accumulated in their tissues, altering their physiology and reproduction (Kirby et al. 2000; van der Oost et al. 2003; Holt and Miller 2010; Rahmanpour et al. 2016; Yu et al. 2019).

The effect of oil pollutants can be assessed by in vivo and in vitro assays where cellular and molecular biomarker responses are implemented as indicators of environmental disturbances (Gold-Bouchot et al. 2017; Praveen Kumar et al. 2017). The presence of abnormal cells in blood smears of fish are useful to detect chromosomic damage produced by a wide range of toxic compounds, specifically by detecting binucleated or polynucleated cells containing micronucleus (MNs) (Ayanda et al. 2018). The MNs are extranuclear bodies that contain damaged chromosome fragments and/or whole chromosomes that were not incorporated into the nucleus after cell division. While microcytes (MCs) are small mature erythrocytes characterized by having a smaller diameter when compared to normal erythrocytes and their presence has been associated to a deficiency of hemoglobin formation linked to anemia and malnourishment

✉ Rossanna Rodríguez-Canul
rossana.rodriguez@cinvestav.mx

[1] Departamento de Recursos del Mar, Centro de Investigación y de Estudios Avanzados del Instituto Politécnico Nacional - Unidad Mérida, Km. 6 Antigua Carretera a Progreso, CORDEMEX, CP 97310 Mérida, Yucatán, Mexico

[2] Unidad de Química Sisal, Facultad de Química, Universidad Nacional Autónoma de México, Puerto de Abrigo S/N, 97356 Sisal, Yucatán, Mexico

Published online: 23 March 2021

(Alkaladi et al. 2015). The MNs assay are biomarkers used to detect chromosomic damage induced by a variety of genotoxic agents (Sommer et al. 2020). Fish challenged with Cd showed a time-dependent increase in the frequency of MNs and nuclear abnormalities that had high correlation with cytoplasmic cell alterations including MCs (Jindal and Verma 2015).

The objectives of this study were to report the presence of MNs and MCs in seven species of fish flounder from two areas of the GoM as well as to associate these values with oil pollutants from sediments and fish muscle.

Methods and Materials

A permit for collection (PPF/DGOPA-070/16) was issued by Comisión Nacional de Acuacultura y Pesca. Flounder fish were collected from two areas of the GoM; 41 fish from eight locations (4 to 6 fish per location) from the Perdido Foldbelt (PF). Sampling was done aboard the oceanographic cruise "Justo Sierra"-UNAM using benthic sled trawlers with 2.40 m of width, 0.90 m of height, 5 m of coded large and mesh size of 0.0254 m. Trawling of approximately one mile was performed at depths of 44 to 107 m between the 12th to 19th in May 2016. In the Yucatan Platform (YP), 108 fishes were collected using a boat from the commercial shrimp fleet implemented with net shrimp trawls of 18.3 m and 0.034 m of mesh size. Sampling was done on two occasions: In 2015 (November 17th to 18th) five locations were surveyed and in 2016 (April 13th to 16th), 13 locations were surveyed. In both cases four to 10 organisms per location were collected in trawlers of approximately one mile done at depths of 40 to 200 m (Fig. 1) (Supplementary Table 1).

After capture, ≈ 20 µL of blood was collected from the caudal vein of each fish using a hypodermic syringe and dropped on glass-slides to perform two blood smears from each fish. Smears were air dried and fixed in absolute methanol (Hycel®) for 10 min (Al-Sabti and Metcalfe 1995). Subsequently, fishes were euthanized using a sharp scalpel on the fish head. After that, they were dissected, and their sex was identified by visual inspection. Muscle tissue was collected above the lateral line from both sides of the fish avoiding the abdominal cavity. Muscle samples were stored at − 20°C for pollutants analyses: PAHs (polycyclic aromatic hydrocarbons) and heavy metals (V, Ni, Cd and Pb). Also, approximately 200 g of sediments from nine locations (G34, H39, I43, J48, L59, O73, P78, P79 and P80) from the YP and from each location of the PF (B1, B2, C1, C2, D1, D2, F1 and F2) were collected for contaminant (PAHs, Al, V, Ni, Cd and Pb) analyses. For heavy metals quantifications, sediments samples were placed in plastic bags previously washed with a 1 M HNO_3 (Sigma-Aldrich pure grade) solution and deionized water, while for PAHs quantification, they were placed in glass containers previously washed with hexane and acetone (both Sigma-Aldrich chromatographic grade). Both samples were kept at 4ºC until further analysis.

For the MNs and MCs assay, glass slides were stained with 10% Giemsa solution for 8 min, air dried, and then analysed under bright-field microscopes Olympus BX51 at 100× (Baršiene et al. 2004). The MNs and MCs were scored from 3000 mature erythrocytes analysed in each sample. MNs were identified as small nuclei separated from the main nucleus. MCs were identified from normal erythrocytes by having sizes less than one-third of the normal erythrocyte. Results were expressed as frequency (MNs/3000 mature erythrocytes and MCs/3000 mature erythrocytes), mean value (an average of n numbers computed by adding some

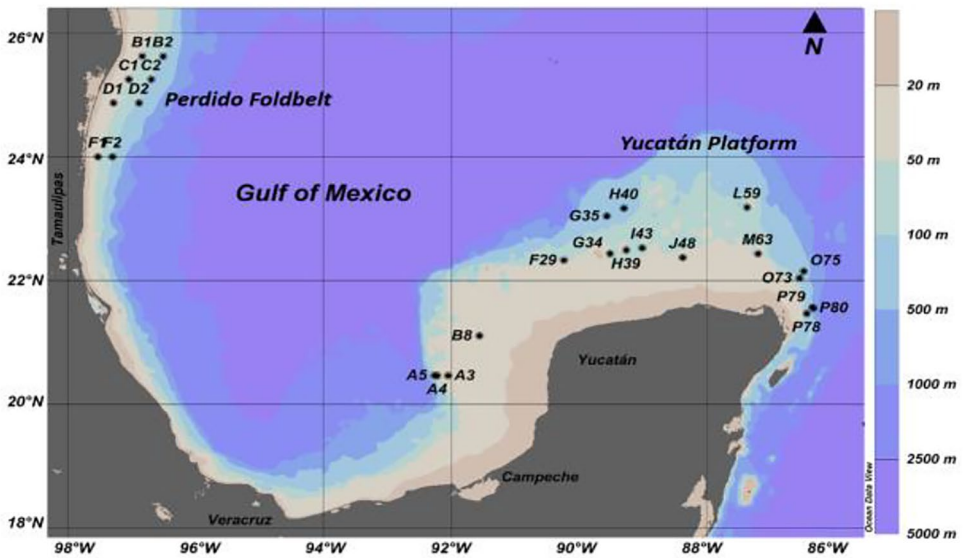


Fig. 1 Sampling locations at the Perdido Foldbelt (PF) and the Yucatan Platform (YP)

function of the numbers and dividing by some function of n), prevalence (%) (the number of organisms with MNs or MCs, divided by the total number of organisms in the sample), and mean intensity (the mean number of MNs or MCs found in infected hosts in a particular population (see Jindal and Verma 2015).

PAHs concentration in muscle was measured in the dry-freeze tissue following the procedures described by MacLeod et al. (1985) and Wade et al. (1988), and in the dry-freeze sediments following Wade et al. (1988). Previous to column chromatographic extraction, samples were fortified using a surrogate solution of deuterated PAHs (1–3 dimethyl-2 nitrobenzene; acenaphthene δ10, phenanthrene δ10, pyrene δ10, triphenyl phosphate, chrysene δ10 and perylene δ10 from UltraScientific). Detection limits for PAHs ranged from 0.0152 (benzo[a]anthracene) to 0.4039 (benzo[a]pyrene) and all recovery percentages were above 60%. Compounds were identified and quantified using a Clarus 500 Perkin Elmer gas chromatographer coupled to a mass spectrometer detector by the full scan and the ion selected methods (Wang et al. 1994).

For heavy metals analysis, samples were acid-digested (MARS 6-CEM, EPA method 3052) with nitric acid trace metal grade. Cd, Ni, V and Pb quantification was done using an ICP-MS iCAP Q Thermo Scientific using the ions ^{51}V, ^{60}Ni, ^{112}Cd and ^{208}Pb. Multielement solutions in several concentrations were prepared from a multistandard solution according to manufacturer instructions. PACS3 and MESS4 (National Research Council Canada) certified reference material was used to determine precision and accuracy of the analysis. Recovery percentages (RP) were 89.5% for ^{51}V, 86% for ^{60}Ni, 111% for ^{112}Cd, and 118% for ^{208}Pb; the coefficient of variation (CV) for V = 20.4%, for Ni = 21.1%, for Cd = 23.7% and for Pb = 24.5% in PACS3. In CRM MESS4, RP were 83% for ^{51} V, 81% for ^{60}Ni, 95% for ^{112}Cd, and 112% for ^{208}Pb with a CV of 15.2% for V, for Ni = 22.1%, 8.9% for Cd and for Pb = 22.5%. Detection limits (DL) were 0.0043 μg g^{-1} for V, 0.0049 μg g^{-1} for Ni, 0.0039 μg g^{-1} for Cd, and 0.0053 μg g^{-1} for Pb, calculated as three times the standard deviation of the measured concentrations of 12 blanks.

The data mapping of all locations was geopositioned with Ocean Data View software version 4.7.9. The variability of MNs and MCs frequencies between localities and fish species were compared with Fisher´s exact test using the Statistica 8.0 software. The association of variables like frequency, mean number, prevalence and intensity of MNs and MCs with sex, fish species, depth and pollutants (PAHs, heavy metals) from fish tissues and sediments were established using the generalised additive models for location of scale and shape (GAMLSS) (Rigby and Stasinopoulos 2005). Previously, the multicollinearity of variables was evaluated through a variance inflation factor index (VIF) and Spearman's correlation, for which the usdm package of R was used (Naimi et al. 2014). To decide which variables were discarded, a threshold of VIF < 4 was established and considered in the GAMLSS analysis (Zuur et al. 2010). The Akaike information criterion (AIC) value in the model setting GAMLSS package in R was used to fit the models. The best statistical model was selected by performing a forward procedure using the stepGAIC (generalised Akaike information criterion) function in the GAMLSS package, as this assessed the contribution of each variable and their combinations in the final model through an iterative process. This function chooses the best model based on the lowest AIC value. In addition, the goodness of fit of each model was evaluated through the explained deviance (ED), expressed as a percentage (Rigby and Stasinopoulos 2005). Finally, similitude of mean MNs for the species was tested with MVSP 3.1 software.

Results and Discussion

This study represents the first approach of using MNs and MCs from flounder fish collected from the YP and PF of the GoM. The values of MNs and MCs were associated with data of pollutants obtained from fish muscle and sediments. Fish collected from location C1 of PF were the most affected. Three to nine MNs/fish were observed, 100% of prevalence and mean intensity of 5. Also 60% of sampled fish harbored MCs. The location M63 from YP was the least affected because MNs were not detected there (Table 1). Likewise, we found significant differences in MCs frequencies between locations in the YP ($F_{17;108} = 32.952$; $p = 0.011$) but not in locations from the PF ($F_{7;41} = 0.976$; $p = 0.995$). No significant differences of MNs frequencies were found among locations from the YP ($F_{17;108} = 24.939$; $p = 0.096$), but significant differences were found in locations from the PF ($F_{7;41} = 16.17$; $p = 0.024$). MNs are useful biomarkers associated to genotoxicity (Jindal and Verma 2015; Shah et al. 2020), and the MCs are more related to health status of fish, but in controlled studies the frequency of MCs showed a high time-dependent correlation to the frequency of MNs (Jindal and Verma 2015). Based in the results of this study, we could speculate that MCs can be also useful biomarkers, but this topic needs further considerations in laboratory and field studies. For instance, MNs are used in monitoring studies and the variation on their frequencies is associated with anthropogenic pressure and pollution like the presence of heavy metals in each location (Rebok et al. 2017). Our statistical analysis suggested that MNs and MCs frequencies could be subjected to the environmental condition of localities sampled, in special, because the PF areas are used for petroleum exploration and exploitation.

Table 1 MNs and MCs values from flounder fish collected in the YP and PF

Cruise	Location	N	Mean ± S.D		Range		Prevalence (%)		Mean intensity	
			MNs	MCs	MNs	MCs	MNs	MCs	MNs	MCs
YP	A3	5	2.4 ± 2.7	0.6 ± 0.55*	0–7	0–1	80	60	3	1
YP	A4	5	0.4 ± 0.55	0	0–1	0	40	0	1	0
YP	A5	4	2.25 ± 1.71	0	0–4	0	75	0	3	0
YP	B8	5	1.8 ± 1.79	0	0–4	0	60	0	3	0
YP	F29	5	1.8 ± 2.05	0.2 ± 0.45*	0–4	0–1	60	20	3	1
YP	G34	10	0.3 ± 0.67	2.8 ± 5.27*	0–2	0–15	20	50	1.5	5.6
YP	G35	10	0.3 ± 0.48	2 ± 3.27*	0–1	0–9	30	50	1	4
YP	H39	5	0.8 ± 1.79	0.4 ± 0.89*	0–4	0–2	20	20	4	2
YP	H40	2	1 ± 0	2 ± 1.41*	1	1–3	100	100	1	2
YP	I43	5	0.6 ± 1.34	3 ± 3*	0–3	0–8	20	80	3	3.75
YP	J48	5	1 ± 1.73	0.6 ± 0.55*	0–4	0–1	40	60	2.5	1
YP	L59	5	0.4 ± 0.55	0	0–1	0	40	0	1	0
YP	M63	5	0	0.6 ± 1.34*	0	0–3	0	20	0	3
YP	O73	10	0.1 ± 0.32	0.7 ± 1.34*	0–1	0–4	10	30	1	2.33
YP	O75	7	0.14 ± 0.38	0.71 ± 1.11*	0–1	0–3	14.29	42.86	1	1.67
YP	P78	10	0.7 ± 0.82	0.2 ± 0.63*	0–2	0–2	50	10	1.4	2
YP	P79	5	0.4 ± 0.89	1.8 ± 1.92*	0–2	0–5	20	80	2	2.25
YP	P80	5	0.6 ± 1.34	0.8 ± 0.84*	0–3	0–2	20	60	3	1.33
PF	B1	5	0.4 ± 0.89**	2.8 ± 3.11	0–2	0–7	20	60	2	4.67
PF	B2	5	0.8 ± 0.84**	1.2 ± 1.79	0–2	0–4	60	40	1.33	3
PF	C1	5	5 ± 2.35**	1.6 ± 1.82	3–9	0–4	100	60	5	2.67
PF	C2	5	1.4 ± 1.14**	0.6 ± 0.89	0–3	0–2	80	40	1.75	1.5
PF	D1	6	0.83 ± 1.17**	0.83 ± 0.98	0–3	0–2	50	50	1.67	1.67
PF	D2	4	0.75 ± 1.5**	0.75 ± 0.96	0–3	0–2	25	50	3	1.5
PF	F1	6	0.33 ± 0.52**	2.17 ± 3.49	0–1	0–9	33.33	50	1	4.33
PF	F2	5	2.2 ± 1.3**	0.8 ± 1.3	1–4	0–3	100	40	2.2	2

*,**$p < 0.001$

Table 2 Metals and PAHs concentration (μg kg^{-1}) detected in flounder fish tissue (FT) and in the sediment (Sed) from locations of the YP and the PF

Sample	Pollutants	YP		PF	
		Mean ± S.D	Range	Mean ± S.D	Range
FT	V	30.11 ± 30.55	0–192.9	173.41 ± 144.16	19.34–591.32
FT	Ni	327.81 ± 435.58	0–2354.63	799.97 ± 1143.64	70.39–7399.06
FT	Cd	8.44 ± 18.04	0–96.82	4.97 ± 13.83	0–89.2
FT	Pb	21.62 ± 70.99	0–496.32	2070 ± 3044.53	0–15,253.52
FT	PAHs	2248.14 ± 2153.39	2.11–14,324.99	124.5 ± 124.23	2.87–486.97
Sed	V	530.59 ± 419.62	0–1279.67	114,732.79 ± 37,732.37	72,631.88–164,299.22
Sed	Ni	1124.04 ± 808.16	289.22–2699.98	23,657.21 ± 6750.1	15,442.04–31,847.44
Sed	Cd	217.32 ± 36.5	173.55–273.79	99.53 ± 65.74	34.43–211.65
Sed	Pb	530.93 ± 332.98	14.86–950.43	20,383.63 ± 4163.1	16,447.21–27,855.68
Sed	Al	259,182.5 ± 247,080	779,752.6–43,388.47	11,828,539.21 ± 9,245,092.95	27,342,167.63–2,751,901.14
Sed	PAHs	18.59 ± 15.72	0.41–42.53	82.16 ± 60.38	2.02–195.61

Heavy metal values detected herein in fish muscle (except for Cd) were higher in the PF than in the YP, however PAHs values were higher in the YP than in PF (Table 2). Our data values differ with previous reports in the GoM. During 2012–2014 the muscle tissues of flounder fish *Syacium gunteri* presented lower values of heavy metals and PAHs in YP than in PF (Quintanilla-Mena et al. 2019). Whereas in 2016, higher PAHs concentrations were detected in tissues of the fish *Lopholatilus chamaeleonticeps* from the YP than the ones from the PF (Snyder et al. 2020). Probably the higher values of PAHs and heavy metals found in PF were related to remains of the Macondo oil spill occurred in 2010. Fish have a high capacity to metabolize PAHs through a well-developed enzymatic system (Baali et al. 2016), thus, the PAHs values decrease in fish muscle. Nevertheless, the higher values observed in this study in the YP, could be related to bioaccumulation of contaminants from different sources.

In sediments the heavy metals (except for Cd) and PAHs concentration were higher in PF than in YP (Table 2). PAHs concentration for the YP (18.59 ± 15.72 μg kg^{-1}) and the PF (82.16 ± 60.38 μg kg^{-1}) are considered low according to the classification suggested by Recabarren-Villalón et al. (2019) (PAHs low values from 10 to 100 μg kg^{-1}). The variations of contaminants levels in sediments between YP and PF could be reflecting remains of contaminants from the oil spill occurred in 2010 in the northern GoM (McNutt et al. 2012) although, local inputs such as extraction, production, and transport of petroleum, among others (Murawski and Hogarth 2013), and contaminant transport related with the hydrology and the hydrodynamics, could also be contributing to the contaminant load (Botello et al. 2015).

The GAMLSS models of MCs and MNs frequency correlated with heavy metals from fish muscle, fish species and the locality, with a 20.37% and 25.15% contribution to the explained deviance, respectively (Table 3). Heavy metals are bioaccumulated in the muscle of fish and it is widely known that Cd, V, Pb and Ni induce the formation of MNs and MCs and that the values are different between species (Singh et al. 2019; Shah et al. 2020).

The variability of metals levels found in fish tissue can be subjected to feeding habits, habitats (location) and behaviour of species (Ergene et al. 2007). In this sense, our results indicate that the MNs and MCs have a multifactorial induction and can be closely related to the biology of the species. The MNs and MCs prevalence, intensity and mean values correlated with Al, PAHs, depth, and locality (31.08%, 59.13% and 33.19% explained deviance to MNs, and 43.22%, 60.45% and 76.37% to MCs respectively) (Table 3). The contaminants detected in sediments from this study could persist for decades in the subsurface layers and organisms could bioaccumulate and incorporate them into their food chains, increasing the risk for humans through fish and seafood consumption (Bianchini and Morrissey 2018). Likewise, the PAHs and heavy metals mutagenicity affect directly on DNA damage, and also induce MCs formation through cytoplasmic alterations (Shah et al. 2020). PAHs are considered the most toxic components of crude oil and have genotoxic effects including DNA damage and DNA mutation (Santana et al. 2018). The exposure of fish to PAHs of oil-contaminated sediment can cause severe adverse effects like metabolic changes, energy imbalance, alterations in organs structure, altered intestinal microbiome structures and increased mortality (Brown-Peterson et al. 2015). Our results suggest that the variation of PAHs and heavy metals in the location samples at different depths are important factors in the variation of the prevalence, mean and intensity of the MNs and MCs because they are associated with the habitat and fish behaviour (Ergene et al. 2007).

Table 3 GAMLSS model

Model	Df	GD	ED (%)	AIC	FD
FMNs ~ cs(Ni) + cs(Pb) + cs(Species) + cs(Loc)*	17	337.89	25.15	371.90	PO
FMCs ~ cs(Ni) + cs(Pb) + cs(V) + cs(Cd) + cs(Species) + cs(Loc)*	25	516.71	20.37	568.71	NO
PMNs ~ cs(Al) + cs(PAHs) + cs(Depth) + cs(Loc)*	17	110.95	31.08	146.95	NO
IMN ~ cs(Al) + cs(PAHs) + cs(Depth)*	14	23.64	59.13	51.64	GU
MMN ~ cs(PAHs) + cs(Depth)*	10	52.26	33.19	72.26	NO
PMCs ~ cs(PAHs) + cs(Depth) + cs(Loc)*	13	175.41	43.22	201.41	PO
IMC ~ cs(PAHs) + cs(Depth) + cs(Loc)*	13	23.51	60.45	51.50	NO
MMC ~ cs(Depth) + cs(Loc)*	10	11.83	76.37	31.83	GU

AIC Akaike information criterion, *cs* cubic spline smooth function, *df* degrees of freedom, *GD* global deviance, *FD* family distribution, *P* p value, *%ED* percent of explained deviance, *FMNs* frequency of MNs, *PMNs* prevalence of MNs, *IMN* intensity of MNs, *MMN* mean of MNs, *FMCs* frequency of MCs, *PMCs* prevalence of MCs, *IMC* intensity of MCs, *MMC* mean of MCs, *Species* fish species, *Loc* localities, *PO* Poisson distribution, *NO* Normal distribution, *GU* Gumbel distribution

*$p < 0.001$

From the 149 flounders collected we detected 7 species: *Syacium papillosum* (n = 81), *Cyclopsetta chittendeni* (n = 18), *Cyclopsetta fimbriata* (n = 11), *Syacium micrurum* (n = 4), *Ancyclopsetta dilecta* (n = 8), *Syacium gunteri* (n = 22), and *Trichopsetta ventralis* (n = 5). In all species MNs were detected (range = 0–9 MNs per fish), as well as MCs (range = 0–15 MCs per fish), but the highest number of MNs was found in *C. chittendeni* ($F_{6;149} = 18.99$; $p = 0.004$). In this sense, fish response to xenobiotics as the PAHs differs among the species and stages (Recabarren-Villalón et al. 2019). In contrast, the MCs frequency variation was not significant for the different species ($F_{6;149} = 5.23$; $p = 0.515$). The MVPS analysis showed 45.68% of similitude of the mean MNs among *S. papillosum* and *C. chittendeni*. These results might indicate different susceptibility among species. Therefore, the selection of sentinel species to assess xenobiotics impact in aquatic environments is a key factor that needs to be evaluated deeply to obtain more accurate information. The presence of environmental contaminants has different accumulation rates that depend on their type, lifetime, and toxicity.

In conclusion, this study represents the first effort to evaluate the presence of MNs and MC in flounders from two important areas of the GoM. Their values were related to PAHs and heavy metals exposition and showed a negative association to health or fitness of fish. MNs and MCs frequencies were more evident in the PF than in the YP. PAHs found in fish muscle were higher than the values found in sediments. MNs frequency showed a species-specific association being *C. chittendeni* the most affected organism. Also, we consider that MNs and MCs inductions are multifactorial with a probable relevant influence of the fish biology. Our results support the potential use of MNs and MCs as biomarkers in environmental pollution studies.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s00128-021-03176-w.

Acknowledgements Special thanks are conveyed to the personnel of the laboratory of Geochemistry (CINVESTAV-Unidad Merida) for processing samples. Research funded by the National Council of Science and Technology of Mexico – Mexican Ministry of Energy – Hydrocarbon Trust, project 201441. This is a contribution of the Gulf of Mexico Research Consortium (CIGoM) and PEMEX. We acknowledge PEMEX´s specific request to the Hydrocarbon Fund to address the environmental effects of oil spills in the Gulf of Mexico. The authors are grateful to the comments of the anonymous reviewers.

References

Alkaladi A, El-Deen NAMN, Afifi M, Zinadah OAA (2015) Hematological and biochemical investigations on the effect of vitamin E and C on *Oreochromis niloticus* exposed to zinc oxide nanoparticles. Saudi J Biol Sci 22:556–563. https://doi.org/10.1016/j.sjbs.2015.02.012

Allan SE, Smith BW, Anderson KA (2012) Impact of the Deepwater Horizon oil spill on bioavailable polycyclic aromatic hydrocarbons in Gulf of Mexico coastal waters. Environ Sci Technol 46(4):2033–2039. https://doi.org/10.1021/es202942q

Al-Sabti K, Metcalfe CD (1995) Fish micronuclei for assessing genotoxicity in water. Mutat Res Toxicol 343:121–135. https://doi.org/10.1016/0165-1218(95)90078-0

Ayanda IO, Yang M, Yu Z, Zha J (2018) Cytotoxic and genotoxic effects of perfluorododecanoic acid (PFDoA) in *Japanese medaka*. Knowl Manag Aquat Ecosyst. https://doi.org/10.1051/kmae/2017058

Baali A, Kammann U, Hanel R et al (2016) Bile metabolites of polycyclic aromatic hydrocarbons (PAHs) in three species of fish from Morocco. Environ Sci Eur 28:25. https://doi.org/10.1186/s12302-016-0093-6

Baršiene J, Lazutka J, Šyvokiene J et al (2004) Analysis of micronuclei in blue mussels and fish from the Baltic and North Seas. Environ Toxicol 19:365–371. https://doi.org/10.1002/tox.20031

Bejarano AC (2018) Critical review and analysis of aquatic toxicity data on oil spill dispersants. Environ Toxicol Chem 37(12):2989–3001. https://doi.org/10.1002/etc.4254

Bianchini K, Morrissey CA (2018) Assessment of shorebird migratory fueling physiology and departure timing in relation to polycyclic aromatic hydrocarbon contamination in the Gulf of Mexico. Environ Sci Technol 52:13562–13573. https://doi.org/10.1021/acs.est.8b04571

Bolognesi C, Perrone E, Roggieri P, Sciutto A (2006) Bioindicators in monitoring long term genotoxic impact of oil spill: Haven case study. Mar Environ Res 62:S287–S291. https://doi.org/10.1016/j.marenvres.2006.04.047

Botello AV, Soto LA, Ponce-Velez G, Villanueva-F S (2015) Baseline for PAHs and metals in NW Gulf of Mexico related to the Deepwater Horizon oil spill. Estuar Coast Shelf Sci 156:124–133. https://doi.org/10.1016/j.ecss.2014.11.010

Brown-Peterson NJ, Krasnec M, Takeshita R et al (2015) A multiple endpoint analysis of the effects of chronic exposure to sediment contaminated with *Deepwater Horizon* oil on juvenile Southern flounder and their associated microbiomes. Aquat Toxicol 165:197–209. https://doi.org/10.1016/j.aquatox.2015.06.001

Conti ME, Iacobucci M (2008) Marine organisms as biomonitors. . WIT Transact State-of-the-art Sci Eng 30:81–110. https://doi.org/10.2495/978-1-84564-002-6/04

Ergene S, Cavas T, Celik A et al (2007) Monitoring of nuclear abnormalities in peripheral erythrocytes of three fish species from the Goksu Delta (Turkey): genotoxic damage in relation to water pollution. Ecotoxicology 16:385–391. https://doi.org/10.1007/s10646-007-0142-4

Gold-Bouchot G, Rubio-Piña J, Montero-Muñoz J et al (2017) Pollutants and biomarker responses in two reef fish species (*Haemulon aurolineatum* and *Ocyurus chrysurus*) in the Southern Gulf of Mexico. Mar Pollut Bull 116:249–257. https://doi.org/10.1016/j.marpolbul.2016.12.073

Gregson BH, McKew BA, Holland RD, Nedwed TJ, Prince RC, McGenity TJ (2021) Marine oil snow, a microbial perspective. Front Mar Sci 8:619484. https://doi.org/10.3389/fmars.2021.619484

Holt EA, Miller SW (2010) Bioindicators: using organisms to measure environmental impacts. Nature 3(10):8–13

Jindal R, Verma S (2015) In vivo genotoxicity and cytotoxicity assessment of cadmium chloride in peripheral erythrocytes of *Labeo rohita* (Hamilton). Ecotoxicol Environ Saf 118:1–10. https://doi.org/10.1016/j.ecoenv.2015.04.005

Kirby MF, Morris S, Hurst M, Kirby SJ, Neall P, Tylor T, Fagg A (2000) The use of cholinesterase activity in flounder (*Platichthys flesus*) muscle tissue as a biomarker of neurotoxic

contamination in UK estuaries. Mar Pollut Bull 40(9):780–791. https://doi.org/10.1016/S0025-326X(00)00069-2

MacLeod WD, Brown DD, Friedman AJ et al (1985) Standard analytical procedures of the NOAA (National Oceanic and Atmospheric Administration) national analytical facility, 1985–1986: extractable toxic organic compounds, 2nd edn. S. Department of Commerce, NOAA/NMFS, NOAA Tech Memo NMFS F/NWRC-92

McNutt MK, Chu S, Lubchenco J et al (2012) Applications of science and engineering to quantify and control the *Deepwater Horizon* oil spill. Proc Natl Acad Sci USA 109:20222–20228. https://doi.org/10.1073/pnas.1214389109

Murawski SA, Hogarth WT (2013) Enhancing the ocean observing system to meet restoration challenges in the Gulf of Mexico. Oceanography 26(1):10–16. https://doi.org/10.5670/oceanog.2013.12

Murawski SA, Hollander DJ, Gilbert S, Gracia A (2020) Deep-water oil and gas production in the Gulf of Mexico, and related global trends. In: Murawski SA, Ainsworth C, Gilbert S, Hollander D, Paris CB, Schlüter M et al (eds) Scenarios and responses to future deep oil spills—fighting the next war. Springer, Cham

Naimi B, Hamm NAS, Groen TA et al (2014) Where is positional uncertainty a problem for species distribution modelling? Ecography 37:191–203

Perez CR, Moye JK, Cacela D et al (2017) Low level exposure to crude oil impacts avian flight performance: The *Deepwater Horizon* oil spill effect on migratory birds. Ecotoxicol Environ Saf 146:98–103. https://doi.org/10.1016/j.ecoenv.2017.05.028

Praveen Kumar MK, Shyama SK, D'Costa A et al (2017) Evaluation of DNA damage induced by gamma radiation in gill and muscle tissues of *Cyprinus carpio* and their relative sensitivity. Ecotoxicol Environ Saf 144:166–170. https://doi.org/10.1016/j.ecoenv.2017.06.022

Quintanilla-Mena M, Gold-Bouchot G, Zapata-Pérez O et al (2019) Biological responses of shoal flounder (*Syacium gunteri*) to toxic environmental pollutants from the southern Gulf of Mexico. Environ Pollut. https://doi.org/10.1016/j.envpol.2019.113669

Rahmanpour S, Ashtiyani SML, Ghorghani NF (2016) Biomonitoring of heavy metals using bottom fish and crab as bioindicator species, the Arvand River. Toxicol Ind Health 32:1208–1214. https://doi.org/10.1177/0748233714554410

Rebok K, Jordanova M, Slavevska-Stamenković V et al (2017) Frequencies of erythrocyte nuclear abnormalities and of leucocytes in the fish *Barbus peloponnesius* correlate with a pollution gradient in the River Bregalnica (Macedonia). Environ Sci Pollut Res 24:10493–10509. https://doi.org/10.1007/s11356-017-8665-6

Recabarren-Villalón T, Ronda AC, Arias AH (2019) Polycyclic aromatic hydrocarbons levels and potential biomarkers in a native South American marine fish. Reg Stud Mar Sci 29:100695. https://doi.org/10.1016/j.rsma.2019.100695

Rigby RA, Stasinopoulos DM (2005) Generalized additive models for location, scale and shape. J R Stat Soc Ser C Appl Stat 54:507–554

Santana MS, Sandrini-Neto L, Filipak Neto F et al (2018) Biomarker responses in fish exposed to polycyclic aromatic hydrocarbons (PAHs): systematic review and meta-analysis. Environ Pollut 242:449–461. https://doi.org/10.1016/j.envpol.2018.07.004

Shah N, Khan A, Ali R, Marimuthu K et al (2020) Monitoring Bioaccumulation (in Gills and Muscle Tissues), Hematology, and Genotoxic Alteration in *Ctenopharyngodon idella* Exposed to Selected Heavy Metals. Biomed Res Int. https://doi.org/10.1155/2020/6185231

Singh M, Khan H, Verma Y, Rana SVS (2019) Distinctive fingerprints of genotoxicity induced by As, Cr, Cd, and Ni in a freshwater fish. Environ Sci Pollut Res 26:19445–19452. https://doi.org/10.1007/s11356-019-05274-z

Snyder SM, Olin JA, Pulster EL, Murawski SA (2020) Spatial contrasts in hepatic and biliary PAHs in Tilefish (*Lopholatilus chamaeleonticeps*) throughout the Gulf of Mexico, with comparison to the Northwest Atlantic. Environ Pollut 258:113775. https://doi.org/10.1016/j.envpol.2019.113775

Sommer S, Buraczewska I, Kruszewski M (2020) Micronucleus assay: the state of art, and future directions. Int J Mol Sci 21:1–19. https://doi.org/10.3390/ijms21041534

Van der Oost R, Beyer J, Vermeulen NPE (2003) Fish bioaccumulation and biomarkers in environmental risk assessment: a review. Environ Toxicol Pharmacol 13(2):57–149. https://doi.org/10.1016/S1382-6689(02)00126-6

Wade TL, Atlas EL, Brooks JM et al (1988) NOAA Gulf of Mexico status and trends program: Trace organic contaminant distribution in sediments and oysters. Estuaries 11:171–179. https://doi.org/10.2307/1351969

Wang Z, Fingas M, Li K (1994) Fractionation of a light crude oil and identification and quantitation of aliphatic, aromatic, and biomarker compounds by GC-FID and GC-MS, part I. J Chromatogr Sci 32:361–366. https://doi.org/10.1093/chromsci/32.9.361

Ward CH, Tunnell JW (2017) Habitats and Biota of the Gulf of Mexico: An Overview. In: Ward C (ed) Habitats and biota of the Gulf of Mexico: before the Deepwater Horizon oil spill. Springer, New York, pp 1–54

Yu Z, Lin Q, Gu Y, Du F, Wang X, Shi F, Ke Ch, Xiang M, Yu Y (2019) Bioaccumulation of polycyclic aromatic hydrocarbons (PAHs) in wild marine fish from the coastal waters of the northern South China Sea: risk assessment for human health. Ecotoxicol Environ Saf 180:742–748. https://doi.org/10.1016/j.ecoenv.2019.05.065

Zuur AF, Ieno EN, Elphick CS (2010) A protocol for data exploration to avoid common statistical problems. Methods Ecol Evol 1:3–14

Injection-Induced Earthquakes

William L. Ellsworth

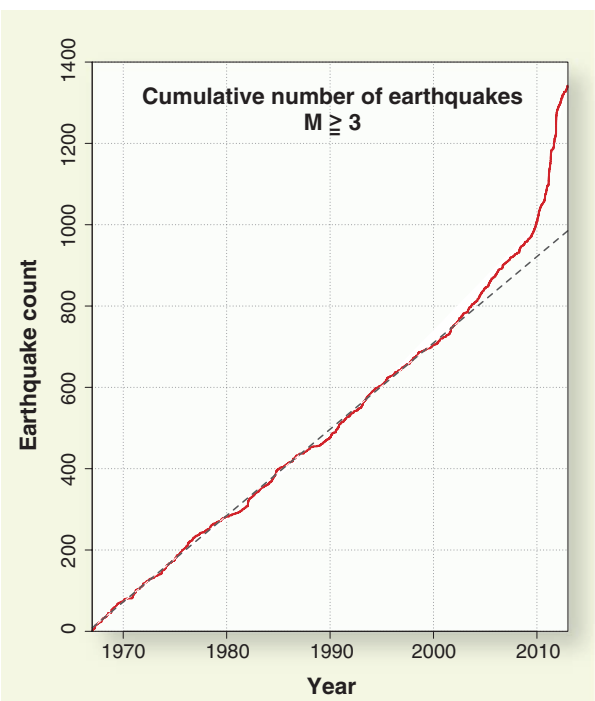
READ THE FULL ARTICLE ONLINE
http://dx.doi.org/10.1126/science.1225942

Cite this article as W. L. Ellsworth, *Science* **341**, 1225942 (2013).
DOI: 10.1126/science.1225942

Background: Human-induced earthquakes have become an important topic of political and scientific discussion, owing to the concern that these events may be responsible for widespread damage and an overall increase in seismicity. It has long been known that impoundment of reservoirs, surface and underground mining, withdrawal of fluids and gas from the subsurface, and injection of fluids into underground formations are capable of inducing earthquakes. In particular, earthquakes caused by injection have become a focal point, as new drilling and well-completion technologies enable the extraction of oil and gas from previously unproductive formations.

Advances: Microearthquakes (that is, those with magnitudes below 2) are routinely produced as part of the hydraulic fracturing (or "fracking") process used to stimulate the production of oil, but the process as currently practiced appears to pose a low risk of inducing destructive earthquakes. More than 100,000 wells have been subjected to fracking in recent years, and the largest induced earthquake was magnitude 3.6, which is too small to pose a serious risk. Yet, wastewater disposal by injection into deep wells poses a higher risk, because this practice can induce larger earthquakes. For example, several of the largest earthquakes in the U.S. midcontinent in 2011 and 2012 may have been triggered by nearby disposal wells. The largest of these was a magnitude 5.6 event in central Oklahoma that destroyed 14 homes and injured two people. The mechanism responsible for inducing these events appears to be the well-understood process of weakening a preexisting fault by elevating the fluid pressure. However, only a small fraction of the more than 30,000 wastewater disposal wells appears to be problematic—typically those that dispose of very large volumes of water and/or communicate pressure perturbations directly into basement faults.

Outlook: Injection-induced earthquakes, such as those that struck in 2011, clearly contribute to the seismic hazard. Quantifying their contribution presents difficult challenges that will require new research into the physics of induced earthquakes and the potential for inducing large-magnitude events. The petroleum industry needs clear requirements for operation, regulators must have a solid scientific basis for those requirements, and the public needs assurance that the regulations are sufficient and are being followed. The current regulatory frameworks for wastewater disposal wells were designed to protect potable water sources from contamination and do not address seismic safety. One consequence is that both the quantity and timeliness of information on injection volumes and pressures reported to regulatory agencies are far from ideal for managing earthquake risk from injection activities. In addition, seismic monitoring capabilities in many of the areas in which wastewater injection activities have increased are not capable of detecting small earthquake activity that may presage larger seismic events.



Earthquakes with magnitude (M) ≥ 3 in the U.S. midcontinent, 1967–2012. After decades of a steady earthquake rate (average of 21 events/year), activity increased starting in 2001 and peaked at 188 earthquakes in 2011. Human-induced earthquakes are suspected to be partially responsible for the increase.

Earthquake Science Center, U.S. Geological Survey, Menlo Park, CA 94025, USA.
E-mail: ellsworth@usgs.gov

ARTICLE OUTLINE

ADDITIONAL RESOURCES

The following resources provide an introduction to earthquake hazards and risk, the science of induced earthquakes, and strategies for managing the risk.

C. Nicholson, R. L. Wesson, "Earthquake hazard associated with deep well injection: A report to the U.S. Environmental Protection Agency," *U.S. Geol. Surv. Bull. 1951* (1990); http://pubs.usgs.gov/bul/1951/report.pdf.

Committee on Induced Seismicity Potential in Energy Technologies, *Induced Seismicity Potential in Energy Technologies* (National Research Council, Washington, DC, 2012); http://dels.nas.edu/Report/Induced-Seismicity-Potential-Energy-Technologies/13355.

S. Horton, Disposal of hydrofracking waste fluid by injection into subsurface aquifers triggers earthquake swarm in central Arkansas with potential for damaging earthquake. *Seismol. Res. Lett.* **83**, 250–260 (2012). doi:10.1785/gssrl.83.2.250

Tutorial material on probabilistic seismic hazard analysis (PSHA): www.opensha.org/sites/opensha.org/files/PSHA_Primer_v2_0.pdf

M. D. Zoback, Managing the seismic risk posed by wastewater disposal. *Earth Magazine* **57**, 38–43 (2012).

Injection-Induced Earthquakes

William L. Ellsworth

Earthquakes in unusual locations have become an important topic of discussion in both North America and Europe, owing to the concern that industrial activity could cause damaging earthquakes. It has long been understood that earthquakes can be induced by impoundment of reservoirs, surface and underground mining, withdrawal of fluids and gas from the subsurface, and injection of fluids into underground formations. Injection-induced earthquakes have, in particular, become a focus of discussion as the application of hydraulic fracturing to tight shale formations is enabling the production of oil and gas from previously unproductive formations. Earthquakes can be induced as part of the process to stimulate the production from tight shale formations, or by disposal of wastewater associated with stimulation and production. Here, I review recent seismic activity that may be associated with industrial activity, with a focus on the disposal of wastewater by injection in deep wells; assess the scientific understanding of induced earthquakes; and discuss the key scientific challenges to be met for assessing this hazard.

Earthquakes are expected within tectonically active regions such as along plate boundaries or within distributed zones of deformation. Recent seismic activity across the coterminous United States, for example, concentrates along the plate boundaries of the West Coast and within the intermountain West (Fig. 1). Within such actively deforming zones, elastic strain energy accumulates in the crust, sometimes for centuries, before being released in earthquakes. The potential for earthquakes also exists within continental interiors, despite very low deformation rates (*1*). This is because shear stress levels within the interior of plates or near plate boundaries are commonly found to be near the strength limit of the crust (*2*). Under these conditions, small perturbations that effect fault stability can and do trigger earthquakes (*3*–*6*). For example, the injection of water under high pressure into impermeable basement rocks beneath Basel, Switzerland, to develop an enhanced geothermal system beneath the city induced four moment magnitude (M_w) 3 earthquakes in 2006 and 2007 (*7*) (earthquake magnitudes measured using other scales are denoted by M). These small earthquakes led to the abandonment of the project, loss of the investment, and ongoing litigation over compensation for damage. The extraction of natural gas from shallow deposits in the Netherlands also causes earthquakes (*8*). A recent M 3.4 event near Loppersum damaged scores of homes in the area, resulting in large losses for the property owners (*9*).

Within the central and eastern United States, the earthquake count has increased dramatically over the past few years (Fig. 2). More than 300 earthquakes with $M \geq 3$ occurred in the 3 years from 2010 through 2012, compared with an average rate of 21 events/year observed from 1967 to 2000. States experiencing elevated levels of seismic activity included Arkansas, Colorado, New Mexico, Ohio, Oklahoma, Texas, and Virginia. The greatest rise in activity occurred in 2011 when 188 $M \geq 3$ earthquakes occurred. Although earthquake

Earthquake Science Center, U.S. Geological Survey, Menlo Park, CA 94025, USA.
E-mail: ellsworth@usgs.gov

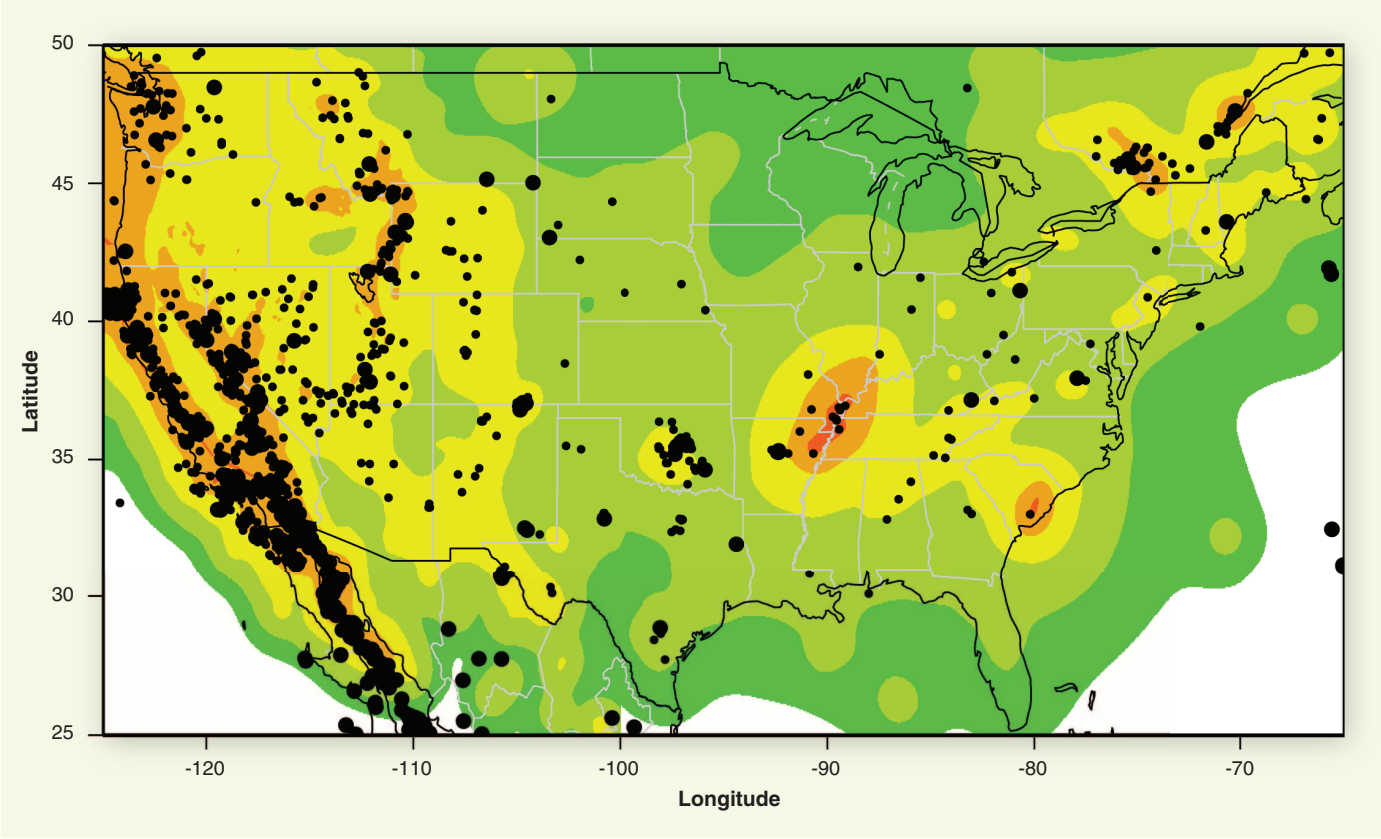


Fig. 1. Seismicity of the coterminous United States and surrounding regions, 2009–2012. Black dots denote seismic events. Only earthquakes with $M \geq 3$ are shown; larger symbols denote events with $M \geq 4$. Background colors give the probability of peak ground acceleration with a 2% probability of exceedance in 50 years, from the U.S. National Seismic Hazard Map (*1*). Red, ≥ 1*g*; orange, 0.3 to 1*g*; yellow, 0.1 to 0.3*g*; light green, 0.03 to 0.1*g*; darker green, 0.03 to 0.1*g*.

detection improved for $M < 3$ as the USArray transportable seismograph array began to pass through the region starting in 2008 (*10*), a recent report on seismicity in the central and eastern United States found that the probability of missing $M \geq 3$ earthquakes in the region has been near zero for decades (*11*). Consequently, the increased earthquake count represents a temporal change in earthquake rate. Because the hazard of damaging ground shaking is fundamentally related to the rate of earthquake occurrence (*1*), regions where the rate increased may be more hazardous than forecast by the 2008 version of the U.S. National Seismic Hazard Map (Fig. 1) (*1*). Understanding why seismicity increased and how this increase affects the hazard have become a priority for the earthquake-research community.

A number of these recent earthquakes occurred in areas where specific types of nearby industrial activities raise the possibility that these events were induced by human activity. Here, I will use the term "induced" to include both earthquakes triggered by anthropogenic causes that primarily release tectonic stress and those that primarily release stresses created by the industrial activity (*4*). Understanding which earthquakes may have been induced and, if so, how are challenging problems to solve in the current data-poor environment.

Several examples since 2011 highlight the difficulty in determining whether earthquakes were induced by human activity. The M_w 4.0 earthquake on 31 December 2011 in Youngstown, Ohio, appears to have been induced by injection of wastewater in a deep Underground Injection Control (UIC) class II well (*12*). The M_w 4.7 27 February 2011 central Arkansas earthquake has also been linked to deep injection of wastewater (*13*). The M_w 4.4 11 September 2011 earthquake near Snyder, Texas, occurred in an oil field where injection for secondary recovery has been inducing earthquakes for years (*14*). The M_w 4.8 10 October 2011 earthquake near Fashing, Texas, occurred in a region where long-term production of gas has been linked to earthquake activity (*15*). For others, such as the M_w 5.7 6 November 2011 central Oklahoma earthquake (*16*) or the M_w 4.9 17 May 2012 east Texas earthquake (*17*), where active wastewater-injection wells are located near their respective epicenters, the question of natural versus induced remains an active topic of research.

The potential association between deep wastewater disposal wells and earthquakes has received considerable attention due to the association of this activity with the development of tight shale formations for gas and petroleum by hydraulic fracturing, or "fracking" (*5*). Wells used in the U.S. petroleum industry to inject fluids are regulated as UIC class II wells. Approximately 110,000 of these wells are used for enhanced oil recovery. In addition, 30,000 class II wells in the United States are used for wastewater disposal. Of these wells, most have no detected seismicity within tens of kilometers, although a few are correlated with seismicity (*18*). However, this can be said with confidence only for earthquakes $M_w \geq 3$, as smaller earthquakes are not routinely reported in the central and eastern United States. So it is possible that smaller earthquakes could be more common in the vicinity of these wells. In California, where the completeness threshold is below M_w 2, the majority of the 2300 active wastewater-injection wells are located in regions of low seismicity. As with elsewhere in the United States, a small fraction of the California wastewater wells coincide with earthquakes, which raises the question of what factors distinguish those seismically active wells from the majority of wells if the earthquakes and injection activities are related.

Mechanics of Induced Earthquakes

Earthquakes release stored elastic strain energy when a fault slips. A fault will remain locked as long as the applied shear stress is less than the strength of the contact. The failure condition to initiate rupture is usually expressed in terms of the effective stress $\tau_{crit} = \mu(\sigma_n - P) + \tau_o$, where the critical shear stress τ_{crit} equals the product of the coefficient of friction μ and the effective normal stress given by the difference between the applied normal stress σ_n and the pore pressure P (*3*, *19*, *20*). For almost all rock types, μ lies between 0.6 and 1.0, and the cohesive strength of the sliding surface, τ_o, is negligible under typical crustal conditions. Increasing the shear stress, reducing the normal stress, and/or elevating the pore pressure can bring the fault to failure, triggering the nucleation of the earthquake (Fig. 3). Once initiated, sliding resistance drops and seismic waves radiate away, driven by the imbalance between the elastic stress stored in the surrounding rock mass and the frictional resistance of the dynamically weakened sliding surface. Rupture will continue to propagate, as long as the wave-mediated stress at the rupture front exceeds the static strength, and may extend into regions where the ambient stresses are below the failure threshold.

Rocks fail in tension when the pore pressure exceeds the sum of the least principal stress, σ_3, and the tensile strength of the rock, forming an opening-mode fracture that propagates in the plane normal to σ_3. The industrial process of hydraulic fracturing commonly involves both tensile and shear failure. Depending on the local stress state, hydraulically conductive fractures may be induced to fail in shear before $P = \sigma_3$. A successful "frac job" may create a fracture network dominated by pathways created by shear failure (*21*).

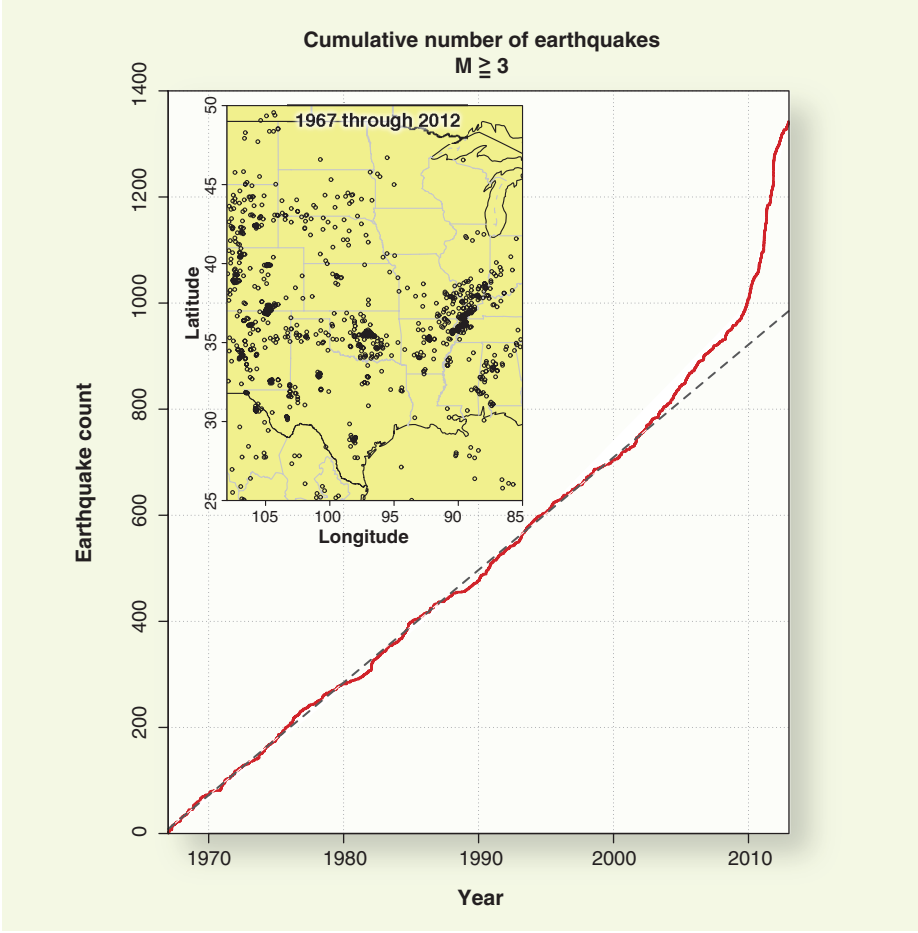


Fig. 2. Cumulative count of earthquakes with $M \geq 3$ in the central and eastern United States, 1967–2012. The dashed line corresponds to the long-term rate of 21.2 earthquakes/year. (**Inset**) Distribution of epicenters in the region considered here.

Earthquakes are known to be induced by a wide range of human activities (3–5) that modify the stress and/or pore pressure (Fig. 3). At present, with the use of seismological methods, it is not possible to discriminate between man-made and natural tectonic earthquakes. Induced earthquakes sometimes occur at the source of the stress or pressure perturbation; at other times, these events take place deep below and kilometers away from the source. When removed from the source, induced earthquakes typically release stored tectonic stress on preexisting faults, as do natural earthquakes. Sometimes induced events occur shortly after the industrial activity begins, but in other cases they happen long after it has been under way or even ceased. Factors that should enhance the probability of a particular stress or pore-pressure perturbation inducing earthquakes include the magnitude of the perturbation, its spatial extent, ambient stress condition close to the failure condition, and the presence of faults well oriented for failure in the tectonic stress field. Hydraulic connection between the injection zone and faults in the basement may also favor inducing earthquakes, as the tectonic shear stress increases with depth in the brittle crust (2). In addition, the larger the fault, the larger the magnitude of earthquakes it can host.

Methods for anticipating the time of failure have long been the "holy grail" of seismology (22). Though short-term prediction remains an elusive goal, it has been proposed that critically loaded faults have enhanced triggering susceptibility to dynamic stresses from distant earthquakes (23). Specifically, some but not all of the sites where fluid-injection–induced earthquakes are suspected of contributing to the recent increase in seismicity in the midcontinent (Fig. 2) experienced increased rates of microearthquakes in the days immediately after three recent great earthquakes (23).

Earthquakes Induced by Hydraulic Fracturing

The industrial process of hydraulic fracturing involves the controlled injection of fluid under pressure to create tensile fractures, thereby increasing the permeability of rock formations. It has been used for well over half a century to stimulate the recovery of hydrocarbons. For many decades, the primary application was to improve the output of aging oil and gas reservoirs. Beginning in the late 1990s, technologies for extracting natural gas and oil from tight shale formations led to the development of new natural gas fields in many parts of the central and eastern United States, western Canada, and Europe. Global development of oil and gas from shale will undoubtedly continue, as the resource potential is high in many parts of the world.

Extracting hydrocarbons from shale requires the creation of a network of open fractures connected to the borehole. Horizontal drill holes extending up to several kilometers within the shale formation undergo a staged series of hydraulic fractures, commonly pressurizing a limited section of the cased well at a time to stimulate the flow of gas or oil into the well. Each stage involves the high-pressure injection of water into the formation. Fracking intentionally induces numerous microearthquakes, the vast majority with $M_w < 1$.

Several cases have recently been reported in which earthquakes large enough to be felt but too small to cause structural damage were associated directly with fracking. These cases are notable because of the public concern that they raised, despite maximum magnitudes far too small to cause structural damage. Investigation of a sequence of felt events with maximum M 2.9 in south central Oklahoma revealed a clear temporal correlation between fracking operations in a nearby well and the seismic activity (24). Available data were insufficient to definitely rule out a natural cause due to the occurrence of some natural seismicity in the general area. In April and May 2012, a series of induced earthquakes with maximum M 2.3 occurred near Blackpool, United Kingdom (25), during fracking to develop a shale gas reservoir.

One of the major shale plays in the United States—the Marcellus Shale of the Appalachian Basin in Pennsylvania, West Virginia, Ohio, and New York—lies within a region characterized by low levels of natural seismic activity (Fig. 1). The regional seismographic network operated by Lamont Doherty Earth Observatory (LDEO) systematically catalogs all earthquakes with $M \geq 2$ in Pennsylvania (Fig. 4). Although thousands of hydraulic fractures were done in Pennsylvania since major development of the field began in 2005, only six earthquakes with $M \geq 2$ were detected by the LDEO network within the footprint of the Marcellus Shale, the largest of which was just M 2.3. The largest earthquake in the region since the development of shale gas happened across the Ohio border in Youngstown, where it was induced by injection (12), much of the fluid apparently coming from wells in Pennsylvania.

Beginning in 2009, an unusual sequence of earthquakes was noted in the Horn River Basin of British Columbia, including 21 events with M_w 3.0 and larger. Only the largest, at M_w 3.6, was reported as felt by workers in this remote area where it did no damage (26). The investigation into the cause of these events by the BC Oil and Gas Commission (26) concluded that the events "were caused by fluid injection during hydraulic fracturing in proximity of pre-existing faults." Two of the hydrofrac treatments were recorded by dense seismometer deployments at the surface. Precise hypocentral locations showed that the induced earthquakes occurred on previously unknown faults located outside of the stimulation interval that were well oriented for failure in the ambient stress field. Apparently, fracture pressure was quickly communicated through hydraulically conductive pathways and induced slip on critically stressed faults via reduction of the effective normal stress.

Earthquakes Induced by Deep Injection

There has been a growing realization that the principal seismic hazard from injection-induced earthquakes comes from those associated with disposal of wastewater into deep strata or basement formations (5). Before 2011, the M_w 4.8 event on 9 August 1967 near Denver, Colorado, was the largest event widely accepted in the scientific community as having been induced by wastewater injection (5). The hazard landscape of what is possible has shifted due to the role that wastewater injection into a depleted oil field may have played in the M_w 5.7 6 November 2011 central Oklahoma earthquake (16), although a consensus on its origin has not yet been reached (27). This earthquake damaged homes and unreinforced masonry buildings in the epicentral

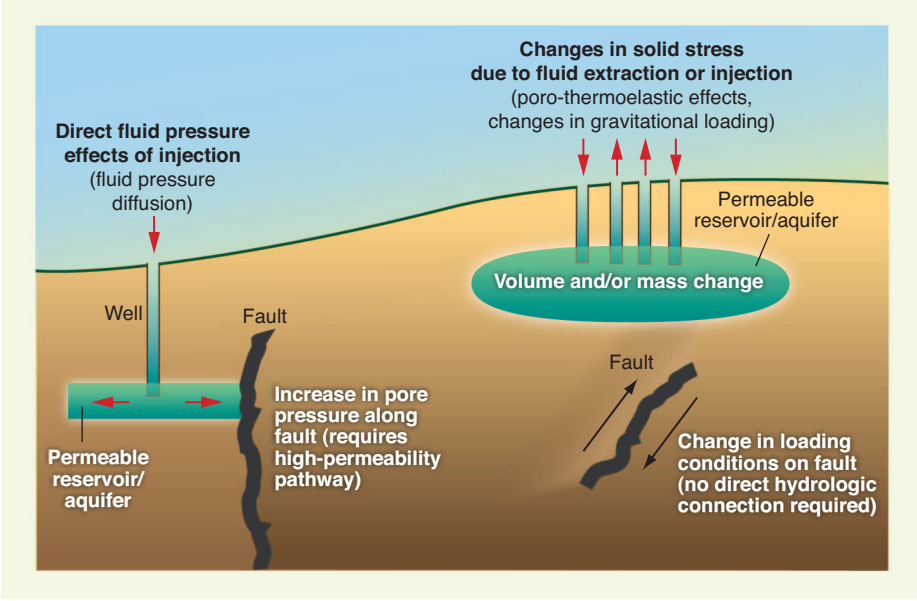


Fig. 3. Schematic diagram of mechanisms for inducing earthquakes. Earthquakes may be induced by increasing the pore pressure acting on a fault (**left**) or by changing the shear and normal stress acting on the fault (**right**). See (4).

area and was felt as far as 1000 km away in Chicago, Illinois.

The November 2011 central Oklahoma earthquake sequence initiated very close to a pair of wastewater-injection wells where disposal operation began 18 years earlier (*16*). No unusual seismicity was detected in this historically quiet region, where only a few events of $M < 2$ were noted, until a M_w 4.1 earthquake occurred near the wells in early 2010. Aftershocks of this event continued sporadically through 2010 and into mid-2011. This decaying sequence was shattered by a M_w 5.0 earthquake on 5 November 2011, followed 20 hours later by the M_w 5.7 mainshock. With the initiating point of the November sequence within 1.5 km of the injection wells and some earthquake hypocenters at the same depth as injection, the potential for a causal connection between injection and the earthquakes is clear. The long delay between the start of injection and the earthquakes, however, deviates from the pattern seen in other documented cases of injection-induced seismicity, such as the 2011 Youngstown, Ohio, earthquake where there was, at most, a few months of delay before induced seismicity began. In the Oklahoma case, years of injection may have been needed to raise the pore pressure above the preproduction level in this depleted oil field before fault strength was exceeded (*16*).

Much of the concern about earthquakes and fracking centers on the injection of wastewater, composed of flowback fluids and coproduced formation brine in deep wells, and not on fracking itself. Wastewater disposal appears to have induced both the 2011 central Arkansas earthquake (*13*) and the 2011 Youngstown, Ohio, earthquake (*12*), as mentioned above. Unprecedented levels of seismicity have also been seen in the Barnett Shale in north central Texas, where commercial development of shale gas was pioneered. Since development began in late 1998, nine earthquakes of $M \geq 3$ occurred, compared with none in the preceding 25 years. A notable sequence occurred in the Dallas–Fort Worth area from October 2008 through May 2009. A detailed investigation of this sequence concluded that the earthquakes were most probably caused by disposal of shale gas wastewater in a UIC class II disposal well at the Dallas/Fort Worth International Airport (*28*), although as with the Oklahoma earthquake, not all investigators agree that the case is proven (*29*). Because routine earthquake reporting in the region is incomplete for events of $M < 3$, the passage of the USArray Transportable Array through the region over an 18 month period in 2009–2011 made it possible to improve magnitude completeness to M 1.5 and location accuracy by several fold. Epicenters for the most reliable locations were clustered in eight groups, all within 3 km of high-rate (>25,000 m^3/month) wastewater-injection wells (*18*). These results suggest that the injection rate, as well as the total volume of injection, may be a predictor of seismic potential.

Lessons from Three Case Studies of Deep, High-Volume Injection

Conclusions about the cause of many of the recent earthquakes suspected of being induced by injection are complicated by incomplete information on the hydrogeology, the initial state of stress and pore pressure, the pumping history of the well(s), and where pressure changes are being communicated at depth. Routine earthquake locations with uncertainties of 5 to 10 km and a high magnitude-detection threshold are of limited use. Three particularly well-documented cases of injection-induced seismicity from Colorado illustrate what can be learned when more is known about the pre-injection stress state and seismicity, as well as the injection history.

Rocky Mountain Arsenal

In 1961, a deep injection well was drilled at the Rocky Mountain Arsenal (RMA) northeast of Denver, Colorado, to dispose of hazardous chemicals produced at this defense plant (*30*). Within several months of the start of routine injection in the 3.6-km-deep well in March 1962, residents of the northeastern Denver area began to report earthquakes, and events registered on two nearby seismic stations. Between the start of injection and its termination in February 1966, a total of 13 earthquakes with body wave magnitudes (m_b) 4 and larger occurred. The following year, the three largest of the Denver earthquakes occurred, including the M_w 4.8 event on 9 August 1967 that caused minor structural damage near the epicenter. By this time, the earthquakes had migrated as far as 10 km from the injection point (*31*). Hydrologic modeling showed that the migrating seismicity would track a critical pressure front of 3.2 MPa (*32*). Although declining, earthquake activity continued for the next two decades, including a m_b 4.3 earthquake on 2 April 1981. The RMA earthquakes demonstrate how the diffusion of pore pressure within an ancient fault system can initiate earthquakes many kilometers from the injection point, delayed by months or even years after injection ceased.

Rangely

The insights gained from RMA led to the suggestion that earthquakes could be controlled by modulating the fluid pressure in the fault, according to the effective-stress relation (*19*). In 1969, the U.S. Geological Survey (USGS) began an experiment to test the effective-stress hypothesis in the Rangely oil field in northwestern Colorado (*20*). Water injected into the reservoir under high pressure had been used to enhance oil production at Rangely since 1957. The operator, Chevron Oil Company, gave USGS permission to regulate the fluid pressure in a portion of the field that was known to be seismically active. Laboratory measurements of the coefficient of friction on core samples of the reservoir rocks and in situ determination of the state of stress led to the prediction that a critical fluid pressure of 25.7 MPa would be required to induce earthquakes. Two cycles of fluid injection and withdrawal were conducted between 1969 and 1973. When the pressure in a monitoring well exceeded the target pressure, earthquake activity increased; when pressure was below the threshold, earthquake activity decreased. In particular, the earthquake activity ceased within 1 day of the start of backflow in May 1973, providing strong evidence that the rate of seismicity could be controlled by adjusting the pore pressure at the depth where earthquakes initiate, if stress conditions and the strength of the faulted rock mass were known. The rapid response of seismicity at the onset of backflow also emphasized the importance of understanding the geohydrology and, in particular, the importance of hydraulically conductive faults and fractures for transmitting pore pressure within the system.

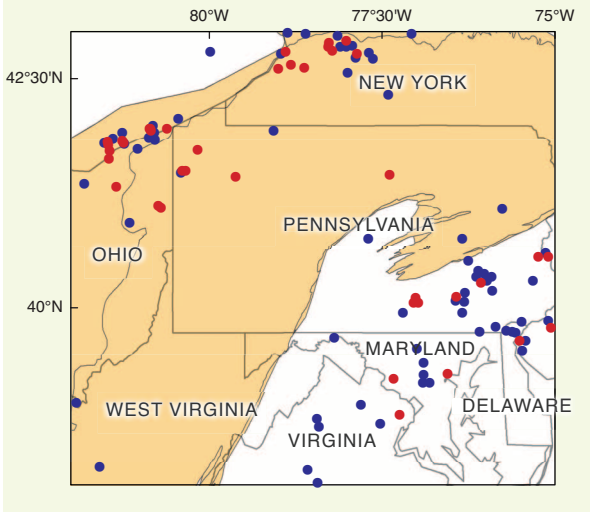


Fig. 4. Seismicity of Pennsylvania and surrounding regions, 1970–2012. Shading indicates areas underlain by deposits of the Marcellus Shale. Blue dots, earthquakes before 2005; red dots, after 2005. Seismicity was determined by the Lamont Doherty Earth Observatory (*45*).

Paradox Valley

An ongoing fluid-injection project has been under way since 1996 in Paradox Valley in southwestern Colorado, where the saline shallow water table is being suppressed by pumping to prevent salt from entering the Dolores River as it crossed the valley and, eventually, the Colorado River further downstream (*33*). In its natural state, the Dolores River picks up salt from the groundwater as it crosses Paradox Valley. After extensive study of alternatives, the U.S. Bureau of Reclamation determined that high-pressure injection of brine into a deep disposal well (UIC class V) provided the best method for reducing the salinity of the Dolores River. Injection occurs in a tight, but highly fractured dolomitic limestone with a fracture-dominated porosity of less than 6% located 4.3 km

below land surface. To date, more than 7×10^6 m^3 of brine have been injected. One operational objective, based on both the RMA and Rangely experiences, was the need to minimize the magnitude of earthquakes induced by injection.

A local seismic network was established in 1985 to determine background levels of seismicity before the drilling of the well and initial injection tests. Between 1985 and June 1996, only three tectonic earthquakes were detected within 15 km of the well and just 12 within 35 km (*33*). However, hundreds of earthquakes were induced during injection tests conducted between 1991 and 1995. Most of these earthquakes were concentrated within 1 km of the injection point, although a few were located 3 to 4 km from this site. All events were below *M* 3. The occurrence of induced earthquakes is not notable here, as injection required a bottom hole pressure in excess of the hydraulic fracture pressure of 70 MPa.

High injection pressure was needed to keep pace with the disposal requirements; consequently, induced earthquakes were expected when disposal operations went into production in 1996. Continuous monitoring of injection pressures and volumes, along with seismicity, is being conducted to insure the safe operation of the project. During the first few years of operations, several of the induced earthquakes exceeded *M* 3, necessitating changes in injection procedures in an attempt to limit the maximum magnitude. The dimension of the activated zone also grew, with earthquakes as far as 8 km from the injection point appearing within a year and events to beyond 12 km several years later (Fig. 5). Because seismicity rapidly abated after each injection test, it was hypothesized that occasional shutdowns of 20 days would allow the fluid pressure to equilibrate, reducing the potential for larger events (*33*). By itself, this procedure proved inadequate, as a *M* 4.3 event was induced in May 2000.

After this earthquake, a new procedure was introduced in 2000 that involved periodic 20-day shutdowns and a 33% reduction in the injection volume, which initially reduced the required bottom hole pressure to 78 MPa. Over the following decade, the pressure required to inject that volume steadily increased to more than 84 MPa in 2012, drawing the revised strategy into question, as a steadily increasing injection pressure is not sustainable in the long term. On 24 January

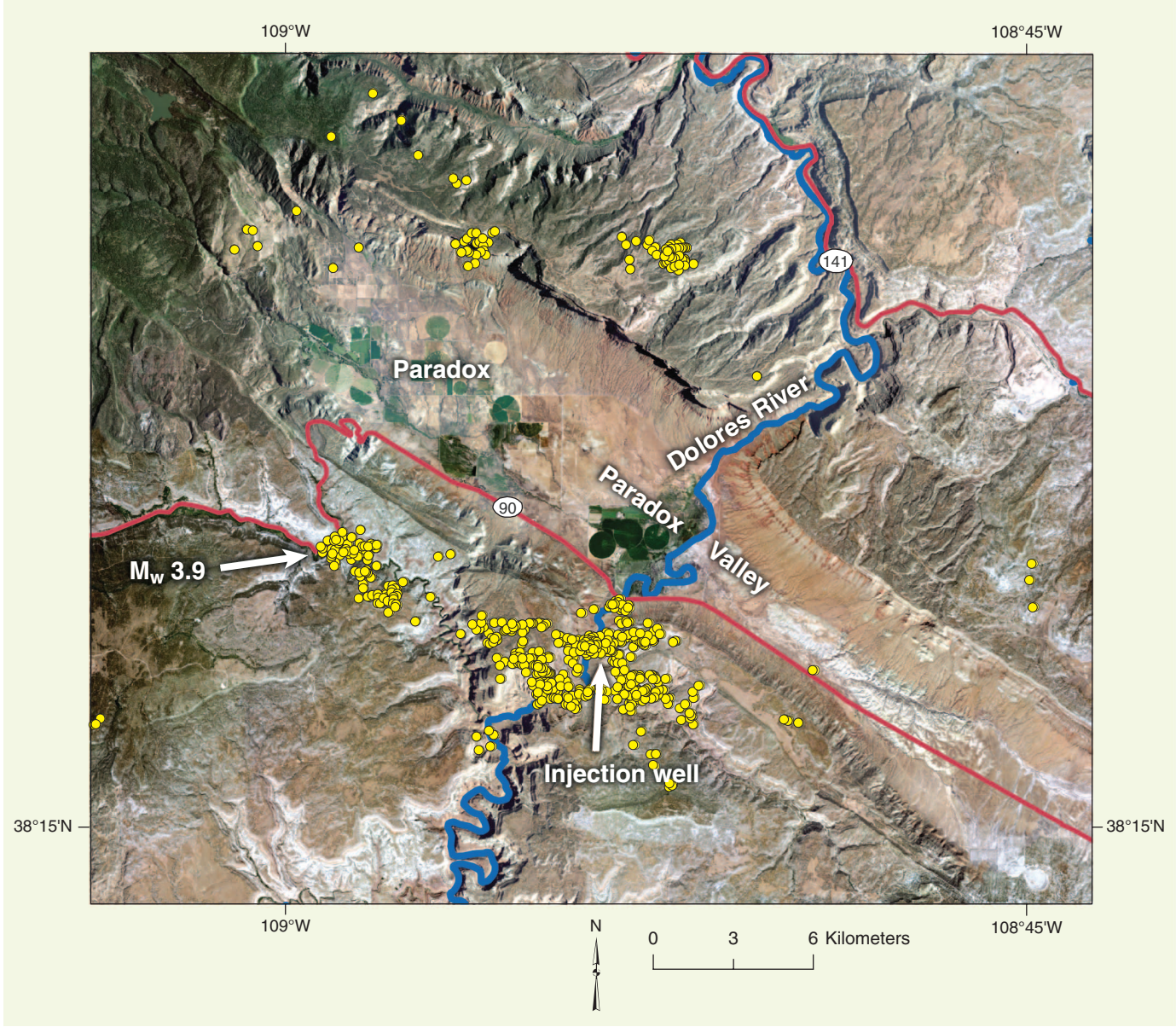


Fig. 5. Seismicity near Paradox Valley, Colorado. The U.S. Bureau of Reclamation extracts saline groundwater from shallow wells where the Dolores River crosses Paradox Valley to prevent its entry into the Colorado River system. Since 1996, the brine has been disposed of by injection into a 4.3-km-deep UIC class V well. Injection has induced more than 1500 earthquakes with $M \geq 1$, including the M_w 3.9 earthquake on 25 January 2013, which was located 8 km northwest of the well.

2013, a M_w 3.9 earthquake occurred 8 km northwest of the well in a previously active cluster, causing strong shaking in the town of Paradox, Colorado (Fig. 5). As a consequence, injection was halted for 12 weeks before restarting at a reduced rate. The Paradox Valley experience illustrates how long-term, high-volume injection can lead to the continued expansion of the seismically activated region and the triggering of large-magnitude events many kilometers from the injection well more than 15 years after observation of the initial seismic response. This case study also illustrates the challenges for managing the risk once seismicity has been induced.

Other Causes of Induced Earthquakes

According to the effective-stress model described above, earthquakes can be induced by either reducing the effective normal stress or raising the shear stress (*3*–*5*). It has been known for decades that large reservoirs can induce earthquakes either from the effect of the elastic load of the reservoir or by diffusion of elevated pore pressure (*34*). Well-known examples include the deadly 1967 M 6.3 earthquake in Koyna, India (*35*). Yet, establishing a causal connection can be difficult when natural seismicity occurs nearby. For example, the debate about the role of the Zipingpu reservoir in triggering the M_w 7.9 2005 Wenchuan, China, earthquake may never be resolved (*36*, *37*). What is clear, however, is that deep reservoirs in tectonically active zones carry a real risk of inducing damaging earthquakes.

Earthquakes throughout the world are also recognized to be associated with mining, petroleum and gas production, and geothermal energy extraction. Withdrawal of large volumes of fluid or gas from a reservoir or creation of a void space in a mine may modify the state of stress sufficiently to induce earthquakes that relax the stress perturbations (*4*). Production may also release tectonic stress. The long-term pumping of groundwater may have induced the deadly M_w 5.1 earthquake in Lorca, Spain, on 11 May 2011 (*38*). Pore-pressure changes alone can also induce seismicity, such as by waterflooding for secondary recovery of oil or to maintain the fluid level in a geothermal reservoir, or when a mine is abandoned and allowed to flood (*3*, *4*). The physical connection between operational parameters such as injected volume and the seismic response can be complex. In the Salton Sea Geothermal Field, for example, the seismicity rate positively correlates with the net volume of produced fluid (extraction minus injection) rather than net injection, as would be expected if seismicity rate simply tracked pore pressure (*39*). This underscores the importance of geomechanical modeling for transferring understandings developed in one setting to others.

Hazard and Risk of Induced Earthquakes

The hazard from earthquakes depends on proximity to potential earthquake sources, their magnitudes, and rates of occurrence and is usually expressed in probabilistic terms (*1*, *40*). The U.S. National Seismic Hazard Map, for example, gives the exceedance probabilities for a variety of ground-motion measures from which the seismic design provisions in the building codes are derived (Fig. 1) (*1*). Our understanding of the hazard will evolve as new information becomes available about the underlying earthquake sources, which are ideally derived from a combination of fault-based information and historical seismicity. Accounting for the hazard of induced earthquakes, however, presents some formidable challenges.

In the current U.S. map (Fig. 1), for example, the estimated hazard in most parts of the central and eastern regions of the country derives exclusively from historical seismicity. How should increases in the earthquake rate since 2009 (Fig. 2) be incorporated in the model? Should identified or suspected induced earthquakes be treated the same as or differently than natural events? In particular, do induced earthquakes follow the same magnitude-frequency distribution models as natural earthquakes? This issue has particular importance, as the high end of the magnitude distribution, where events are infrequent, contributes disproportionately to both the hazard and risk. Although injection-induced earthquakes have done only minor damage in the United States to date (*5*), the 2011 central Oklahoma earthquake was the same magnitude as the 1986 San Salvador, El Salvador, tectonic earthquake that killed more than 1500 people, injured more than 10,000, and left 100,000 homeless (*41*). Losses on this scale are unlikely in North America and northern Europe, where a catastrophic building collapse in a M_w 5.7 earthquake is unlikely, but the same cannot be said for large portions of the world where nonductile concrete frame or unreinforced masonry buildings are prevalent. The earthquake that killed nine and caused serious damaged Lorca, Spain, was even smaller at M_w 5.1 (*40*). The heavy losses in this possibly induced earthquake resulted from the exposure of many fragile buildings to strong shaking from this very shallow-focus earthquake (*42*). This event should serve as a reminder that risk is the product of the hazard, exposure, and vulnerability.

Unknown Knowns

Ignorance of the things that we understand we should know but do not leaves us vulnerable to unintended consequences of our actions. The effective-stress model provides straightforward guidance for avoiding induced earthquakes but requires knowledge that we rarely possess of the stress state and pore pressure acting on the fault. Quantitative predictions from the model depend on knowing initial stress and pore-pressure conditions and how perturbations to those conditions due to injection will affect the surroundings. For example, pore-pressure changes in a fault kilometers from the injection point depend on the hydrologic characteristics of connecting pathways that will, in all likelihood, be poorly known. The seismic response might not take place immediately, and decades may elapse before a damaging event occurs, as illustrated by the recent Paradox Valley earthquake and possibly the central Oklahoma earthquake as well. Simply injecting water by gravity feed (pouring it down the well with no surface pressure) sounds safe enough. But if the deep aquifer system was originally underpressured and the faults were in frictional equilibrium with the stress (*2*), this apparently benign type of injection can bring faults to failure by raising the water table and, hence, the pore pressure acting on the faults.

The fact that the great majority of UIC class II injection wells in the United States appear to be aseismic, at least for earthquakes $M_w > 3$, suggests that ambient conditions in geologic formations commonly approved for disposal are far enough removed from failure that injection can be done with low risk, provided that the pressure perturbation remains confined within the intended formation. The largest injection-induced events have all involved faulting that is considerably deeper than the injection interval (*13*, *16*, *30*, *43*), suggesting that transmission of increased pressure into the basement elevates the potential for inducing earthquakes. Consequently, detection of seismicity in the vicinity of the well or changes in seismicity in the neighborhood should prompt reevaluation of the hazard.

License and operational requirements for UIC class II wells in the United States are regulated under the Safe Drinking Water Act, by the U.S. Environmental Protection Agency or by delegation of authority to state agencies. The law's provisions are primarily directed toward protection of potable aquifers by requiring injection into formations deep below and geologically isolated from drinking water sources. As such, the law focuses on well integrity, protection of impermeable barriers above the injection zone, and setting operational injection pressure limits to avoid hydraulically fracturing the well. Diffusion of pore pressure into basement faults or injection pressure that would raise critically stressed faults to failure is not considered in U.S. federal regulations. From a scientific standpoint, measuring the initial stress state and pore pressure, tracking of injection history, and careful seismic monitoring would be of great value. At present, little more is required by regulation than an estimate of the fracture pressure (not to be exceeded) and monthly reporting of total injection volume and average injection pressure. In most cases, this information is not sufficient to apply the effective-stress model or gain an understanding of the hazard posed by injection activity.

Reducing the Risk of Injection-Induced Earthquakes

How can the risk of inducing damaging earthquakes through human activity be minimized in an information-poor environment? Long-term and high-volume injection in deep wells clearly carries some risk (*18*), even though most wells are apparently aseismic (*5*). In contrast, earthquakes induced during hydraulic fracturing have lower risk because of their much smaller magnitudes. The largest fracking-induced earthquakes

(*24*, *26*) have all been below the damage threshold for modern building codes.

One approach for managing the risk of injection-induced earthquakes involves setting seismic activity thresholds that prompt a reduction in injection rate or pressure or, if seismic activity increases, further suspension of injection (*44*). Such "traffic-light" systems have been used selectively, going back to at least the RMA well pump tests in 1966–1967. The traffic-light system used in in Basel, Switzerland (*7*), did not stop the four M_w 3 earthquakes from happening but might have prevented larger events. The decision to stop injection in the Youngstown, Ohio, well, based on the seismicity (*12*) and made the day before the M_w 4.0 event, resulted in seismicity near the well declining within a month. All of these examples feature better seismic monitoring capabilities than currently exist in much of the United States or most of the rest of the world. Lowering the magnitude-detection threshold in regions where injection wells are concentrated to below M_w 2 would certainly help, as a traffic-light system using the current U.S. detection threshold of M_w 3 in many of these areas would have limited value. Improvements in the collection and timeliness of reporting of injection data to regulatory agencies would provide much-needed information on hydrologic conditions potentially associated with induced seismicity. In particular, daily reporting of volumes, peak, and mean injection pressures would be a step in the right direction, as would measurement of the pre-injection formation pressure.

Ultimately, better knowledge of the stress and pressure conditions at depth; the hydrogeolgic framework, including the presence and geometry of faults; and the location and mechanisms of natural seismicity at a few sites will be needed to develop a predictive understanding of the hazard posed by induced earthquakes. Industry, regulatory agencies, and the public are all aware that earthquakes can be induced by fluid injection. Industry needs clear requirements under which to operate, regulators must have a firm scientific foundation for those requirements, and the public needs assurance that the regulations are adequate and are being observed.

References and Notes

1. M. D. Petersen, A. D. Frankel, S. C. Harmsen, C. S. Mueller, K. M. Haller, R. L. Wheeler, R. L. Wesson, Y. Zeng, O. S. Boyd, D. M. Perkins, N. Luco, E. H. Field, C. J. Wills, K. S. Rukstales, "Documentation for the 2008 update of the United States National Seismic Hazard Map," *U.S. Geol. Surv. Open-File Rep. 2008–1128* (2008).
2. J. Townend, M. D. Zoback, How faulting keeps the crust strong. *Geology* **28**, 399–402 (2000). doi: 10.1130/0091-7613(2000)28<399:HFKTCS>2.0.CO;2
3. C. Nicholson, R. L. Wesson, "Earthquake hazard associated with deep well injection: A report to the U.S. Environmental Protection Agency," *U.S. Geol. Surv. Bull. 1951* (1990); http://pubs.usgs.gov/bul/1951/report.pdf.
4. A. McGarr, D. Simpson, L. Seeber, "Case histories of induced and triggered seismicity," *International Handbook of Earthquake and Engineering Seismology* (Academic Press, Waltham, MA, 2002), vol. 8, chap. 40.
5. Committee on Induced Seismicity Potential in Energy Technologies, *Induced Seismicity Potential in Energy Technologies* (National Research Council, Washington, DC, 2012); http://dels.nas.edu/Report/Induced-Seismicity-Potential-Energy-Technologies/13355.
6. K. F. Evans, A. Zappone, T. Kraft, N. Deichmann, F. Moia, A survey of the induced seismic responses to fluid injection in geothermal and CO_2 reservoirs in Europe. *Geothermics* **41**, 30–54 (2012). doi: 10.1016/j.geothermics.2011.08.002
7. N. Deichmann, D. Giardini, Earthquakes induced by the stimulation of an enhanced geothermal system below Basel (Switzerland). *Seismol. Res. Lett.* **80**, 784–798 (2009). doi: 10.1785/gssrl.80.5.784
8. T. van Eck, F. Goutbeek, H. Haak, B. Dost, Seismic hazard due to small-magnitude, shallow-source induced earthquakes in The Netherlands. *Eng. Geol.* **87**, 105–121 (2006). doi: 10.1016/j.enggeo.2006.06.005
9. J. Tagliabue, "Parts of low country are now quake country," *New York Times*, 26 March 2013, p. A6.
10. F. L. Vernon, L. Astiz, Seismicity in the mid-continental U.S. as recorded by the Earthscope USArray Transportable Array. *Geol. Soc. Am. Abstr. Programs* **44**, 511 (2012).
11. "Technical report: Central and eastern United States seismic source characterization for nuclear facilities," Appendix B (Electric Power Research Institute, Palo Alto, CA; U.S. Department of Energy and U.S. Nuclear Regulatory Commission, Washington, DC; 2012).
12. W.-Y. Kim, Induced seismicity associated with fluid injection into a deep well in Youngstown, Ohio. *J. Geophys. Res.* 10.1002/jgrb.50247 (2013).
13. S. Horton, Disposal of hydrofracking waste fluid by injection into subsurface aquifers triggers earthquake swarm in central Arkansas with potential for damaging earthquake. *Seismol. Res. Lett.* **83**, 250–260 (2012). doi: 10.1785/gssrl.83.2.250
14. S. D. Davis, W. D. Pennington, Induced seismic deformation in the Cogdell oil field of west Texas. *Bull. Seismol. Soc. Am.* **79**, 1477–1495 (1989).
15. W. D. Pennington, S. D. Davis, The evolution of seismic barriers and asperities caused by the depressuring of fault planes in oil and gas fields of south Texas. *Bull. Seismol. Soc. Am.* **76**, 939–948 (1986).
16. K. M. Kerenan, H. M. Savage, G. A. Abers, E. S. Cochran, Potentially induced earthquakes in Oklahoma, USA: Links between wastewater injection and the 2011 M_w 5.7 earthquake sequence. *Geology* **41**, 699–702 (2013). doi: 10.1130/G34045.1
17. W. A. Brown, C. Frohlich, Investigating the cause of the 17 May 2012 M 4.8 earthquake near Timpson, east Texas (abstr.). *Seismol. Res. Lett.* **84**, 374 (2013).
18. C. Frohlich, Two-year survey comparing earthquake activity and injection-well locations in the Barnett Shale, Texas. *Proc. Natl. Acad. Sci. U.S.A.* **109**, 13934–13938 (2012). doi: 10.1073/pnas.1207728109; pmid: 22869701
19. M. K. Hubbert, W. W. Rubey, Role of fluid pressure in mechanics of overthrust faulting. *Geol. Soc. Am. Bull.* **70**, 115–206 (1959). doi: 10.1130/0016-7606(1959)70[115:ROFPIM]2.0.CO;2
20. C. B. Raleigh, J. H. Healy, J. D. Bredehoeft, An experiment in earthquake control at Rangely, Colorado. *Science* **191**, 1230–1237 (1976). doi: 10.1126/science.191.4233.1230; pmid: 17737698
21. C. Barton, D. Moos, K. Tezuka, Geomechanical wellbore imaging: Implications for reservoir fracture permeability. *AAPG Bull.* **93**, 1551–1569 (2009). doi: 10.1306/06180909030
22. W. H. Bakun *et al.*, Implications for prediction and hazard assessment from the 2004 Parkfield earthquake. *Nature* **437**, 969–974 (2005). doi: 10.1038/nature04067; pmid: 16222291
23. N. J. van der Elst, H. M. Savage, K. M. Keranen, G. A. Abers, Enhanced remote earthquake triggering at fluid-injection sites in the midwestern United States. *Science* **341**, 164–167 (2013).
24. A. Holland, Earthquakes triggered by hydraulic fracturing in south-central Oklahoma. *Bull. Seismol. Soc. Am.* **103**, 1784–1792 (2013).
25. C. A. Green, P. Styles, "Preese Hall shale gas fracturing: Review and recommendations for induced seismicity mitigation" (2012); www.gov.uk/government/uploads/system/uploads/attachment_data/file/15745/5075-preese-hall-shale-gas-fracturing-review.pdf.
26. "Investigation of observed seismicity in the Horn River Basin" (BC Oil and Gas Commission, Victoria, British Columbia, Canada, 2012); www.bcogc.ca/node/8046/download?documentID=1270.
27. G. R. Keller, A. Holland, "Oklahoma Geological Survey evaluation of the Prague earthquake sequence of 2011" (Oklahoma Geological Survey, Norman, OK, 2013); www.ogs.ou.edu/earthquakes/OGS_PragueStatement201303.pdf.
28. C. Frohlich, C. Hayward, B. Stump, E. Potter, The Dallas–Fort Worth earthquake sequence: October 2008 through May 2009. *Bull. Seismol. Soc. Am.* **101**, 327–340 (2011). doi: 10.1785/0120100131
29. E. Janská, L. Eisner, Ongoing seismicity in the Dallas-Fort Worth area. *Leading Edge* **31**, 1462–1468 (2012). doi: 10.1190/tle31121462.1
30. J. H. Healy, W. W. Rubey, D. T. Griggs, C. B. Raleigh, The Denver earthquakes. *Science* **161**, 1301–1310 (1968). doi: 10.1126/science.161.3848.1301; pmid: 17831340
31. R. B. Herrmann, S.-K. Park, The Denver earthquakes of 1967-1968. *Bull. Seismol. Soc. Am.* **71**, 731–745 (1981).
32. P. A. Hsieh, J. S. Bredehoeft, A reservoir analysis of the Denver earthquakes: A case of induced seismicity. *J. Geophys. Res.* **86**, 903–920 (1981). doi: 10.1029/JB086iB02p00903
33. J. Ake, K. Mahrer, Deep-injection and closely monitored induced seismicity at Paradox Valley, Colorado. *Bull. Seismol. Soc. Am.* **95**, 664–683 (2005). doi: 10.1785/0120040072
34. D. W. Simpson, W. S. Leith, Two types of reservoir-induced seismicity. *Bull. Seismol. Soc. Am.* **78**, 2025–2040 (1988).
35. H. Gupta, A review of recent studies of triggered earthquakes by artificial water reservoirs with special emphasis on earthquakes in Koyna, India. *Earth Sci. Rev.* **58**, 279–310 (2002). doi: 10.1016/S0012-8252(02)00063-6
36. S. Ge, M. Liu, N. Lu, J. W. Godt, N. Luo, Did the Zipingpu Reservoir trigger the 2008 Wenchuan earthquake? *Geophys. Res. Lett.* **36**, L20315 (2009). doi: 10.1029/2009GL040349
37. K. Deng *et al.*, Evidence that the 2008 Mw 7.9 Wenchuan earthquake could not have been induced by the Zipingpu Reservoir. *Bull. Seismol. Soc. Am.* **100**, 2805–2814 (2010). doi: 10.1785/0120090222
38. P. J. González, K. F. Tiampo, M. Palano, F. Cannavó, J. Fernández, The 2011 Lorca earthquake slip distribution controlled by groundwater crustal unloading. *Nat. Geosci.* **5**, 821–825 (2012). doi: 10.1038/ngeo1610
39. E. E. Brodsky, L. J. Lajoie, Anthropogenic seismicity rates and operational parameters at the Salton Sea Geothermal Field. *Science* 10.1126/science.1239213 (2013). doi: 10.1126/science.1239213
40. R. K. McGuire, "Seismic hazard and risk analysis," *Earthquake Engineering Research Institute Monograph MNO-10* (2004).
41. D. H. Harlow, R. A. White, The San Salvador earthquake of 10 October 1986 and its historical context. *Bull. Seismol. Soc. Am.* **83**, 1143–1154 (1993).
42. J.-P. Avouac, Earthquakes: Human-induced shaking. *Nat. Geosci.* **5**, 763–764 (2012). doi: 10.1038/ngeo1609
43. J. L. Rubinstein, W. L. Ellsworth, The 2001 - present triggered seismicity sequence in the Raton basin of southern Colorado/Northern New Mexico. *Seismol. Res. Lett.* **84**, 374 (2013).
44. M. D. Zoback, Managing the seismic risk posed by wastewater disposal. *Earth Magazine* **57**, 38–43 (2012).
45. www.ldeo.columbia.edu/LCSN/index.php.

Acknowledgments: This paper is a contribution of the USGS's John Wesley Powell Center Working Group on Understanding Fluid Injection Induced Seismicity. I thank L. Block, C. Frohlich, S. Hickman, W. Leith, A. McGarr, J. Rubinstein, and an anonymous reviewer for insightful comments during the development of this paper.

10.1126/science.1225942

8,300,000 Metric Tons **of Carbon Dioxide (CO_2) equivalent**

This is equivalent to greenhouse gas emissions from:

1,788,395 gasoline-powered passenger vehicles driven for one year
<https://epa.gov/energy/greenhouse-gases-equivalencies-calculato-calculations-and-references#vehicles>

20,602,306,153 miles driven by an average gasoline-powered passenger vehicle
<https://epa.gov/energy/greenhouse-gases-equivalencies-calculato-calculations-and-references#miles>

This is equivalent to CO_2 emissions from:

933,948,464 gallons of gasoline consumed
<https://epa.gov/energy/greenhouse-gases-equivalencies-calculator-calculations-and-references#gasoline>

815,324,165 gallons of diesel consumed
<https://epa.gov/energy/greenhouse-gases-equivalencies-calculator-calculations-and-references#diesel>

9,183,196,553 pounds of coal burned
<https://epa.gov/energy/greenhouse-gases-equivalencies-calculator-

calculations-and-references#lbscoal>

109,876 **tanker trucks' worth of gasoline**

<https://epa.gov/energy/greenhouse-gases-equivalencies-calculator-calculations-and-references#tankers>

1,045,493 **homes' energy use for one year**

<https://epa.gov/energy/greenhouse-gases-equivalencies-calculator-calculations-and-references#houseenergy>

1,614,969 **homes' electricity use for one year**

<https://epa.gov/energy/greenhouse-gases-equivalencies-calculator-calculations-and-references#houseelec>

45,830 **railcars' worth of coal burned**

<https://epa.gov/energy/greenhouse-gases-equivalencies-calculato calculations-and-references#railcars>

19,216,255 **barrels of oil consumed**

<https://epa.gov/energy/greenhouse-gases-equivalencies-calculator-calculations-and-references#oil>

338,928,280 **propane cylinders used for home barbeques** <https://epa.gov/energy/greenhouse-gases-equivalencies-calculator-calculations-and-references#propane>

2.2 **coal-fired power plants in one year** <https://epa.gov/energy/greenhouse-gases-equivalencies-calculator-calculations-and-references#coalplant>

20.9 **natural gas-fired power plants in one year** <https://epa.gov/energy/greenhouse-gases-equivalencies-calculator-calculations-and-references#gasplant>

1,009,633,964,2 **number of smartphones charged** <https://epa.gov/energy/greenhouse-gases-equivalencies-calculator-calculations-and-references#smartphones>

This is equivalent to greenhouse gas emissions avoided by:

2,871,972 **tons of waste recycled instead of landfilled** <https://epa.gov/energy/greenhouse-gases-equivalencies-calculator-calculations-and-references#recycle>

410,282 **garbage trucks of waste recycled instead of landfilled** <https://epa.gov/energy/greenhou gases-equivalencies-calculator-calculations-and-references#gtrucks>

359,270,800 **trash bags of waste recycled instead of landfilled** <https://epa.gov/energy/greenhouse gases-equivalencies-calculator-calculations-and-references#trash>

2,256 **wind turbines running for a year** <https://epa.gov/energy/greenhouse-gases-equivalencies-calculator-calculations-and-references#wind>

314,578,404 **incandescent lamps switched to LEDs** <https://epa.gov/energy/greenhouse-gases-equivalencies-calculator-calculations-and-references#lights>

This is equivalent to carbon sequestered by:

137,241,214 **tree seedlings grown for 10 years** <https://epa.gov/energy/greenhouse-gases-equivalencies-calculator-calculations-and-references#seedlings>

9,822,514 **acres of U.S. forests in one year**

<https://epa.gov/energy/greenhouse-gases-equivalencies-calculator-calculations-and-references#pineforests>

55,984 **acres of U.S. forests preserved from conversion to cropland in one year**

<https://epa.gov/energy/greenhouse-gases-equivalencies-calculator-calculations-and-references#deforestation>

label // // //

Controlling Air Pollution from the Oil and Natural Gas Industry

CONTACT US <https://epa.gov/controlling-air-pollution-oil-and-natural-gas-industry/forms/contact-us-about-controlling-air>

Basic Information about Oil and Natural Gas Air Pollution Standards

The oil and natural gas industry includes a wide range of operations and equipment, from wells to natural gas gathering lines and processing facilities, to storage tanks, and transmission and distribution pipelines.

The industry is a significant source of emissions of methane <https://epa.gov/ghgemissions/overview-greenhouse-gases#methane>, a potent greenhouse gas with a global warming potential more than 25 times that of carbon dioxide.

It also is the largest industrial source of emissions of volatile organic compounds (VOCs), a group of chemicals that contribute to the formation of ground-level ozone (smog) <https://epa.gov/ozone-pollution/health-effects-ozone-pollution>. Exposure to ozone is linked to a wide range of health effects, including aggravated asthma, increased emergency room visits and hospital admissions, and premature death.

In addition to helping form ozone, VOC emissions from the oil and gas industry include air toxics <https://epa.gov/haps> such as benzene, ethylbenzene, and n-hexane, also come from this industry. Air toxics are pollutants known, or suspected of causing cancer and other serious health effects.

Oil and Natural Gas Air Standards Home <https://epa.gov/controlling-air-pollution-oil-and-natural-gas-industry>

Basic Information

Actions and Notices <https://epa.gov/controlling-air-pollution-oil-and-natural-gas-industry/actions-and-notices-about-oil-and-natural>

Implementation <https://epa.gov/controlling-air-pollution-oil-and-natural-gas-industry/implementation-oil-and-natural-gas-air>

Contact Us <https://epa.gov/controlling-air-pollution-oil-and-natural-gas-industry/forms/contact-us-about-controlling-air> to ask a question, provide feedback, or report a problem.

LAST UPDATED ON SEPTEMBER 29, 2022


UNITED STATES
ENVIRONMENTAL PROTECTION AGENCY

Discover.

Accessibility Statement <https://epa.gov/accessibility/epa-accessibility-statement>

Budget & Performance <https://epa.gov/planandbudget>

Contracting <https://epa.gov/contracts>

EPA www Web Snapshot <https://epa.gov/utilities/wwwepagov-snapshots>

Grants <https://epa.gov/grants>

No FEAR Act Data <https://epa.gov/ocr/whistleblower-protections-epa-and-how-they-relate-non-disclosure-agreements-signed-epa>

Plain Writing <https://epa.gov/web-policies-and-procedures/plain-writing>

Privacy <https://epa.gov/privacy>

Privacy and Security Notice <https://epa.gov/privacy/privacy-and-security-notice>

Connect.

Data.gov <https://www.data.gov/>

Inspector General <https://epa.gov/office-inspector-general/about-epas-office-inspector-general>

Jobs <https://epa.gov/careers>

Newsroom <https://epa.gov/newsroom>

Open Government <https://epa.gov/data>

Regulations.gov <https://www.regulations.gov/>

Subscribe <https://epa.gov/newsroom/email-subscriptions-epa-news-releases>

USA.gov <https://www.usa.gov/>

White House <https://www.whitehouse.gov/>

Ask.

Contact EPA <https://epa.gov/home/forms/contact-epa>

EPA Disclaimers <https://epa.gov/web-policies-and-procedures/epa-disclaimers>

Hotlines <https://epa.gov/aboutepa/epa-hotlines>

Hotlines <https://epa.gov/aboutepa/epa-hotlines>

FOIA Requests <https://epa.gov/foia>

Frequent Questions <https://epa.gov/home/frequent-questions-specific-epa-programstopics>

Follow.

Ecotoxicology (2020) 29:1399–1408
https://doi.org/10.1007/s10646-020-02255-8

Ultraviolet-assisted oiling assessment improves detection of oiled birds experiencing clinical signs of hemolytic anemia after exposure to the Deepwater Horizon oil spill

Jesse A. Fallon[1] · Eric P. Smith[2] · Nina Schoch[3] · James D. Paruk[4] · Evan M. Adams[5] · David C. Evers[5] · Patrick G. R. Jodice[6] · Marie Perkins[7] · Dustin E. Meattey[5] · William A. Hopkins[1]

Accepted: 11 July 2020 / Published online: 12 August 2020

Abstract

While large-scale oil spills can cause acute mortality events in birds, there is increasing evidence that sublethal oil exposure can trigger physiological changes that have implications for individual performance and survival. Therefore, improved methods for identifying small amounts of oil on birds are needed. Because ultraviolet (UV) light can be used to identify thin crude oil films in water and on substrate that are not visually apparent under normal lighting conditions, we hypothesized that UV light could be useful for detecting small amounts of oil present on the plumage of birds. We evaluated black skimmers (*Rynchops niger*), brown pelicans (*Pelecanus occidentalis*), clapper rails (*Rallus crepitans*), great egrets (*Ardea alba*), and seaside sparrows (*Ammodramus maritimus*) exposed to areas affected by the Deepwater Horizon oil spill in the Gulf of Mexico as well as from reference areas from 20 June, 2010 to 23 February, 2011. When visually assessed without UV light, 19.6% of birds evaluated from areas affected by the spill were determined to be oiled (previously published data), whereas when examined under UV light, 56.3% of the same birds were determined to have oil exposure. Of 705 individuals examined in areas potentially impacted by the spill, we found that fluorescence under UV light assessment identified 259 oiled birds that appeared to be oil-free on visual exam, supporting its utility as a simple tool for improving detection of modestly oiled birds in the field. Further, UV assessment revealed an increase in qualitative severity of oiling (approximate % of body surface oiled) in 40% of birds compared to what was determined on visual exam. Additionally, black skimmers, brown pelicans, and great egrets exposed to oil as determined using UV light experienced oxidative injury to erythrocytes, had decreased numbers of circulating erythrocytes, and showed evidence of a regenerative hematological response in the form of increased reticulocytes. This evidence of adverse effects was similar to changes identified in birds with oil exposure as determined by visual examination without UV light, and is consistent with hemolytic anemia likely caused by oil exposure. Thus, UV assessment proved useful for enhancing detection of birds exposed to oil, but did not increase detection of birds experiencing clinical signs of anemia compared to standard visual oiling assessment. We conclude that UV light evaluation can help identify oil exposure in many birds that would otherwise be identified visually as unexposed during oil spill events.

Keywords Deepwater horizon · Oil spill · Heinz bodies · Ultraviolet fluorescence · Hemolytic anemia

Supplementary information The online version of this article (https://doi.org/10.1007/s10646-020-02255-8) contains supplementary material, which is available to authorized users.

✉ Jesse A. Fallon
jfallon@vt.edu

[1] Department of Fish and Wildlife Conservation, Virginia Tech, Blacksburg, VA, USA

[2] Department of Statistics, Virginia Tech, Blacksburg, VA, USA

[3] Adirondack Center for Loon Conservation, Ray Brook, NY, USA

[4] Saint Joseph's College of Maine, Portland, ME, USA

[5] Biodiversity Research Institute, Portland, ME, USA

[6] U.S. Geological Survey South Carolina Cooperative Fish and Wildlife Research Unit, Clemson University, Clemson, SC, USA

[7] John A. Paulson School of Engineering and Applied Sciences, Harvard University, Cambridge, MA, USA

Introduction

Acute avian mortality associated with large-scale oil spills is well documented, with seabirds, waterfowl, and colonial waterbirds at particular risk (e.g., Piatt et al. 1990; Iverson and Esler 2010; Munilla et al. 2011; USFWS 2011). However, there is increasing evidence that sublethal exposure (modest external oiling that does not result in rapid mortality) can have important impacts upon individual health and may affect population dynamics, which could influence damage assessments and subsequent restoration and mitigation efforts (Seiser et al. 2000; Trust et al. 2000; Golet et al. 2002; Alonso-Alvarez et al. 2007; Harr et al. 2017; Fallon et al. 2018). Consequently, it is important not only to identify birds that have died from oil exposure, but also to identify birds with sublethal exposure to better estimate the number of birds at risk following both small- and large-scale oil spill events. However, identifying small amounts of oil on feathers can be difficult, especially in birds with dark plumage, under natural lighting conditions. Failure to detect modest oiling may result in inaccurate estimates of the number of birds exposed during oil spills events. Therefore, there is a need for simple, reliable techniques to identify small amounts of oil on birds.

Crude oil fluoresces under ultraviolet (UV) light (Burlamacchi et al. 1983; Colligan and LaManna 1993; Fingas and Brown 2014). This attribute has led to its use to improve detection of oil in several abiotic matrices. For example, UV light can be used to enhance detection of oil on or below the surface of a body of water as well as on snow and ice (Fingas and Brown 2000; Fingas and Brown 2013). Remote UV sensors are commonly used to monitor oil films associated with spills in very thin layers down to 0.1 µm (Fingas and Brown 1997; Brekke and Solberg 2005; Jha et al. 2008). Thus, we hypothesized that UV light may also be useful to detect small amounts of oil on the feathers of captured birds that might not be apparent under normal light conditions.

Birds experiencing modest exposure to crude oil can experience myriad physiological effects, including inflammation, immunosuppression, and oxidative damage to cells (Fry et al. 1986; Leighton 1986; Leighton 1995; Briggs et al. 1996; Golet et al. 2002). These sublethal effects can negatively impact growth, alter organ function, reduce reproductive success, and likely increase risk of disease (Briggs et al. 1996; Esler et al. 2000; Giese et al. 2000; Eppley and Rubega 1990; Alonso-Alvarez et al. 2007). Of the sublethal, physiological impacts resulting from avian exposure to oil spills, oxidative damage to erythrocytes and subsequent anemia are of particular interest during oil spill investigations, as such injury can be evaluated in blood samples taken from live birds. Hemolytic anemia has been demonstrated in several species of birds exposed to crude oil under both experimental (e.g., Leighton et al. 1983, 1985; Fry and Lowenstine 1985; Harr et al. 2017) and natural conditions (Yamato et al. 1996; Troisi et al. 2007; Fallon et al. 2018). Although most work has focused on severe oiling, recent evidence indicates that small amounts of visible oiling correlate with oxidative injury to erythrocytes in several species of birds (Fallon et al. 2018).

In this investigation, we evaluated the utility of UV fluorescence as a tool to identify birds with small amounts of oil on their plumage that might otherwise be missed by traditional visual oiling assessment during and in the immediate aftermath of the 2010 Deepwater Horizon oil spill in the Gulf of Mexico, USA. To achieve this objective, we determined the presence and severity of oiling (% of body surface oiled) by visual inspection under natural light (visual oiling assessment) and then again under UV light (UV oiling assessment) in black skimmers (*Rynchops niger*, BLSK), brown pelicans (*Pelecanus occidentalis*, BRPE), great egrets (*Ardea alba, GREG*), clapper rails (*Rallus crepitans*, CLRA), and seaside sparrows (*Ammodramus maritimus*, SESP). Second, we evaluated relationships between severity of visible oiling, UV oiling, and a suite of hematologic parameters characteristic of adverse effects from oil exposure in BLSK, BRPE, and GREG. We evaluated these relationships to determine whether UV oiling assessment improved detection of the number of birds experiencing adverse clinical signs compared to those detected through visual assessment alone.

Methods

Study area and focal species

Our five focal species represent a diversity of ecological niches, which could influence their relative susceptibility to oil exposure. Clapper rails and SESP are year-round residents along the Gulf Coast, inhabit salt marshes surrounded by open water, and are omnivorous, eating seeds and marine invertebrates (Post and Greenlaw 2020; Rush et al. 2020). Black skimmers, recognized as a species with declining populations (Vieira et al. 2018), were selected because of their unique surface water foraging strategy and because their nesting habits (sand and shell beaches and islands) put them at high risk for exposure to oil (Gochfeld et al. 2020). Brown pelicans eat mostly fish and are at high risk of dermal exposure to oil because they capture their food most often by diving after prey (Shields 2020). Great egrets forage for food in a wide range of habitats and are unique among the other species evaluated in this study because they are a wading bird that has diverse prey items, including fishes, insects, marine invertebrates, small mammals, reptiles, and amphibians (McCrimmon et al. 2020).

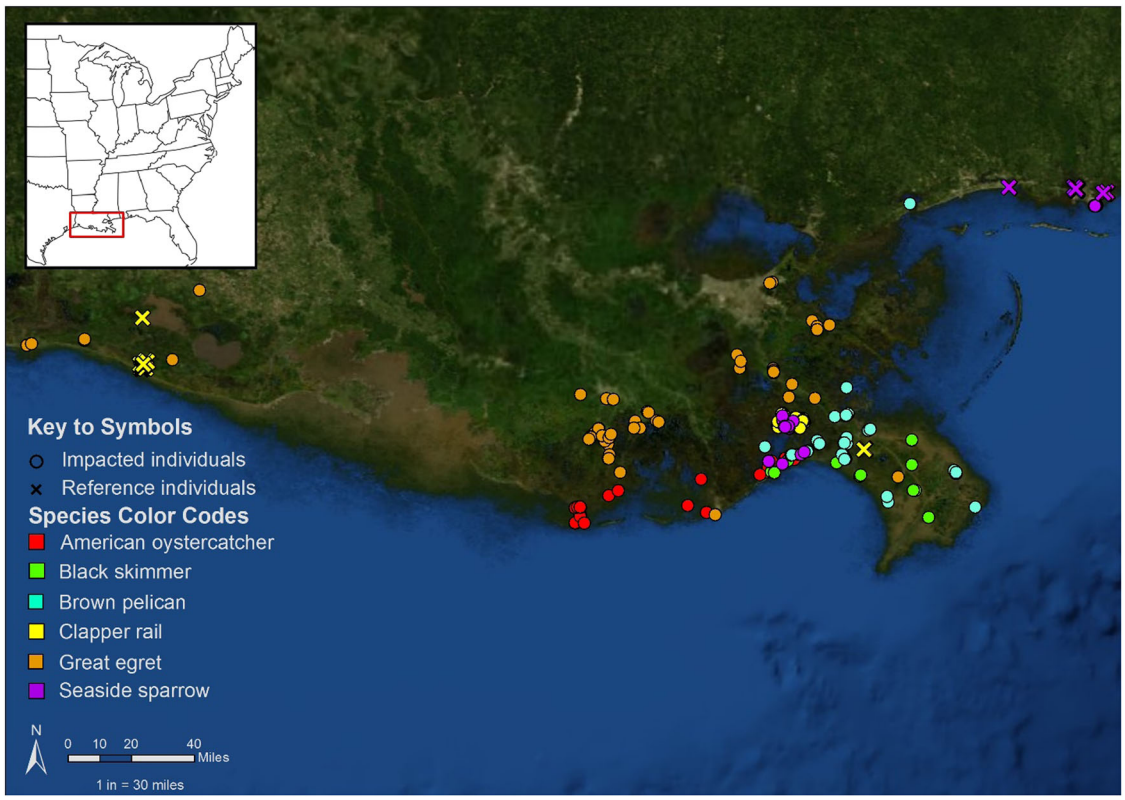


Fig. 1 Area of potential impact capture locations in Louisiana, Mississippi and Alabama for American oystercatcher, black skimmer, brown pelican, and great egret. Also shown are reference capture locations and area of potential impact capture locations for clapper rail and seaside sparrow. Because these two species maintain small home ranges, we classified *Juncus* marshes, saline *Spartina* marshes, and brackish *Phragmites* marshes with no known direct connectivity to the oil spill area as reference sites

The procedures involving animals were conducted by Biodiversity Research Institute with approval from the US Fish and Wildlife Service. We captured BLSK ($n = 120$), BRPE ($n = 66$), CLRA ($n = 100$), GREG ($n = 54$), and SESP ($n = 365$) from reference areas and areas impacted by the Deepwater Horizon spill from 20 June, 2010 until 23 February, 2011. We captured BLSK with noose mats, box traps, and cannon-nets, BRPE with noose traps, padded leghold traps, and net guns, CLRA by hand with night lighting from airboats as well as with drift fences leading to box traps, GREG with net guns, and SESP with targeted mist netting (Mills and Ryder 1979; Crozier and Gawlik 2003; Herring et al. 2008; Perkins et al. 2010).

Sites affected by the Deepwater Horizon spill included locations along coastal Louisiana, with five BRPE collected from coastal Mississippi, where exposure to oil from the Deepwater Horizon spill was likely (Fig. 1). Reference sites for BLSK, BRPE, and GREG included various locations along coastal South Carolina and Georgia, USA where no recent oiling events had been recorded (Fig. 2). Because CLRA and SESP maintain small home ranges, reference sites for these two species included saline *Juncus* marshes, saline *Spartina* marshes, and brackish *Phragmites* marshes with no visible oil along coastal Louisiana, Mississippi, and Alabama (Fig. 1). We banded all birds with leg bands appropriate for each species and released them at the capture location after we completed oiling assessments and sample collection.

Visible and UV oiling assessment

We evaluated the majority of birds captured in both oiled and reference sites for evidence of visible oiling under natural lighting conditions, and assigned a visible oiling score of none (0% of plumage affected with visible oil), trace (<5% plumage affected), light (6–20% plumage affected) moderate (21–40% plumage affected) or heavy (>40% plumage affected; see Supplementary Figs. 1, 2). We competed this examination under physical restraint appropriate for each species with wings in both extension and normal standing posture. We then placed each bird under an opaque canvas cover to block out natural light and exposed the plumage to UV light (Labino compact PH135 UV spotlight, Labino AB Solna, Sweden, 365 nm peak UV-A). We assigned a separate UV oiling score using the same five oil score categories. Thus, the same birds were categorized using two independent techniques (visible and UV oiling) to determine whether the use of a UV light source improved

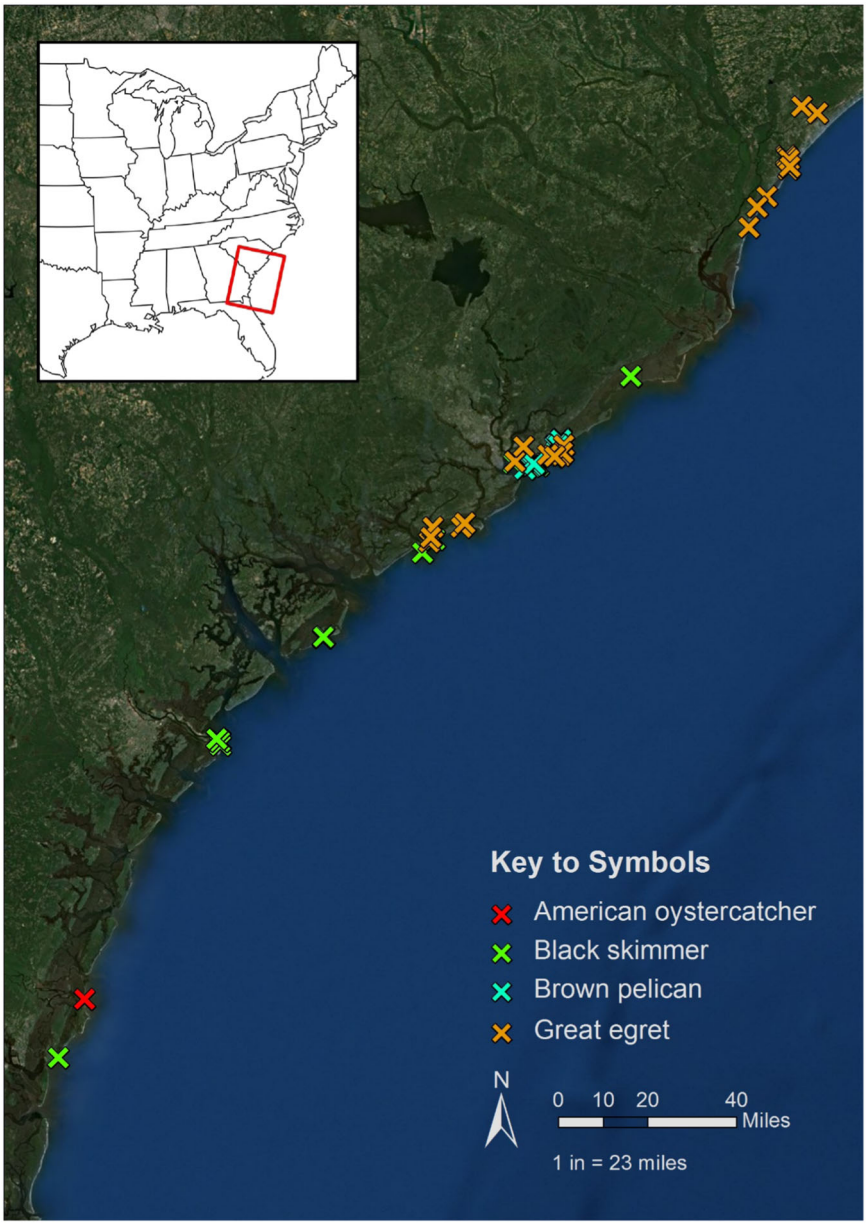


Fig. 2 Reference capture locations in South Carolina for American oystercatcher, black skimmer, brown pelican, and great egret

detection and severity of oiling (see example in Supplementary Fig. 3).

Blood collection and sample handling

We collected blood in a subset of individual BLSK, BRPE, and GREG from the medial metatarsal vein or superficial ulnar vein using a 21G or 23G butterfly catheter and lithium heparin and ethylenediaminetetraacetic acid (EDTA) vacutainers. These three species were selected for hematological analyses due to ease of blood collection and their relatively large body size. Immediately following collection, we filled two heparinized hematocrit tubes for packed cell volume (PCV) analysis. At this time, we also prepared new methylene blue-stained blood smears to quantify Heinz bodies and reticulocytes as described below. We placed remaining blood samples on ice and transferred them to the field laboratory. Once in the field laboratory, we prepared two additional EDTA-treated blood smears for complete blood cell analysis using a standard two-slide technique (Aird 2010).

Hematologic parameters

The hematological assessment methods and results, including our new methylene blue staining technique as well as reticulocyte and Heinz body identification, have been described in detail previously (Fallon et al. 2018). For

BLSK, BRPE, and GREG, we prepared new methylene blue-stained blood smears in the field after incubating for 20 min (Fallon et al. 2013). We evaluated 1000 erythrocytes under 1000X light microscopy, counting the number of cells affected by Heinz bodies as well as the number of reticulocytes (Johns et al. 2008). The individual performing these analyses (JAF) was blinded to oiling status, capture location, and results of other analyses.

We determined PCV and hemoglobin (Hb) from heparinized samples within 12 h of collection. Packed cell volume (%) was calculated using a standard hematocrit reader following centrifugation at $11{,}865 \times g$ for 5 min. Total Hb (g/dl) was quantified using a Hemocue Hb Analyzer Hb201 (Velguth et al. 2010). Red blood cell count (RBC, cells/mm^3) was estimated via standard manual methodology using a hemocytometer at a commercial laboratory (Avian and Exotics Clinical Pathology Laboratory, Wilmington, OH, USA) (Campbell 1995). Individuals performing these analyses were blinded to oiling status, capture location, and results of other analyses. Hematological results of these parameters in birds from reference sites and birds with visible oiling were first reported in Fallon et al. (2018), but are reanalyzed here in relation to the current UV oiling assessment.

Statistical analyses

We used SAS software (version 9.3 SAS Institute Inc., Cary, NC, USA) for all analyses. Where appropriate, we evaluated normality and homogeneity of variance using Shapiro–Wilk and Levene's tests, respectively. We used univariate statistical tests for physiological variable analyses, as this dataset contained missing values for one or more values in several birds (for more details, see Fallon et al. 2018). To account for the lack of independence of physiological responses compared in our univariate models, we applied a conservative $\alpha \leq 0.01$ to assess statistical significance in these models, while also noting cases where $\alpha > 0.01$ and $\alpha \leq 0.05$.

To determine the utility of UV light assessment as a tool to identify birds with small amounts of oil on the plumage that would otherwise be missed by visual oiling assessment, we used McNemar's exact test to compare the number of birds with visible oil to the number of birds with UV oiling from areas affected by the Deepwater Horizon spill within each species. Additionally, we calculated the number of birds that increased one or more category in oiling severity under the application of UV light (e.g., a bird that was categorized as light oiling under visual assessment appeared as moderate oiling under UV assessment) and compared the effect size of this change in severity using Cliff's delta (Cliff 1993, Romano et al. 2006, Macbeth et al. 2011). Cliff's delta is a measure of the degree of overlap between two populations, and ranges from −1 to +1, with ±0.147 representing a small effect (percent of non-overlap is 14.7%), ±0.33 representing a moderate effect (percent of non-overlap is 33%), and ≥±0.474 representing a large effect (percent of non-overlap is 47.4% (Cohen 1988).

To determine whether UV oiling assessment improved detection of birds experiencing adverse clinical signs compared to those detected through visual assessment alone, we first determined the effects of UV-detectable oiling on Heinz body formation, reticulocytes, PCV, Hb, and RBC using Kruskal–Wallis tests for each species with subsequent post-hoc analysis (SAS Multtest procedure, Sas Institute Inc 2011). Additionally, we used Mann–Whitney tests to compare physiologic parameters (Heinz bodies, reticulocytes, PCV, and Hb) from birds with visible oiling to the subset of birds that had no evidence of visible oiling but tested positive for oiling under UV light. Because we had physiological data on a limited number of birds with UV oil but no visible oil, we pooled species for this analysis (Heinz bodies: BLSK $n = 18$, BRPE $n = 8$, GREG $n = 5$ [$n = 31$ total]; reticulocytes: BLSK $n = 18$, BRPE $n = 8$, GREG $n = 5$, [$n = 31$ total]; PCV: BLSK $n = 19$, BRPE $n = 8$, GREG $n = 7$, [$n = 34$ total]; Hb: BLSK $n = 11$, BRPE $n = 6$, GREG $n = 7$, [$n = 24$ total]).

Results

UV oiling assessment

From our sample of 705 birds from areas impacted by the Deepwater Horizon spill, we identified 138 birds with evidence of visible oiling. However, the number of oiled birds from the same sample population increased by 259 individuals to 397 birds once we viewed them under UV light. Therefore, use of UV light increased the overall percentage of oiled birds in our sample population from 19.6 to 56.3% (Table 1, Fig. 3). Importantly, improved detection with the aid of a UV light occurred in all five species (Table 1,

Table 1 Number of individual black skimmer (BLSK), brown pelican (BRPE), clapper rail (CLRA), great egret (GREG), and seaside sparrow (SESP) from areas affected by the Deepwater Horizon oil spill with McNemar's test comparing visible oiling and UV fluorescence (*statistical significance)

	Total (*N*)	Visible oil	UV oil	Chi^2	*p* value
BLSK	120	47	92	45.00	<0.001*
BRPE	66	21	35	14.00	<0.001*
CLRA	100	14	82	65.06	<0.001*
GREG	54	38	46	8.00	0.005*
SESP	365	18	142	142.00	0.001*
Total	705	138	397		

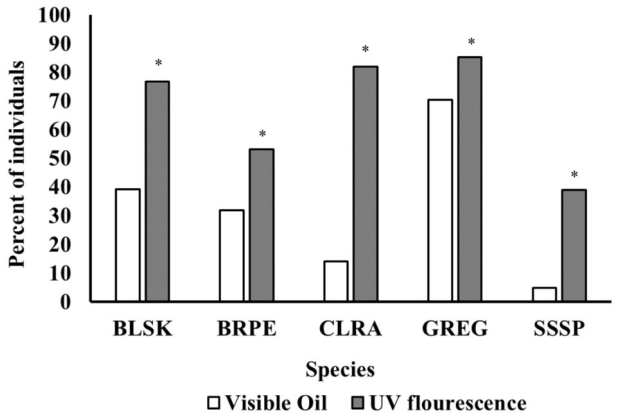


Fig. 3 Percent of black skimmer (BLSK), brown pelican (BRPE), clapper rail (CLRA), great egret (GREG), and seaside sparrow (SESP) in areas of potential impact from the Deepwater Horizon oil spill with visible oiling and oil detected under UV fluorescence. Asterisks indicate statistically significant difference ($p < 0.05$) between visible and UV detection techniques

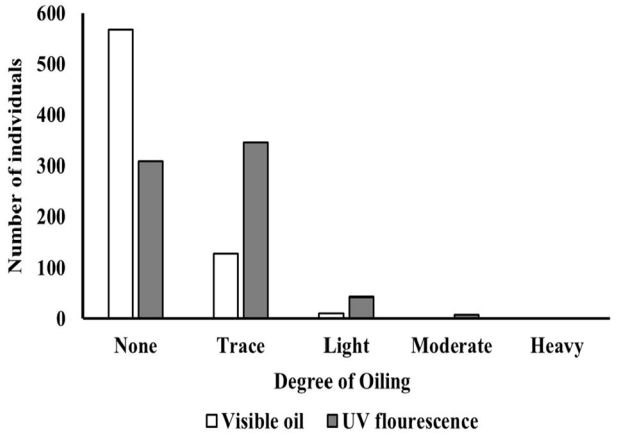


Fig. 4 Severity of visible oiling (none is 0% of plumage affected with UV oiling, trace <5% plumage affected, light 6–20% plumage affected, moderate 21–40% plumage affected or heavy >40% plumage affected) and oil detected under ultraviolet fluorescence in birds from areas affected by the Deepwater Horizon spill

Fig. 3). Additionally, we found evidence that the qualitative categorization of oiling severity increased when we evaluated birds under UV light; 40% of birds increased by at least one category (e.g., an increase from lightly to moderately oiled) after using UV compared to a standard visual exam (Fig. 4). Cliff's delta analysis revealed a moderate effect size on oiling severity with UV evaluation (delta = 0.288). No BLSK ($n = 87$), BRPE ($n = 39$), or SESP ($n = 55$) from reference locations were found to have oil on their plumage either by visual or UV assessment. We found no CPRL ($n = 30$) from reference sites with oil on standard visual assessment, but one individual was found to have trace amounts under UV assessment. In contrast, of 47 GREG from reference sites, 7 individuals had evidence of trace visible oil and 27 had trace or light UV oil.

Physiological correlates of UV oiling

The physiological responses associated with visible oiling were originally reported elsewhere (Fallon et al. 2018). Here, we focus on comparing the physiological responses detectable based on the two oiling assesment protocols. We found significantly more Heinz bodies and reticulocytes in BLSK, BRPE, and GREG with UV oiling compared to reference populations (Table 2). Additionally, we found significantly lower PCV and Hb in BRPE and GREG, as well as decreased RBC in GREG with UV oiling compared to reference populations (Table 2). Based on visual comparison of statistical outcomes of our previous study and the current study, we found that the effect of oiling on Heinz body formation, reticulocytes, PCV, Hb, and RBC counts was similar for both visual (data from Fallon et al. 2018) and UV assessment techniques (Table 3). Additionally, we found no significant difference between the mean number of Heinz bodies, reticulocytes, PCV, or Hb between birds that had only UV oiling and birds with oil apparent on both visual and UV oiling assessment (Table 4).

Discussion

It is important to understand the extent of exposure and injury to birds and other wildlife during oil spill events to develop an accurate damage assessment. While there were thousands of dead birds found in the weeks following the Deepwater Horizon spill, there were many more that were likely exposed to oil but did not immediately succumb (Peterson et al. 2003; USFWS 2011). We hypothesized that the use of hand-held UV lights could enhance the sensitivity of visible oiling assessments, because even trace amounts of oil have been shown to fluoresce (Chase et al. 2005). To test this, we evaluated 705 individuals from sites affected by the Deepwater Horizon oil spill with both visual and UV assessment. Ultraviolet assessment identified oiling on 259 individual birds (a 97% increase in detection) that appeared to be oil-free on initial visual examination. Ultraviolet assessment resulted in a significant increase in the number of individuals determined to be oiled in all species, although this effect was least pronounced in GREG (Table 1, Fig. 3). Adult GREG have exclusively white plumage, which likely makes even trace amounts of oil more apparent on visual exam. Additionally, we found that UV assessment revealed that birds had more extensive exposure than was apparent on visual assessment, with 40% of birds increasing by at least one category of oiling severity after application of the UV light (Fig. 4). Cliff's delta analysis confirmed that this effect size was statistically significant. Together, these findings suggest that UV assessment can more accurately determine the number and severity of birds exposed to oil

Table 2 Summary of the response of birds exposed to oil based on UV oiling assessment from the Deepwater Horizon oil spill

Variable	BLSK			BRPE			GREG			Percent difference
	Mean reference	Mean impacted	*p* value	Percent difference	Mean reference	Mean impacted	*p* value	Percent difference	Mean reference	Mean impacted	*p* value	
Heinz bodies	0	9.34	<0.001*	N/A	0	1.64	0.028**	N/A	0.87	8.1	<0.001*	96.44%
Reticulocytes	53.6	67.3	0.004*	25.6%	44.5	65.6	<0.001*	47.38%	54.3	69.0	0.007*	27.11%
PCV	44.7	43.1	0.137	−3.6%	50.2	43.3	<0.001*	−13.83%	46.1	37.3	<0.001*	−19.02%
Hemoglobin	17.0	16.8	0.841	−1.1%	17.4	19.2	0.006*	−10.29%	15.9	13.9	<0.001*	−12.61%
RBC	2.71	2.64	0.905	−2.6%	2.89	2.85	0.791	−1.55%	2.63	2.43	0.012**	−7.77%

Kruskal–Wallis results are reported for Heinz bodies (cells with Heinz bodies/1000 erythrocytes), reticulocytes (%), PCV (%), hemoglobin (g/dl), and RBC (cells/mm^3) found in black skimmers (BLSK), brown pelicans (BRPE), and great egrets (GREG). Percent differences calculated from the mean values from birds from reference areas (BLSK $n = 57$, BRPE $n = 32$, GREG $n = 46$) and birds from Deepwater Horizon affected (impacted) sites with UV detected oil (BLSK $n = 51$, BRPE $n = 44$, GREG $n = 51$) relative to the reference mean. Asterisk represents significance ($p \leq 0.01$) and **indicates marginal significance ($0.01 < p \leq 0.05$). N/A indicates that results were not determined for this variable in a particular bird except one GREG. Mean reference results previously published in Fallon et al. 2018)

during spill events compared to visual assessment without the aid of UV light.

We found that UV-oiled birds were experiencing adverse effects similar to those we had observed in our prior visual assessment (Fallon et al. 2018). Birds with small amounts of oil on their plumage as determined by UV evaluation had hematological changes consistent with oxidative injury to red blood cells (Tables 2, 3). The presence of Heinz bodies combined with increased reticulocytes found in oiled BLSK, BRPE, and GREG with UV oiling suggests that this method can be used to detect birds with modest oiling that may be experiencing sublethal physiological injury. Additionally, BRPE and GREG from impacted sites had decreased PCV and Hb. These three features—presence of Heinz bodies, decreased PCV or Hb, and increased reticulocytes—are indicative of oxidative injury, anemia, and a physiological regenerative response. This physiologic cascade decreases oxygen availability to tissues (Latimer et al. 2003) which can induce muscle fatigue, lethargy, decreased energy availability for metabolic processes, and adversely affect reproduction (Butler et al. 1986; Piersma et al. 1996; Walton et al. 1997; Ots et al. 1998; Hylton et al. 2006). These physiological changes have implications for survival and fitness, suggesting that sublethal physiological injury associated with modest oil exposure may have important negative long-term repercussions for individuals. Although there is no clearly established threshold for what degree of reduced erythrocyte volume leads to decreased survival, anemia in oiled birds at admission to rehabilitation facilities is correlated with higher mortality rates (Duerr et al. 2016). Our results suggest that UV assessments can be useful in identifying birds with very small amounts of oil that also have experienced adverse effects, and that these hematological changes mirror those found with visible oiling (Fallon et al. 2018).

Although all BLSK, BRPE, and SESP and all but one CPRL from reference sites had no evidence of visual or UV oil on their plumage, GREG appeared to be at increased risk of oil exposure in our reference sites. Of 47 GREG from reference sites evaluated by both visual and UV assessment, 7 birds (15%) had trace visual oiling and 27 (57%) had trace ($n = 26$) or light ($n = 1$) UV oiling. Of our study species, GREG are the only species with exclusively white plumage, which may make small amounts of oil more easily discernible. Additionally, this species is unique among those in this study, as it is a wading bird that spends a great deal of foraging time standing or slowly wading through water, and frequents man-made drainage ponds and pooled, standing water from residential, agricultural, or industrial run-off which may contain petroleum waste (Trail 2006; McCrimmon et al. 2011). Consistent with their exposure, this was the only species in which Heinz bodies were identified in the reference population. Our results suggest that further

Table 3 Summary of results from Kruskal–Wallis analysis of oiled birds as determined by visual assessment under natural lighting conditions (data from Fallon et al. 2018) compared to reference populations and UV-assisted assessment compared to reference populations

	Heinz Bodies	Reticulocytes	PCV	Hb	RBC
BLSK					
UV oiled	<0.001	0.004	0.137	0.841	0.905
Visibly oiled	<0.001	0.004	0.164	0.876	0.613
BRPE					
UV oiled	0.028	<0.001	<0.001	0.006	0.791
Visibly oiled	0.024	<0.001	<0.001	0.002	0.561
GREG					
UV oiled	<0.001	0.007	<0.001	<0.001	0.012
Visibly oiled	0.003	0.010	<0.001	<0.001	0.003

Response variables were Heinz bodies (cells with Heinz bodies/1000 erythrocytes), reticulocytes (%), packed cell volume (PCV, %), hemoglobin (Hb, g/dl), and red blood cell count (RBC, cells/mm^3) found in black skimmers (BLSK), brown pelicans (BRPE), and great egrets (GREG)

Table 4 Mann–Whitney comparison of mean number of Heinz bodies (number of cells/1000 erythrocytes), reticulocytes (%), packed cell volume (PCV, %), and hemoglobin (g/dl) between birds (pooled species including black skimmers, brown pelicans, and great egrets) that tested positive for oiling under both UV and natural (visible) light versus those that tested positive under UV light only from sites impacted by the Deepwater Horizon oil spill

	Visible and UV oil		UV oil only		
Variable	*n*	mean (±SE)	*n*	mean (±SE)	*p* value
Heinz bodies	79	8.22 (1.99)	31	2.87 (1.58)	0.355
Reticulocytes	79	69.08 (2.10)	31	64.84 (3.09)	0.597
PCV	78	40.40 (0.57)	34	40.24 (0.94)	0.730
Hemoglobin	73	14.88 (0.38)	24	15.40 (0.53)	0.320

investigation into the frequency of exposure to petroleum products in this species is warranted.

There are several limitations to consider when incorporating UV light assessment during oil spill events. First, the individual bird must be evaluated under minimal natural light, which is cumbersome with large birds. Second, although application of UV light increased the detection of oiled birds, the majority of birds that appeared to be oil-free on visual examination that were determined to be oiled under UV light application had only trace or light amounts of oil on their plumage (5–20% of plumage affected). Because of this, the severity of hematologic changes in the UV-oiled population of birds was similar to that of visibly oiled birds (Table 3, Fig. 4). Thus, UV assessment proved useful for enhancing detection of birds exposed to oil, but did not increase detection of birds experiencing clinical signs of anemia compared to standard visual assessment. Finally, there is the possibility of false positive fluorescence with naturally occurring oils. Further work in an experimental setting may help determine the frequency of false positive results.

In summary, our results demonstrate that UV assessment can identify small amounts of oil present on birds that appear oil-free on visual exam. Additionally, UV light allowed detection of oiled feathers over a larger proportion of surface area on individuals than can be seen on visual exam. Therefore, UV assessment of individual birds could be considered as an additional tool following both large and small oil spill events to help formulate a more complete damage assessment. This technique may be most useful to categorize birds with trace oiling that would otherwise be missed on visual exam, particularly in birds with dark plumage. Further, UV-oiled birds exposed to the Deepwater Horizon spill had evidence of oxidative injury to erythrocytes, decreased numbers of erythrocytes in circulation, and evidence of an erythrocytic regenerative response, similar to birds with visible oiling. These changes are consistent with formation of Heinz bodies and oxidative hemolytic anemia, a pathological abnormality caused by exposure to oil.

Acknowledgements The authors thank Pete Tuttle and Michael Hooper, for scientific advising, technical support, and assistance during this study. We are indebted to the many dedicated people who assisted with the collection of the avian samples, including Lisa Eggert who helped coordinate the collection of the BLSK samples; Lucas Savoy who coordinated the collection of CLRA, Dr. Peter Frederick and Jason Fidorra who led the collection of the GREG samples; Mike Yates and Jim Dayton who coordinated sample collection from BRPE. Additionally, BLSK, BRPE, GREG samples were processed in BRI's field laboratory by a dedicated team who provided excellent assistance, including Dr. Michelle Walsh, Dr. Lee Friedman, Ruth Valentine, Judi Ellal, Michelle Brown, Alishia Zyer, Tim Watson, and Ken Weber. Jeff Walters, Eric Hallerman, and Todd Katzner provided helpful comments that improved the manuscript. Any use of trade, firm, or product names is for descriptive purposes only and does not imply endorsement by the U.S. Government.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval The procedures involving animals were conducted by Biodiversity Research Institute with approval from the U.S. Fish and

Wildlife Service. All applicable international, national, and/or institutional guidelines for the care and use of animals were followed.

References

Alonso-Alvarez C, Munilla I, López M, Velando A (2007) Sublethal toxicity of the Prestige oil spill on yellow-legged gulls. Environ Intern 33:773–781

Aird B (2010) Clinical and hematologic manifestations of anemia. In: Feldman BF, Zinkl JG, Jain NC (eds) Schalm's veterinary hematology, 5th edn. Blackwell Publishing Ltd, Ames, Iowa, USA, p 140–142

Brekke C, Solberg AHS (2005) Oil spill detection by satellite remote sensing. Proc Spie 95:1–13

Briggs KT, Yoshida SH, Gershwin ME (1996) The influence of petrochemicals and stress on the immune system of seabirds. Regul Toxicol Pharmacol 23:145–55

Burlamacchi P, Cecchi G, Mazzinghi P, Pantani L (1983) Performance evaluation of UV sources for lidar fluorosensing of oil films. Appl Opt 22:48–53

Butler RG, Peakall DB, Leighton FA, Borthwick J, Harmon RS (1986) Effects of crude oil exposure on standard metabolic rate of Leach's storm-petrel. Condor 88:248–249

Campbell TW (1995) Avian hematology and cytology. Iowa State University Press, Ames, Iowa, USA

Chase CR, Van Bibber S, Muniz TP (2005) Development of a non-contact oil spill detection system. Oceans 2:1352–1357

Cliff N (1993) Dominance statistics: ordinal analyses to answer ordinal questions. Psychol Bull 114:494–509

Cohen J (1988) Statistical power analysis for the behavioral sciences, 2nd edn. Hillsdate, New Jersey, USA

Colligan TH, LaManna JM (1993) Using ultraviolet light to investigate petroleum-contaminated soil. Remediat J 3:193–201

Crozier GE, Gawlik DE (2003) The use of decoys as a research tool for attracting wading birds. J Field Ornithol 74:53–58

Duerr RS, Ziccardi MH, Massey JG (2016) Mortality during treatment: factors affecting the survival of oiled, rehabilitated Common Murres (*Uria aalge*). J Wildl Dis 52:495–505

Eppley ZA, Rubega MA (1990) Indirect effects of an oil spill: reproductive failure in a population of South Polar skuas following the 'Bahia Paraiso' oil spill in Antarctica. Mar Ecol Prog Ser 67:1–6

Esler D, Schmutz JA, Jarvis RL, Mulcahy DM (2000) Winter survival of adult female harlequin ducks in relation to history of contamination by the Exxon Valdez oil spill J Wildl Manag 64:839–847

Fallon JA, Hopkins WA, Fox L (2013) A practical quantification method for Heinz bodies in birds applicable to rapid response field scenarios. Environ Toxicol Chem 32:401–405

Fallon JA, Smith EP, Schoch N, Paruk JD, Adams EA, Evers DC, Jodice PG, Perkins C, Schulte S, Hopkins WA (2018) Hematological indices of injury to lightly oiled birds from the Deepwater Horizon oil spill. Environ Toxicol Chem 37:451–461

Fingas M, Brown CE (2014) Review of oil spill remote sensing. Mar Pollut Bull 83:9–23

Fingas M, Brown CE (2013) Detection of oil in ice and snow. J Mar Sci Eng 1:10–20

Fingas M, Brown C (2000) A review of the status of advanced technologies for the detection of oil in and with ice. Spill Sci Technol Bull 6:295–302

Fingas MF, Brown CE (1997) Review of oil spill remote sensing. Spill Sci Technol Bull 4:199–208

Fry M, Lowenstine LJ (1985) Pathology of Common Murres and Cassin's Auklets exposed to oil. Arch Environ Contam Toxicol 14:725–737

Fry DM, Swenson J, Addiego LA, Grau CR, Kangt A (1986) Reduced reproduction of wedge tailed shearwaters exposed to weathered Santa Barbara crude oil. Arch Environ Contam Toxicol 15:453–463

Giese M, Goldsworthy SD, Gales R, Brothers N, Hamill J (2000) Effects of the Iron Baron oil spill on little penguins (*Eudyptula minor*): breeding success of rehabilitated oiled birds. Wildl Res 27:583–591

Gochfeld M, Burger J, Lefevre KL (2020) Black Skimmer (*Rynchops niger*), version 1.0. In Birds of the World (S. M. Billerman, Editor). Cornell Lab of Ornithology, Ithaca, NY, USA. https://doi.org/10.2173/bow.blkski.01

Golet GH, Seiser PE, McGuire AD, Roby DD, Fischer JB, Kuletz KL, Irons DB, Dean TA, Jewett SC, Newman SH (2002) Long-term direct and indirect effects of the Exxon Valdez oil spill on pigeon guillemots in Prince William Sound, Alaska. Mar Ecol Prog Ser 241:287–304

Harr KE, Cunningham FL, Pritsos CA, Pritsos KL, Muthumalage T, Dorr BS, Horak KE, Hanson-Dorr KC, Dean KM, Cacela D, McFadden AK (2017) Weathered MC252 crude oil-induced anemia and abnormal erythroid morphology in double-crested cormorants (*Phalacrocorax auritus*) with light microscopic and ultrastructural description of Heinz bodies. Ecotox Environ Saf 146:29–39

Herring G, Gawlik DE, Beerens JM (2008) Evaluating two new methods for capturing large wetland birds. J Field Ornithol 79:102–110

Hylton RA, Frederick PC, De La Fuente TE, Spalding MG (2006) Effects of nestling health on postfledging survival of wood storks. Condor 108:97–106

Iverson SA, Esler D (2010) Harlequin Duck population injury and recovery dynamics following the 1989 Exxon Valdez oil spill. Ecol Appl 20:1993–2006

Jha MN, Levy J, Gao Y (2008) Advances in remote sensing for oil spill disaster management: state-of-the-art sensors technology for oil spill surveillance. Sensors 8:236–255

Johns JL, Shooshtari MP, Christopher MM (2008) Development of a technique for quantification of reticulocytes and assessment of erythrocyte regenerative capacity in birds. Am J Vet Res 89:1067–1072

Latimer KS, Mahaffey EA, Prasse KW (2003) Duncan and Prasse's veterinary laboratory medicine: clinical pathology, 4th edn. Blackwell Publishing, Ames, Iowa, USA

Leighton FA (1986) Clinical gross and histological findings in Herring Gulls and Atlantic Puffins that ingested Prudhoe Bay oil. Vet Pathol 23:254–263

Leighton FA (1995) The toxicity of petroleum oils to birds: an overview. In: Frink L, Weir KB, Smith C (eds) Wildlife and oil spills. Tri-state Bird Rescue and Research, Inc., Newark, Delaware, USA, p 10–22

Leighton FA, Lee YZ, Rahimtula AD, O'Brien PJ, Peakall DB (1985) Biochemical and functional disturbances in red blood cells of herring gulls ingesting Prudhoe Bay crude oil. Toxicol Appl Pharmacol 81:25–31

Leighton FA, Peakall DB, Butler RG (1983) Heinz-body hemolytic anemia from the ingestion of crude oil: a primary toxic effect in marine birds. Science 4599:871–873

Macbeth G, Razumiejczyk E, Ledesma RD (2011) Cliff's delta calculator: a non-parametric effect size program for two groups of observations. Univ Psychol 10:545–555

McCrimmon, DA, Ogden JC, Bancroft GT, Martínez-Vilalta A, Motis A, Kirwan GM, Boesman PFD (2020) Great Egret (A*rdea alba*), version 1.0. In Birds of the World (S. M. Billerman, Editor).

Cornell Lab of Ornithology, Ithaca, NY, USA. https://doi.org/10.2173/bow.greegr.01
Mills JA, Ryder JP (1979) Trap for capturing shore and seabirds. Bird Band 50:121–123
Munilla I, Arcos JM, Oro D, Álvarez D, Leyenda PM, Velando A (2011) Mass peak mortality of seabirds in the aftermath of the Prestige oil spill. Ecosphere 2:1–14
Ots I, Murumägi A, Horak P (1998) Haematological health state indices of reproducing great tits: methodology and sources of natural variation. Funct Ecol 12:700–707
Perkins M, King SL, Linscombe J (2010) Effectiveness of capture techniques for rails in emergent marsh and agricultural wetlands. Waterbirds 33:376–380
Peterson CH, Rice SD, Short JW, Esler D, Bodkin JL, Ballachey BE, Irons DB (2003) Long term ecosystem response to the Exxon Valdez oil spill. Science 302:2082–2086
Piatt JF, Lensink CJ, Butler W, Kendziorik M, Nysewander DR (1990) Immediate impact of the Exxon Valdez oil spill on marine birds. Auk 107:387–97
Piersma T, Everaarts TM, Jukema J (1996) Build-up of red blood cells in refueling bar-tailed godwits in relation to individual migratory quality Condor 98:363–370
Post W, Greenlaw JS (2020) Seaside Sparrow (*Ammospiza maritima*), version 1.0. In Birds of the World (P. G. Rodewald, Editor). Cornell Lab of Ornithology, Ithaca, NY, USA. https://doi.org/10.2173/bow.seaspa.01
Romano J, Kromrey JD, Coraggio J, Skowronek J, Devine L (2006) Exploring methods for evaluating group differences on the NSSE and other surveys: are the t-test and Cohen's d indices the most appropriate choices? In: Proceedings of the Annual Meeting of the Southern Association for Institutional Research, Arlington, VA, USA, pp 14–17
Rush SA, Gaines KF, Eddleman WR, Conway CJ (2020) Clapper Rail (*Rallus crepitans*), version 1.0. In Birds of the World (P. G. Rodewald, Editor). Cornell Lab of Ornithology, Ithaca, NY, USA. https://doi.org/10.2173/bow.clarai11.01
SAS Institute Inc (2011) Base SAS® 9.3 procedures guide. SAS Institute Inc, Cary, NC
Seiser PE, Duffy LK, McGuire AD, Roby DD, Golet GH, Litzow MA (2000) Comparison of pigeon guillemot, *Cepphus columba*, blood parameters from oiled and unoiled areas of Alaska eight years after the Exxon Valdez oil spill. Mar Pollut Bull 40:152–164
Shields M (2020) Brown Pelican (*Pelecanus occidentalis*), version 1.0. In Birds of the World (A. F. Poole, Editor). Cornell Lab of Ornithology, Ithaca, NY, USA. https://doi.org/10.2173/bow.brnpel.01
Trail PW (2006) Avian mortality at oil pits in the United States: a review of the problem and efforts for its solution. Environ Manag 38:532–544
Troisi GM, Borjesson L, Bexton S, Robinson I (2007) Biomarkers of polycyclic aromatic hydrocarbon (PAH)-associated hemolytic anemia in oiled wildlife. Environ Res 105:324–329
Trust KA, Esler D, Woodin BR, Stegeman JJ (2000) Cytochrome P450 1A induction in sea ducks inhabiting nearshore areas of Prince William Sound, Alaska. Mar Pollut Bull 40:397–403
[USFWS] US Fish and Wildlife Service (2011) Deepwater Horizon bird impact data from the DOI-ERDC NRDA Database 12 May 2011. http://www.fws.gov/home/dhoilspill/pdfs/Bird%20Data%20Species%20Spreadsheet%2005122011.pdf. Accessed 4 Aug 2012
Vieira BP, Furness RW, Nager RG (2018) What do we know about Black Skimmers? review on its annual-cycle and life-history main events. Ardea 106:119–130
Velguth KE, Payton ME, Hoover JP (2010) Relationship of hemoglobin concentration to packed cell volume in avian blood samples. J Avian Med Surg 24:115–121
Yamato O, IKoto I, Maede Y (1996) Hemolytic anemia in wild seaducks caused by marine oil pollution. J Wildl Dis 32:381–384
Walton RM, Brown DE, Hamar DW, Meador VP, Horn JW, Thrall MA (1997) Mechanisms of echinocytosis induced by *Crotalus atrox* venom. Vet Pathol 34:442–449

City of Long Beach Oil and Gas Extraction

April 1, 2022

This summary report describes oil and gas extraction activity occurring within the boundaries of the city of Long Beach. See the map in Figure 2 for a visual description of the study area.

Well Status: There are a total of 2,762 operational wells within the city limits of Long Beach. Active wells account for 71.3%, idle wells for 27.3%, and new wells 1.4%.

Well Type: The majority of wells are oil and gas production wells (2,031). Production from these wells is enhanced via 705 waterflood injection wells.

Location: About half of the operational well count is considered onshore (1,365), and the other half offshore (1,397). For many of the wells on the docks of the Long Beach harbor, the distinction is arbitrary. There are 1,390 operational wells located on the four THUMS/Astronaut Islands.

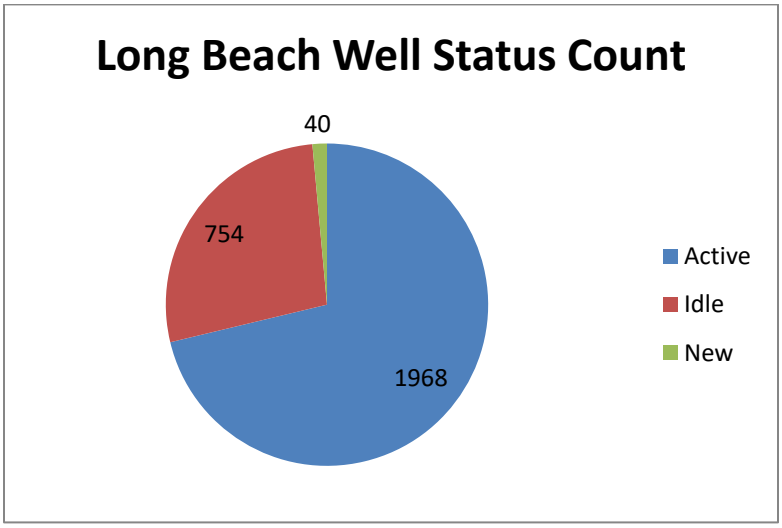


Figure 1. Chart of CalGEM oil and gas well statuses. The pie chart shows the breakdown of well statuses for operational wells within the boundaries of the city of Long Beach.

Operators: There are 92 individual operators in the City of Long Beach. The majority of wells are operated by subsidiaries of California Resources Corporation; California Resources Long Beach, Inc (88), THUMS Long Beach Co (1,562) and Tidelands Oil Production Co (768). Other operators include Signal Hill Petroleum (93), Synergy Oil &Gas LLC (61) in the Seal Beach field, Warren E&P (42) in the Wilmington Field and The Termo Company (22) in several fields.

Production: In total, the operational wells within the Long Beach city limits produced a total 893,247 barrels of crude oil/condensate in 2020. Only 16 of the 93 operators with operational wells produced oil in 2020.

Table 1. Production by operator for 2020.

Operator	2020 Production (Bbls)
THUMS Long Beach Co.	6,561,448
Tidelands Oil Production Co.	2,324,338
Signal Hill Petroleum, Inc.	258,010
California Resources Long Beach, Inc.	232,179
Synergy Oil & Gas, LLC	112,622
Warren E&P, Inc.	76,548
E & T Limited Liability Co.	38,498
The Termo Company	35,899
The Lansdale Co.	23,609
Arrowhead Operating, Inc.	8,885
Herley-Kelley LLC	3,983
P&M Oil Company, Inc.	3,920
E & B Natural Resources Management Corporation	3,681
TJ Scott Family Investments, LLC	2,164
S & C Oil Co., Inc.	2,154
Mitchell-Grossu Oil Co.	1,686

Age: The age of the wells vary widely and a distribution is difficult to generate as spud data is sparsely included in CalGEM's "AllWells" dataset. It is also difficult to pinpoint the oldest well still operating within the city limits, but records dating back to the 1930's are not uncommon.

Population: There is an estimated 463,569 people living in the City of Long Beach (U.S. Census Bureau. American Community Survey, 5-year, 2013-2019). An estimated 140,138 individuals live within 3,200' of an operational oil and gas well within the city limits. This is about 30.2% of the population. Of those, 101,498 (72.4%) identify as non-white, including Latina/Hispanic origin. This is slightly higher than the citywide average (71.7% non-white)

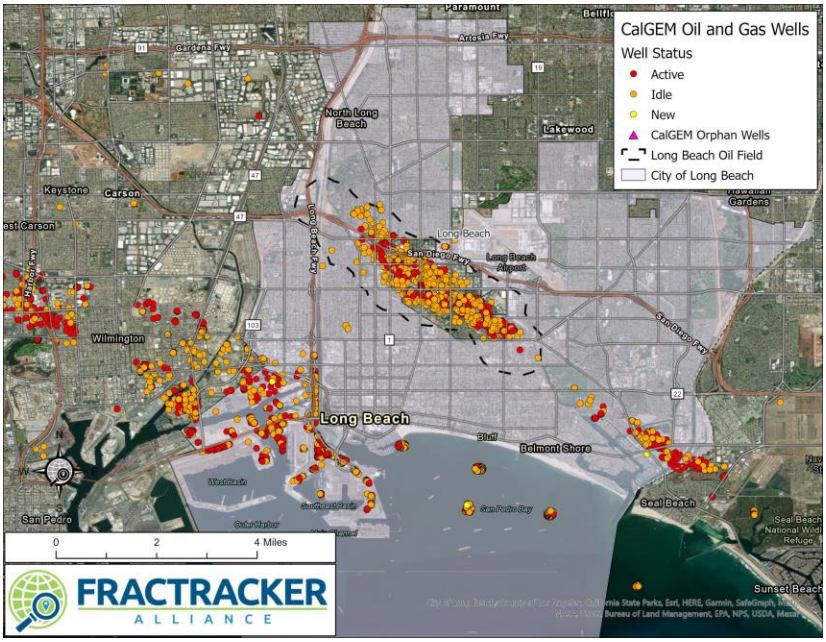


Figure 1. Map of Long Beach Oil and Gas Extraction Locations. The map of the City of Long Beach shows the locations of CalGEM's operational oil and gas wells.

Recovery Plan for the

Pacific Coast Population of the

Western Snowy Plover

(*Charadrius alexandrinus nivosus*)

Volume 1: Recovery Plan

California/Nevada Operations Office
U.S. Fish and Wildlife Service
Sacramento, California

Approved: _____

Manager, California/Nevada Operations Office,
U.S. Fish and Wildlife Service

Date: 8/13/2007

PRIMARY AUTHORS

This final recovery plan was prepared by Kelly Hornaday, Ina Pisani, and Betty Warne of our Sacramento Fish and Wildlife Office. Ruth Pratt of the Sacramento Fish and Wildlife Office coordinated preparation of the draft recovery plan and acted as Recovery Team Manager.

We gratefully acknowledge the efforts of the Pacific Coast Western Snowy Plover Recovery Team in preparing this recovery plan. Special acknowledgment is also given to Nadav Nur, Point Reyes Bird Observatory, Stinson Beach, California, for his work on the population viability analysis.

DISCLAIMER

Recovery plans delineate reasonable actions that are believed to be required to recover and/or protect listed species. We, the U.S. Fish and Wildlife Service, publish recovery plans, sometimes preparing them with the assistance of recovery teams, contractors, State agencies, and others. Recovery teams serve as independent advisors to the U.S. Fish and Wildlife Service. Objectives of the recovery plan will be attained and necessary funds made available subject to budgetary and other constraints affecting the parties involved, as well as the need to address other priorities. Recovery plans do not obligate other parties to undertake specific actions, and may not represent the views or the official positions or approval of any individuals or agencies involved in the recovery plan formulation other than our own. They represent our official position **only** after they have been signed by the Director, Regional Director, or Operations Manager as **approved**. Approved recovery plans are subject to modification as dictated by new findings, changes in species status, and the completion of recovery actions.

Literature Citation Should Read As Follows:
U.S. Fish and Wildlife Service. 2007. Recovery Plan for the Pacific Coast Population of the Western Snowy Plover (*Charadrius alexandrinus nivosus*). In 2 volumes. Sacramento, California. xiv + 751 pages.

An electronic version of this recovery plan also will be made available at http://www.fws.gov/cno/es/recoveryplans.html and http://endangered.fws.gov/recovery/index.html#plans

ACKNOWLEDGMENTS

The following U.S. Fish and Wildlife Service staff provided valuable review and comments on the recovery plan: John Grettenberger and Martha Jensen, Western Washington Office, Lacey, Washington; Carrie Phillips, Elizabeth Kelly, and Fred Seavey, Newport Fish and Wildlife Office, Newport, Oregon; Jim Watkins, Arcata Fish and Wildlife Office, Arcata, California; Kevin Clark and Gjon Hazard, Carlsbad Fish and Wildlife Office, Carlsbad, California; Steve Henry, Steve Kirkland, and David Pereksta, Ventura Fish and Wildlife Office; Diane Elam, California/Nevada Operations Office, Sacramento, California; and Grant Canterbury, Pacific Regional Office, Portland, Oregon. Robert Mesta, Bureau of Land Management Region 2 Office, Tucson, Arizona also provided valuable review and comments on the recovery plan.

Jennifer Bain, Valary Bloom, Don Hankins, Larry Host, Harry McQuillen, Caroline Prose, Caralee Stevens, Glen Tarr, Kim Turner, Betty Warne, and Dan Welsh of the Sacramento Fish and Wildlife Office, Sacramento, California also assisted in preparation of the final recovery plan.

Maps of snowy plover sites in Appendix L were prepared by Brian Cordone, Cheryl Hickam, and Joni Mitchell, Sacramento Fish and Wildlife Office, Sacramento, California.

The cover illustrations for the front cover and Appendix K are used courtesy of Carleton R. Eyster, Santa Cruz, California.

PACIFIC COAST WESTERN SNOWY PLOVER RECOVERY TEAM

Sarah Allen
National Park Service
Point Reyes, California

Tim Burr
U.S. Department of the Navy
San Diego, California

Jeff Chastain
C & M Stables
Florence, Oregon

Janet Didion
California Dept. of Parks and Recreation
Sacramento, California

David Dixon
California Dept. of Parks and Recreation
Monterey, California

Ivette Loredo
San Francisco Bay National Wildlife Refuge
Newark, California

Richard Griffiths
U.S. Marine Corps
Camp Pendleton, California

Jim Heaney
U.S. Bureau of Land Management
North Bend, Oregon

William Hornbrook
Fish Phone
Eureka, California

Tim Jensen
Monterey Peninsula Regional Park District
Carmel Valley, California

Ron Jurek
California Department of Fish and Game
Sacramento, California

Don Klusman
California Assoc. of 4 Wheel Drive Clubs, Inc.
Yuba City, California

Sean Morton
County of Santa Barbara
Santa Barbara, California

Martin Nugent
Oregon Department of Fish and Wildlife
Portland, Oregon

Gary Page
Point Reyes Bird Observatory
Stinson Beach, California

Kerrie Palermo
U.S. Bureau of Land Management
North Bend, Oregon

Daniel Pearson
Southern California Edison
Rosemead, California

John Phillips (deceased)
Oregon Parks and Recreation Dept.
Coos Bay, Oregon

Abby Powell
U.S.G.S., Biological Resources Division
Fairbanks, Alaska

Jim Raives
California Coastal Commission
San Francisco, California

Nancy R. Francine
U.S. Department of the Air Force
Vandenberg Air Force Base, California

Chris Regan
Washington State Parks and Recreation
Olympia, Washington

Scott Richardson (Max Bahn, altemate)
Washington Department of Fish and Wildlife
Olympia, Washington

Mark Stern
Oregon Natural Heritage Program
Portland, Oregon

EXECUTIVE SUMMARY

CURRENT SPECIES STATUS: The Pacific coast population of the western snowy plover (*Charadrius alexandrinus nivosus*) (western snowy plover) is federally listed as threatened. The current Pacific coast breeding population extends from Damon Point, Washington, south to Bahia Magdalena, Baja California, Mexico (including both Pacific and Gulf of California coasts). The western snowy plover winters mainly in coastal areas from southern Washington to Central America.

HABITAT REQUIREMENTS AND LIMITING FACTORS: The Pacific coast population of the western snowy plover breeds primarily above the high tide line on coastal beaches, sand spits, dune-backed beaches, sparsely-vegetated dunes, beaches at creek and river mouths, and salt pans at lagoons and estuaries. Less common nesting habitats include bluff-backed beaches, dredged material disposal sites, salt pond levees, dry salt ponds, and river bars. In winter, western snowy plovers are found on many of the beaches used for nesting as well as on beaches where they do not nest, in man-made salt ponds, and on estuarine sand and mud flats.

Habitat degradation caused by human disturbance, urban development, introduced beachgrass (*Ammophila* spp.), and expanding predator populations have resulted in a decline in active nesting areas and in the size of the breeding and wintering populations.

RECOVERY OBJECTIVE: The primary objective of this recovery plan is to remove the Pacific coast population of the western snowy plover from the *List of Endangered and Threatened Wildlife and Plants* by: (1) increasing population numbers distributed across the range of the Pacific coast population of the western snowy plover; (2) conducting intensive ongoing management for the species and its habitat and developing mechanisms to ensure management in perpetuity; and (3) monitoring western snowy plover populations and threats to determine success of recovery actions and refine management actions.

RECOVERY PRIORITY: 3C, per criteria published by Federal Register Notice (U.S. Fish and Wildlife Service 1983).

RECOVERY CRITERIA: The Pacific coast population of the western snowy plover will be considered for delisting when the following criteria have been met:

1. An average of 3,000 breeding adults has been maintained for 10 years, distributed among 6 recovery units as follows: Washington and Oregon, 250 breeding adults; Del Norte to Mendocino Counties, California, 150 breeding adults; San Francisco Bay, California, 500 breeding adults; Sonoma to Monterey Counties, California, 400 breeding adults; San Luis Obispo to Ventura Counties, California, 1,200 breeding adults; and Los Angeles to San Diego Counties, California, 500 breeding adults. This criterion also includes implementing monitoring of site-specific threats, incorporation of management activities into management plans to ameliorate or eliminate those threats, completion of research necessary to modify management and monitoring actions, and development of a post-delisting monitoring plan.

2. A yearly average productivity of at least one (1.0) fledged chick per male has been maintained in each recovery unit in the last 5 years prior to delisting.

3. Mechanisms have been developed and implemented to assure long-term protection and management of breeding, wintering, and migration areas to maintain the subpopulation sizes and average productivity specified in Criteria 1 and 2. These mechanisms include establishment of recovery unit working groups, development and implementation of participation plans, development and implementation of management plans for Federal and State lands, protection and management of private lands, and public outreach and education.

ACTIONS NEEDED:

1. Monitor breeding and wintering populations and habitats of the Pacific coast population of the western snowy plover to determine progress of recovery actions to maximize survival and productivity.

2. Manage breeding and wintering habitat of the Pacific coast population of the western snowy plover to ameliorate or eliminate threats and maximize survival and productivity.

3. Develop mechanisms for long-term management and protection of western snowy plovers and their breeding and wintering habitat.

4. Conduct scientific investigations that facilitate the recovery of the western snowy plover.

5. Conduct public information and education programs about the western snowy plover.

6. Review progress towards recovery of the western snowy plover and revise recovery efforts, as appropriate.

7. Dedicate U.S. Fish and Wildlife Service staff to allow the Arcata Fish and Wildlife Office to coordinate western snowy plover recovery implementation.

8. Establish an international conservation program with the government of Mexico to protect western snowy plovers and their breeding and wintering locations in Mexico.

Appendices B and C address Actions 1 and 2, providing site-specific recommendations for breeding numbers and management actions. Appendix J addresses Action 1, providing guidelines for monitoring western snowy plovers during the breeding and wintering seasons. Appendix K addresses Action 5, providing a public information and education plan.

ESTIMATED COST OF RECOVERY: $149,946,000 plus additional costs that cannot be estimated at this time.

DATE OF RECOVERY: Delisting could occur by 2047 if the recovery criteria above have been met.

TABLE OF CONTENTS

LIST OF APPENDICES
(See Volume 2)

LIST OF TABLES

LIST OF FIGURES

I. INTRODUCTION

On March 5, 1993, the Pacific coast population of the western snowy plover (*Charadrius alexandrinus nivosus*) (western snowy plover) was listed as threatened under provisions of the Endangered Species Act of 1973, as amended (16 U.S.C. 1531 *et seq.*). The Pacific coast population is defined as those individuals that nest within 50 miles of the Pacific Ocean on the mainland coast, peninsulas, offshore islands, bays, estuaries, or rivers of the United States and Baja California, Mexico (U.S. Fish and Wildlife Service 1993*a*) (Figure 1). General locations of the western snowy plover's breeding and wintering locations in the United States are shown in Appendix A. Surveys, status reviews, and literature searches have identified 159 current or historical western snowy plover breeding or wintering locations on the U.S. Pacific coast. These localities include 6 in Washington, 19 in Oregon, and 134 in California (Appendix B). In Baja California, breeding western snowy plovers concentrate at coastal wetland complexes as far south as Bahia Magdalena, Mexico (Palacios *et al.* 1994). The locations listed in Appendix B are important for the recovery of the United States Pacific coast population of the western snowy plover because they represent important breeding, feeding, and sheltering habitat for the species.

In Washington, the western snowy plover was listed as endangered under Washington Department of Fish and Wildlife Policy #402 in 1981. In 1990 the Washington Fish and Wildlife Commission (Washington Administrative Code 232-12-014) reaffirmed the endangered status. In 1975, the Oregon Fish and Wildlife Commission listed the western snowy plover as threatened. Its threatened status was reaffirmed in 1989 under the Oregon Endangered Species Act and again in 1993 and 1998 by the Oregon Fish and Wildlife Commission as part of its periodic review process. Since 1978, the California Department of Fish and Game has classified both the inland and coastal population of western snowy plover as a "species of special concern." (Remsen 1978, California Natural Diversity Database 2001).

In August 2002, we received a petition from the Surf Ocean Beach Commission of Lompoc, California to delist the Pacific Coast population of the western snowy

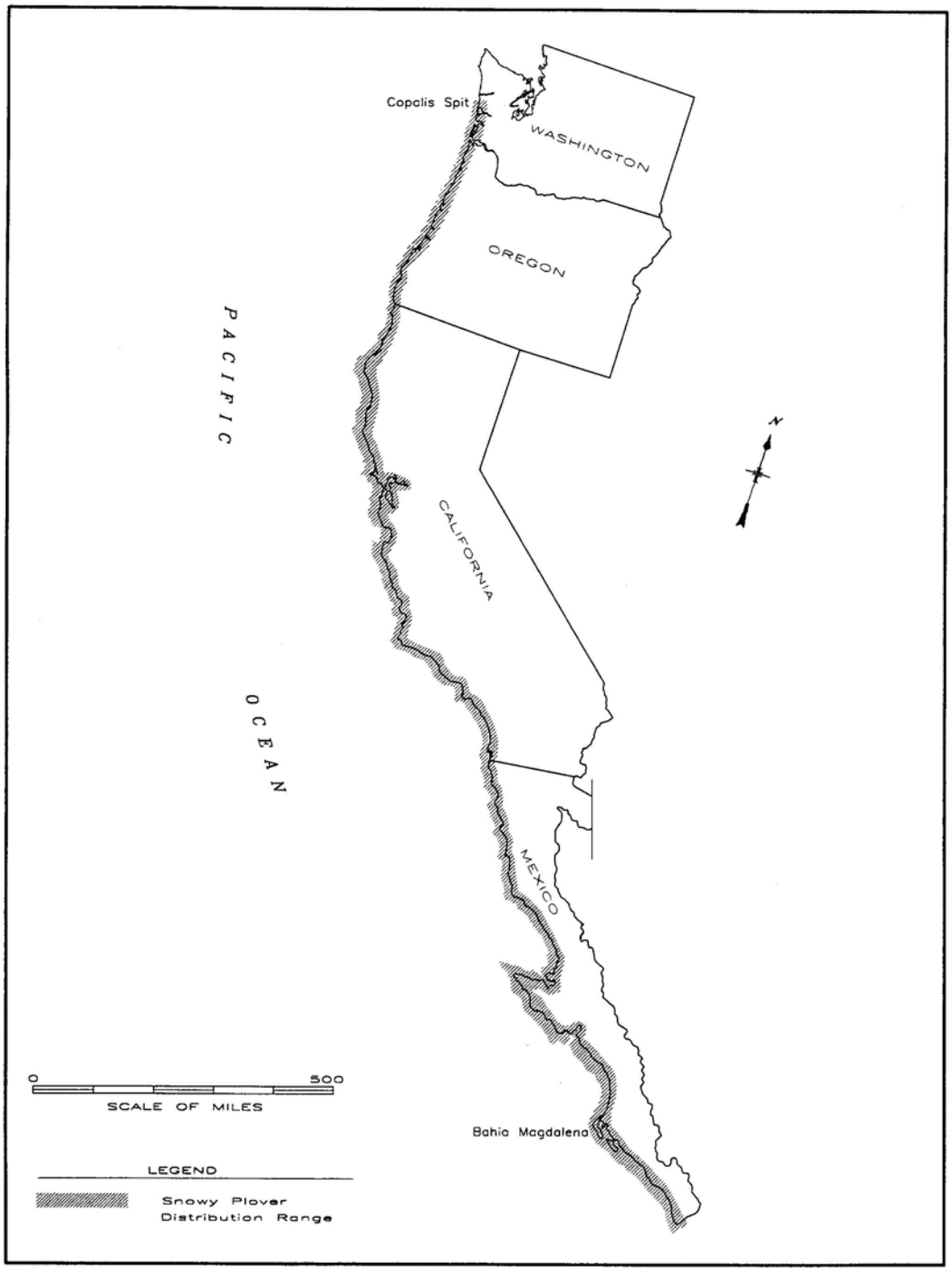


Figure 1. Map of known breeding and wintering distribution of the Pacific coast population of the western snowy plover.

plover. The City of Morro Bay, California submitted substantially the same petition dated May 30, 2003. On March 22, 2004, we published a notice that the petition presented substantial information to indicate that the delisting may be warranted (U.S. Fish and Wildlife Service 2004*a*). This notice also announced our initiation of a 5-year status review for the Pacific coast population of western snowy plover.

Under sections 4(b)(3)(B) and 4(c)(2) of the Endangered Species Act, we conducted a 5-year status review and evaluated whether the petitioned action was warranted. On April 21, 2006, we published a 12-month finding that concluded the petitioned action was not warranted (U.S. Fish and Wildlife Service 2006*a*). We also proposed a special rule pursuant to section 4(d) of the Endangered Species Act (U.S. Fish and Wildlife Service 2006*b*), which would exempt counties that have met western snowy plover recovery goals from most prohibitions on take as long as populations remain above recovery goals. The 5-year status review was completed on June 8, 2006.

Section 4 of the Endangered Species Act of 1973, as amended, requires us to develop a recovery plan for the conservation and survival of a species after it is federally listed as threatened or endangered, unless it is determined that such a plan will not promote the conservation of the species. Recovery is the process of reversing the decline of a listed species, eliminating threats, and ensuring the species' long-term survival. This recovery plan recommends actions necessary to satisfy the biological needs and assure recovery of the Pacific coast population of the western snowy plover. These actions include protection, enhancement, and restoration of all habitats deemed important for recovery; monitoring; research; and public outreach.

This recovery plan will serve as a guidance document for interested parties including Federal, State, and local agencies; private landowners; and the general public. It includes recommendations for western snowy plover management measures for all known breeding and wintering locations (Appendix C). These locations have been divided into six recovery units, as follows: (1) Oregon and Washington; (2) northern California (Del Norte, Humboldt, and Mendocino Counties); (3) San Francisco Bay (locations within Napa, Alameda, Santa Clara,

and San Mateo Counties); (4) Monterey Bay (including coastal areas along Monterey, Santa Cruz, San Mateo, San Francisco, Marin, and Sonoma Counties); (5) San Luis Obispo, Santa Barbara, and Ventura Counties; and (6) Los Angeles, Orange, and San Diego Counties. Designation of these locations and recovery units assists in identifying priority areas for conservation planning across the western snowy plover's breeding and wintering range.

This recovery plan emphasizes management on Federal and State lands, including opportunities to improve or expand upon current efforts. Because of this emphasis on public lands, the cost associated with this emphasis, and potential restrictions of public use on these lands, public support and involvement will be crucial to the recovery of the western snowy plover. Opportunities for public participation in recovery efforts are emphasized in Appendix K (Information and Education Plan).

A. DESCRIPTION AND TAXONOMY

The western snowy plover, a small shorebird in the family Charadriidae, weighs from 34 to 58 grams (1.2 to 2 ounces) and ranges in length from 15 to 17 centimeters (5.9 to 6.6 inches) (Page *et al.* 1995*a*). It is pale gray-brown above and white below, with a white hindneck collar and dark lateral breast patches, forehead bar, and eye patches (Figure 2). The bill and legs are blackish. In breeding plumage, males usually have black markings on the head and breast; in females, usually one or more of these markings are dark brown. Early in the breeding season a rufous crown may be evident on breeding males, but it is not typically seen on females. In non-breeding plumage, sexes cannot be distinguished because the breeding markings disappear. Fledged juveniles have buffy edges on their upper parts and can be distinguished from adults until approximately July through October, depending on when in the nesting season they hatched. After this period, molt and feather wear makes fledged juveniles indistinguishable from adults. Individual birds 1 year or older are considered to be breeding adults. The mean annual life span of western snowy plovers is estimated at about 3 years, but at least one individual was at least 15 years old when last seen (Page *et al.* 1995*a*).

Figure 2. Adult male western snowy plover (photo by Peter Knapp, with permission).

The species was first described in 1758 by Linnaeus (American Ornithologists' Union 1957). Two subspecies of the snowy plover have been recognized in North America (American Ornithologists' Union 1957): the western snowy plover (*Charadrius alexandrinus nivosus*) and the Cuban snowy plover (*C. a. tenuirostris*). The Pacific coast population of the western snowy plover breeds on the Pacific coast from southern Washington to southern Baja California, Mexico. Wintering birds may remain at their breeding sites or move north or south to other wintering sites along the Pacific coast. The interior population of the western snowy plover breeds in interior areas of Oregon, California, Nevada, Utah, New Mexico, Colorado, Kansas, Oklahoma, and north-central Texas, as well as coastal areas of extreme southern Texas, and possibly extreme northeastern Mexico (American Ornithologists' Union 1957). Although previously observed only as a migrant in Arizona, small numbers have bred there in recent years (Monson and Phillips 1981, Davis and Russell 1984). Interior population birds breeding east of the Rockies generally winter along the Gulf coast, while most interior population birds breeding west of the Rockies winter in coastal California and Baja

California, often intermingling with birds from the Pacific coast breeding population. The Cuban snowy plover breeds along the Gulf coast from Louisiana to western Florida and south through the Caribbean (American Ornithologists' Union 1957). More recent works recognize only subspecies *C. a. nivosus* for North America (Hayman *et al.* 1986, Binford 1989, Sibley and Monroe 1990).

A large amount of breeding data indicates that the Pacific coast population of the western snowy plover is distinct from western snowy plovers breeding in the interior (U.S. Fish and Wildlife Service 1993*a*, 2006*a*). A study conducted between 1977 and 1982 reported that western snowy plovers tend to exhibit breeding site fidelity (Warriner *et. al.* 1986). Banding and resighting data show that the Pacific Coast breeding populations and the western interior breeding populations experience limited or rare reproductive interchange (G. Page *in litt.* 2004*a*). Between 1984 and 1995, the period with the most extensive banding studies and search efforts, 907 plovers color-banded in coastal and interior populations were subsequently resighted (excluding birds banded on the coast during winter and birds resighted in their original region without evidence of nesting). Of these, 894 birds (98.6 percent) were observed during the breeding season using the same breeding range in which they were originally banded. Twelve birds (1.3 percent) were banded on the coast and later observed in the interior, only one of which was known to nest in the interior. Only one male (0.1 percent) was banded in the interior (without evidence of nesting) and later found nesting on the coast. Moreover, data from a period of less intensive surveys and banding from 1977 to 1983 corroborate this pattern (G. Page *in litt.* 2004*a*, U.S. Fish and Wildlife Service 2006*a*). During this period, of 400 birds banded in the interior, none were observed on the coast during breeding season, and of 599 birds banded on the coast only one was found nesting in the interior. Finally, 304 retrievals of numbered metal bands reported between 1969 and 2002 show no evidence of movement from interior to coast and only one bird (G. Goldsmith *in litt.* 2004, U.S. Fish and Wildlife Service 2006*a*) that moved from coast to interior (the dates being consistent with a bird from the interior population having been banded on the coast during the non-breeding season).

Thus, intensive banding and monitoring studies have documented only two clear instances of interbreeding between coastal and interior populations, and a few

cases of inter-population movement without confirmed breeding, among thousands of birds observed. These results illustrate that the amount of interchange between coastal and interior populations is likely to be extremely low, though not zero. Movement of birds from coastal to interior populations has been documented more often than the reverse (see also U.S. Fish and Wildlife Service 2006a).

Genetic studies using mitochondrial DNA and microsatellite DNA markers (Gorman 2000, Funk *et al.* 2006) have found no significant genetic differentiation between the Pacific coast and interior populations of the western snowy plover. However, because a small number of dispersing individuals per generation is sufficient to prevent genetic differentiation between two semi-isolated populations (Mills and Allendorf 1996, Funk *et al.* 2006), this result is consistent with the banding data reported above. Because the small number of dispersing individuals indicated by banding data appear insufficient to substantially affect rates of population growth or decline in either population, the two populations evidently function demographically as largely independent of one another. Moreover, the infrequency of observed dispersal from coast to interior further indicates that any declines in the coastal population are not likely to be effectively offset by immigration of interior birds to the coast. Consequently there is no evidence that existing unoccupied habitat along the Pacific coast is currently being or in future would be naturally colonized by birds from the interior population (Funk et al. 2006).

B. LIFE HISTORY AND ECOLOGY

1. Breeding

The Pacific coast population of the western snowy plover breeds primarily on coastal beaches from southern Washington to southern Baja California, Mexico (*e.g.*, Figure 3). Sand spits, dune-backed beaches, beaches at creek and river mouths, and salt pans at lagoons and estuaries are the main coastal habitats for nesting (Stenzel *et al*. 1981, Wilson 1980). This habitat is unstable because of

Figure 3. Coastal beach in Oregon Dunes National Recreational Area (photo by Ruth Pratt, with permission)

unconsolidated soils, high winds, storms, wave action, and colonization by plants. Less common nesting habitats include bluff-backed beaches, dredged material disposal sites, salt pond levees, dry salt ponds, and river bars (Wilson 1980, Page and Stenzel 1981, Powell *et al.* 1996, Tuttle *et al.* 1997).

a. Population Size and Distribution

Population estimates referenced below are based on window surveys as well as on more intensive studies involving repeated surveys of populations with individually identifiable color-banded birds. Window surveys are a one-time pass of a surveyor, or team of surveyors, through potential western snowy plover nesting habitat during May or June (see survey protocol in Appendix J). The surveyor counts all adult western snowy plovers in the habitat and identifies the adults as male or female, when possible. Because window surveys may not detect all birds, they are not directly comparable to more intensive studies. A correction factor can be estimated by comparing window survey data with concurrent population estimates from detailed studies of color-banded populations; currently

the best rangewide estimate of the correction factor is 1.3 (U.S. Fish and Wildlife Service 2006*a*), but it is preferable to determine corrections on a more specific regional or site basis if possible due to differences in survey efficiency in different habitats (see action 4.3.1).

Western snowy plovers concentrate in suitable habitat, with the number of adults at coastal breeding locations ranging from 1 to 315, depending in part, on the size of the area (Appendix B). The largest number of breeding birds occurs from south San Francisco Bay to southern Baja California (Page and Stenzel 1981, Palacios *et al.* 1994).

The locations of the following parenthetical references to western snowy plover breeding and wintering locations in Washington, Oregon, and California are shown in Figures A-1 through A-7 of Appendix A, and mapped in greater detail in Appendix L. Information on the numbers of breeding and wintering western snowy plovers at these locations is described in Appendix B.

Four breeding areas currently exist in southern Washington: Damon Point (Washington location 2 [WA-2]) in Grays Harbor; Midway Beach (WA-4); and Leadbetter Point (WA-5) and Graveyard Spit (discovered in 2006) in Willapa Bay. Prior to the 1998 breeding season, fewer than 25 western snowy plovers and 12 nests were found in Washington during regular, standardized surveys. However, surveys from 1998 through 2006 (Sundstrom 2003, 2005; Brennan and Fernandez 2004*a*, 2006; Pearson *et al.* 2006; Washington Department of Fish and Wildlife unpub. data) indicate greater numbers of western snowy plovers are nesting at Leadbetter Point (WA-5) and Midway Beach (WA-4), with a maximum estimated population of 70 western snowy plovers statewide in 2006.

In Oregon, nesting birds have been recorded at 14 sites since 1990 (Castelein *et al.* 2002, Lauten *et al.* 2006*a*, 2006*b*). Nesting has occurred most frequently at 9 sites, including Sutton (OR-8), Siltcoos (OR-10), Dunes Overlook (OR-10), Tahkenitch (OR-10), Tenmile Spits (OR-12), Coos Bay North Spit (OR-13), Bandon (OR-15), New River (OR-15), and Floras Lake (OR-15). An estimated 177-179 adult western snowy plovers were observed at Oregon sites during the 2006 breeding season. A total of 135 individuals were known to have nested in

2006, with 147 nests located. Individual nests have also been found between 1990 and 2002 at several other Oregon sites, including Necanicum (OR-1); Bayocean Spit (OR-3); North Siuslaw (OR-8); Threemile-Umpqua River (OR-11); and Menasha Spoils, North Bend.

Western snowy plover populations in California have fluctuated between roughly one thousand and two thousand birds over the past 30 years, as detailed in section I.C.1.c below. Eight geographic areas support over three-quarters of the California coastal breeding population: San Francisco Bay (CA-27 to CA-47), Monterey Bay (CA-63 to CA-65), Morro Bay (CA-79 to CA-81), the Callendar-Mussel Rock Dunes area (CA-83), the Point Sal to Point Conception area (CA-84 to CA-88), the Oxnard lowland (CA-96 to CA-99), Santa Rosa Island (CA-93), and San Nicolas Island (CA-100) (Page *et al.* 1991, G. Page *in litt.* 2005*a*).

A survey of breeding western snowy plovers along the Pacific coast of Baja California, Mexico between 1991 to 1992 found 1,344 adults, mostly at four coastal wetland complexes: Bahia San Quintin, Lagunas Ojo de Liebre and Guerrero Negro, Laguna San Ignacio, and Bahia Magdalena (Palacios *et al.* 1994).

b. Arrival and Courtship

Nesting western snowy plovers at coastal locations consist of both year-round residents and migrants (Warriner *et al.* 1986). Migrants begin arriving at breeding areas in southern Washington in early March (Widrig 1980) and in central California as early as January, although the main arrival is from early March to late April (Page *et al.* 1995*a*). Since some individuals nest at multiple locations during the same year, birds may continue arriving through June (Stenzel *et al.* 1994).

Mated birds from the previous breeding season frequently reunite. Pair bonds are associated with territorial defense by males and nest scraping behavior, but early in the season birds begin to associate with one another in pairs within and apart from roosting flocks before nest scraping activity is observed, suggesting that pair bonds can be established prior to overt displays (Warriner *et al.* 1986). A scrape

is a depression in the sand or substrate that a male constructs by leaning forward on his breast and scratching his feet while rotating his body axis (Page *et al.* 1995*a*). Copulations are associated with scraping behavior (Warriner *et al.* 1986). Females choose which scrape becomes the nest site by laying eggs in one of them. In California, pre-nesting bonds and courtship activities are observed as early as mid-February. Similar activities begin by March in Oregon. During courtship, males defend territories and usually make multiple scrapes.

c. Duration of Breeding Season

Along the west coast of the United States, the nesting season of the western snowy plover extends from early March through late September. Generally, the breeding season may be 2 to 4 weeks earlier in southern California than in Oregon and Washington. Fledging (reaching flying age) of late-season broods may extend into the third week of September throughout the breeding range.

The earliest nests on the California coast occur during the first week of March in some years and by the third week of March in most years (Page *et al.* 1995*a*). Peak initiation of nesting is from mid-April to mid-June (Warriner *et al.* 1986; Powell *et al.* 1997). Hatching lasts from early April through mid-August, with chicks reaching fledging age approximately 1 month after hatching (Powell *et al.* 1997). On the Oregon coast nesting may begin as early as mid-March, but most nests are initiated from mid-April through mid-July (Wilson-Jacobs and Meslow 1984); peak nest initiation occurs from mid-May to early July (Stern *et al.* 1990). In Oregon, hatching occurs from mid-April through mid-August, with chicks reaching fledging age as early as mid- to late May. Peak hatching occurs from May through July, and most fledging occurs from June through August. On the Washington coast, most adults arrive during late April, with maximum numbers present from mid-May to late June. Fledging occurs from late June through August (Washington Department of Fish and Wildlife 1995).

d. Nests and Nest Sites

Nests typically occur in flat, open areas with sandy or saline substrates; vegetation and driftwood are usually sparse or absent (Widrig 1980, Wilson 1980,

Stenzel *et al.* 1981). Western snowy plovers also regularly nest on the gravel bars along the Eel River in northern California. In southern California, western snowy plovers nest in areas with 6 to 18 percent vegetative cover and 1 to 14 percent inorganic cover; vegetation height is usually less than six centimeters (2.3 inches) (Powell *et al.* 1995, 1996). Nests consist of a shallow scrape or depression, sometimes lined with beach debris (*e.g.*, small pebbles, shell fragments, plant debris, and mud chips); nest lining increases as incubation progresses. Driftwood, kelp, and dune plants provide cover for chicks that crouch near objects to hide from predators. Invertebrates are often found near debris, so driftwood and kelp are also important for harboring western snowy plover food sources (Page *et al.* 1995*a*). Page and Stenzel (1981) found that nests were usually within 100 meters (328 feet) of water, but could be several hundred meters away when there was no vegetative barrier between the nest and water. They believed the absence of such a barrier is probably important for newly-hatched chicks to have access to the shore. Powell *et al.* (1995, 1996) also reported that nests from southern California were usually located within 100 meters (328 feet) of water, which could be either ocean, lagoon, or river mouth. Although the majority of western snowy plovers are site-faithful, returning to the same breeding area in subsequent breeding seasons, some also disperse within and between years (Warriner *et al.* 1986, Stenzel *et al.* 1994). Western snowy plovers occasionally nest in exactly the same location as the previous year (Warriner *et al.* 1986).

e. Egg Laying, Clutch Size, and Incubation

Initiation (eggs and laying) occurs from mid-February/early March through the third week of July (Wilson 1980, Warriner *et al.* 1986). The approximate periods required for nesting events are: scrape construction (in conjunction with courtship and mating), 3 days to more than a month; egg laying, usually 4 to 5 days; and incubation, 26 to 31 days (mean 27 days) (Warriner *et al.* 1986). The usual clutch size (*e.g.*, number of eggs in one nest) is three (Figure 4) with a range from two to six. (Warriner *et al.* 1986, Page *et al.* 1995*a*). Both sexes incubate the eggs, with the female tending to incubate during the day and the male at night (Warriner *et al.* 1986). Adult western snowy plovers frequently will attempt to lure people and predators from hatching eggs with alarm calls and distraction displays. Occasionally, adults behave similarly during the egg-laying period or

Figure 4. Western snowy plover clutch (photo by Bruce Casler, with permission).

incubation of completed clutches. More typical, however, is for the incubating adult to run away from the eggs without being seen. Incomplete clutches are those in which all eggs have not been laid. Partly-incubated clutches are those clutches having some degree (in days) of incubation.

Western snowy plovers will re-nest after loss of their eggs (Wilson 1980, Warriner *et al.* 1986). Re-nesting occurs 2 to 14 days after failure of a clutch, and up to five re-nesting attempts have been observed for a pair (Warriner *et al.* 1986).

Double brooding with polyandry (meaning the female successfully hatches more than one brood [*i.e.* sibling chicks of a hatched nest] in a nesting season with different mates) is common in coastal California (Warriner *et al.* 1986) and Oregon (Wilson-Jacobs and Meslow 1984). On the California coast, the breeding season is long enough for some females to triple brood and for some males to double brood (Page *et al.* 1995*a*). Triple brooding in a male has, on rare occasion, been recorded; a male triple brooded at Moss Landing salt ponds in 2001 (D. George *in litt.* 2001). After losing a clutch or brood or successfully

hatching a nest, western snowy plovers may re-nest at the same site or move up to several hundred kilometers to nest at other sites (Stenzel *et al.* 1994, Powell *et al.* 1997).

f. Clutch Hatching Success

Widely varying clutch hatching success (percent of clutches hatching at least one egg) is reported in the literature. Clutch hatching success ranging from 0 to 90 percent has been recorded for coastal western snowy plovers (Widrig 1980, Wilson 1980, Saul 1982, Wilson-Jacobs and Dorsey 1985, Warriner *et al.* 1986, Wickham unpubl. data *in* Jacobs 1986). Low clutch hatching success has been attributed to a variety of factors, including predation, human disturbance, high tides, and inclement weather. Heavy recreational beach use coincides with the peak hatching period for western snowy plover eggs (Powell 2001), adding additional pressures to western snowy plover adults and chicks that are more exposed to human disturbance. Observed clutch hatching success ranged from 12.5 to 86.8 percent and averaged 50.6 percent in eight studies of coastal breeding western snowy plovers (Page *et al.* 1995*a*). In San Diego County, estimated nesting success ranged from 43 to 68 percent between 1994 and 1998, averaging 54 percent (Powell *et al.* 2002); nesting western snowy plovers in San Diego County likely benefitted from predator management efforts for snowy plovers and California least terns (*Sternula antillarum browni*) (A. Powell, U.S. Geological Survey, pers. comm. 1998). In Monterey Bay, hatching rate was significantly increased from 43 percent (during 1984-1990) to 68 percent (during 1991-1999) by intensive control of mammalian predators and use of nest exclosures (Neuman *et al.* 2004).

g. Brood-rearing

The first chick hatched remains in or near the nest until other eggs (or at least the second egg) hatch. The adult western snowy plover, while incubating the eggs, also broods the first chick. The non-incubating adult also may brood the first-born chick a short distance from the nest. If the third egg of a clutch is 24 to 48 hours behind the others in hatching, it may be deserted. Western snowy plover chicks are precocial, leaving the nest within hours after hatching to search for

food. They are not able to fly (fledge) for approximately 1 month after hatching; fledging requires 28 to 33 days (Warriner *et al.* 1986). Broods rarely remain in the nesting area until fledging (Warriner *et al.* 1986, Stern *et al.* 1990). Western snowy plover broods may travel along the beach as far as 6.4 kilometers (4 miles) from their natal area (Casler *et al.* 1993).

Adult western snowy plovers do not feed their chicks, but lead them to suitable feeding areas. Adults use distraction displays to lure predators and people away from chicks. With vocalizations, adult western snowy plovers signal the chicks to crouch as another way to protect them (Page *et al.* 1995*a*). They also may lead chicks, especially larger ones, away from predators. Warriner *et al.* (1986) reported that most chick mortality occurs within 6 days after hatching.

Females generally desert mates and broods by the sixth day after hatching and thereafter the chicks are typically accompanied by only the male. While males rear broods, females obtain new mates and initiate new nests (Page *et al.* 1995*a*). Females typically help rear the last brood of the season.

h. Fledging success

The fledging success of western snowy plovers (percentage of hatched young that reach flying age) varies greatly by location and year. Even western snowy plovers nesting on neighboring beach segments may exhibit quite different success in the same year. For example, the percentage of chicks fledged on different beach segments of Monterey Bay in 1997 varied from 11 to 59 percent (average 24 percent) (Page *et al.* 1997). During the prior 13 years, fledging success on Monterey Bay beaches averaged 39 percent (Page *et al.* 1997). From the former Moss Landing salt ponds (now known as the Moss Landing Wildlife Area) in Monterey Bay (CA-64), fledging success ranged from 13.2 percent to 57.1 percent from 1988 to 1997. In San Diego County, fledging success ranged from 32.6 to 51.4 percent (Powell *et al.* 1997). In Oregon, annual fledging success for 1992 to 2006, for all coastal sites combined, ranged from 26 to 55 percent (Lauten *et al.* 2006*a*, 2006*b*). As in California, there is considerable variation among sites within years. For example, in 2005, the fledging success ranged from 24 percent at New River (OR-15) to 70 percent at Coos Bay South

Beach (OR-13). There also is variation at individual sites among years. At the Coos Bay North Spit (OR-13), one of the larger nesting areas in coastal Oregon, annual fledging success for 1992 to 2006 ranged from 38 to 74 percent.

i. Productivity

The productivity information most useful for this recovery plan is reproductive success (the annual number of young fledged per adult male). For the population viability analysis (Appendix D), males were used in the model because their population parameters can be estimated with greater certainty than for females. In addition, it is reasonable to consider that the availability of males is limiting reproductive success because they are responsible for post-hatching parental care, and females can lay clutches for more than one male (Warriner *et al.* 1986).

Chicks are considered fledged at 28 to 33 days after hatching. Estimates of the number of young fledged per adult male are available for Oregon; northern California from Mendocino to Del Norte Counties; Monterey Bay, California; and San Diego County, California. Along the Oregon coast, the average number of young annually fledged per male during the period between 1992 and the initiation of predator management (2002 to 2004 depending on site) was estimated as 0.87 (Lauten *et al.* 2006*b*); this fledging success significantly increased to 1.44 since implementation of predator management. Male fledging success in Oregon has annually ranged between 0.70 and 1.64 (Lauten *et al.* 2006*a*). In northern California, fledging success ranged from 0.8 to 1.7 fledglings per male between 2001-2005, with birds nesting on river gravel bars consistently achieving greater success than those nesting on beaches (Colwell *et al.* 2005). At Monterey Bay, California, from 1984 to 1990, when little effort was made to protect chicks from predators and people, males averaged 0.86 fledglings annually. When intensive efforts were undertaken to control mammalian predators from 1993 to 1999, the number of young fledged per adult male initially increased above 1.1, then declined sharply as avian predation on chicks became increasingly significant (Neuman *et al.* 2004). After live trapping and removal of avian predators was initiated, fledging success again increased in target areas (G. Page *in litt.* 2004b). Over 16 years of study at Monterey Bay, the annual number of young fledged ranged from 0.32 to 1.23 per male (Neuman *et al.* 2004). In San

Diego County from 1994 to 1998, an average of 0.15 to 0.44 young were fledged per male (Powell et al. 2002). Fledging success in Washington cannot be accurately estimated due to lack of banded chicks and adults and variable monitoring effort prior to 2006 (S. Pearson *in litt.* 2006); however it was roughly estimated at between 0.76 and 1.45 young fledged per male in 2006, excluding Leadbetter Point which was insufficiently surveyed but may have had poorer fledging success (Pearson *et al.* 2006).

j. Survival

Annual survival rates for adult and juvenile western snowy plovers have been calculated from studies of color banded birds from the coast of Oregon (M. Stern unpubl. data), the shoreline of Monterey Bay, California (Point Reyes Bird Observatory unpublished data), and the coast of San Diego County, California (A. Powell and J. Terp unpublished data) using the program SURGE (Lebreton *et al.* 1992, Cooch *et al.* 1996). Annual juvenile survival rates for fledged young average 48.5 percent (1992-2002) from the Oregon coast, 45 percent from Monterey Bay, and 45 percent from the San Diego coast. Annual survival rates for adult females and males, respectively, averaged 75 and 75 percent from the Oregon coast, 69 and 75 percent from Monterey Bay, and 72 and 71 percent from the San Diego coast. Differences between males and females were statistically significant only for the Monterey Bay area. Appendix D explains how these survival rates were incorporated into the population viability analysis.

2. Feeding Habitat and Habits

Western snowy plovers are primarily visual foragers, using the run-stop-peck method of feeding typical of *Charadrius* species. They forage on invertebrates in the wet sand and amongst surf-cast kelp within the intertidal zone, in dry sand areas above the high tide, on salt pans, on spoil sites, and along the edges of salt marshes, salt ponds, and lagoons. They sometimes probe for prey in the sand and pick insects from low-growing plants. At the Bolsa Chica wetlands in California, western snowy plovers have been observed pecking small, flying insects from mid-air and shaking one foot in very shallow water to agitate potential prey (Fancher *et al.* 1998). Western snowy plover food consists of immature and adult

forms of aquatic and terrestrial invertebrates. Little quantitative information is available on food habits. In San Diego, California, invertebrates found in western snowy plover feces during the breeding season included rove beetles (Staphylinidae), long-legged flies (Dolichopodidae), shore flies (Ephydridae), water bugs (Saldidae), hymenopterans (Braconidae), and unidentified insect larvae (Tucker and Powell 1999). During the breeding season, Jacobs (1986) observed adult western snowy plovers feeding on sand hoppers (Orchestoidea) and small fish on the Oregon coast. Other food items reported for coastal western snowy plovers include Pacific mole crabs (*Emerita analoga*), striped shore crabs (*Pachygrapsus crassipes*), polychaetes (Neridae, *Lumbrineris zonata, Polydora socialis, Scoloplos acmaceps),* amphipods (*Corophium* ssp., *Ampithoe* spp., *Allorchestes angustus*), tanadacians (*Leptochelia dubia*), shore flies (Ephydridae), beetles (Carabidae, Buprestidae, Tenebrionidae), clams (*Transenella* sp.), and ostracods (Page *et al.* 1995*a*). In salt evaporation ponds in San Francisco Bay, California, the following prey have been recorded: brine flies (*Ephydra cinerea*), beetles (*Tanarthrus occidentalis*, *Bembidion* sp.), moths (*Perizoma custodiata*), and lepidopteran caterpillars (Feeney and Maffei 1991). Opportunities for foraging are directly dependent on salinity levels. Specifically, salt ponds of medium salinity seem to provide the best quality foraging habitat (M. Kolar, San Francisco Bay National Wildlife Refuge, pers. comm. 2004).

3. Migration

While some western snowy plovers remain in their coastal breeding areas year-round, others migrate south or north for winter (Warriner *et al.* 1986, Page *et al.* 1995*a*, Powell *et al.* 1997). In Monterey Bay, California, 41 percent of nesting males and 24 percent of the females were consistent year-round residents (Warriner *et al.* 1986). At Marine Corps Base Camp Pendleton in San Diego County, California, about 30 percent of nesting birds stayed during winter (Powell *et al.* 1995, 1996, 1997). The migrants vacate California coastal nesting areas primarily from late June to late October (Page *et al.* 1995*a*). There is evidence of a late-summer (August/September) influx of western snowy plovers into Washington; it is suspected that these wandering birds are migrants (S. Richardson, Washington Department of Fish and Wildlife, pers. comm. 1998).

Most western snowy plovers that nest inland migrate to the coast for the winter (Page *et al.* 1986, 1995*b*). Thus, the flocks of non-breeding birds that begin forming along the U.S. Pacific coast in early July are a mixture of adult and hatching-year birds from both coastal and interior nesting areas. During migration and winter, these flocks range in size from a few individuals to up to 300 birds (Appendix B).

4. Wintering

a. Distribution and Abundance

In western North America, the western snowy plover winters (here defined as late October to mid-February) mainly in coastal areas from southern Washington to Central America (Page *et al.* 1995*a*). Both coastal and interior populations use coastal locations in winter. Small numbers of western snowy plovers occur at two locations on the Washington coast: Midway Beach (WA-4) (S. Richardson, pers. comm. 1998, J. Grettenberger, U.S. Fish and Wildlife Service, pers. comm. 2004), and Leadbetter Point (WA-5), Willapa Bay (Washington Department of Fish and Wildlife 1995), both in Pacific County. Increasing numbers of wintering western snowy plovers are being documented along the Washington coast, with 32 counted in 2005 (L. Kelly *in litt.* 2005). As many as 97 western snowy plovers were observed wintering on the Oregon coast in 2005 (L. Kelly *in litt.* 2005). During the survey period between 1990 and 2005, at least 9 Oregon locations (Appendix B) have been used by wintering plovers. Probably as many as 2,500 plovers overwinter along the mainland California coast, and hundreds more at San Francisco Bay and in the Channel Islands (Appendix B, Page *et al.* 1986). The majority of wintering western snowy plovers on the California coast are found from Bodega Bay, Sonoma County, southward (Page *et al.* 1986). Appendix B gives the range of years over which each state's data was collected as well as the minimum and maximum number of western snowy plovers inventoried.

Nesting western snowy plovers from the Oregon coast have wintered as far south as Monterey Bay, California; those from Monterey Bay in central California have wintered north to Bandon, Oregon, and south to Laguna Ojo de Liebre, Baja California, Mexico (Page *et al.* 1995*a*); and those from San Diego in southern

California have wintered north to Vandenberg Air Force Base in Santa Barbara County and south to Laguna Ojo de Liebre, Baja California, Mexico (Powell *et al.* 1995, 1996, 1997).

In winter, western snowy plovers are found on many of the beaches used for nesting, as well as some beaches where they do not nest (Appendix B). They also occur in man-made salt ponds and on estuarine sand and mud flats. In California, the majority of wintering western snowy plovers concentrate on sand spits and dune-backed beaches. Some also occur on urban and bluff-backed beaches, which are rarely used for nesting (Page *et al.* 1986). Pocket beaches at the mouths of creeks and rivers on otherwise rocky shorelines are used by wintering western snowy plovers south, but not north, of San Mateo County, California.

b. Site Fidelity

Western snowy plovers that breed on the coast and inland are very site faithful in winter (Point Reyes Bird Observatory unpublished data). For example, after 166 adults and 204 chicks were banded at Lake Abert, Oregon during summer, many were subsequently found along the California and Baja California, Mexico coasts. Of those for which a wintering location was identified, 67 percent of the adult males, 73 percent of the adult females, and 60 percent of the birds banded as chicks (immatures) were found at the same winter location in at least 2 consecutive years; and 33 percent of the males, 32 percent of the females, and 35 percent of the immatures for at least 3 years (Page *et al.* 1995*b*).

c. Behavior

Western snowy plovers are typically gregarious in winter. Although some individuals defend territories on beaches, most usually roost in loose flocks; frequently western snowy plovers also are observed foraging in loose flocks (Page *et al.* 1995*a*). Roosting western snowy plovers usually sit in small depressions in the sand, or in the lee of kelp, other debris, or small dunes (Page *et al.* 1995*a*). Sitting behind debris or in depressions provides some shelter from the wind and probably makes the birds more difficult for predators to detect. When roosting western snowy plovers are disturbed, they frequently run a few meters to

a new spot where they sometimes displace other individuals. Alternatively, the whole flock may fly to a new location.

C. POPULATION STATUS AND TRENDS

1. Historical Trends

Historical records indicate that nesting western snowy plovers were once more widely distributed and abundant in coastal Washington, Oregon, and California.

a. Washington Coast

In Washington, western snowy plovers formerly nested at five coastal locations (Washington Department of Fish and Wildlife 1995). Three of these sites have had active nesting in recent years, as summarized in Table 1. One new site was also recently discovered in 2006. Populations appear to have increased overall since the early 1990s, although consistent, intensive surveys have been conducted only since the mid-1990s. Quantitative comparisons prior to that are not possible because of the inconsistency in surveys. Estimated numbers of breeding adults (Table 1) substantially exceed window survey data (M. Jensen *in litt.* 2006), partially because of adverse weather during window survey periods in recent years.

i. Grays Harbor County

Copalis Spit (WA-1) held 6 to 12 western snowy plover pairs in the late 1950s or early 1960s (Washington Department of Fish and Wildlife 1995). No other information on breeding at Copalis Spit is available. Suitable habitat was judged capable of supporting four pairs in 1984 (Washington Department of Fish and Wildlife 1995). Periodic surveys since 1983 have revealed just a single western snowy plover (Washington Department of Fish and Wildlife unpubl. data). Two post season juvenile western snowy plovers were observed at Copalis Spit in 2001 (Sundstrom 2002*a*). There is no longer vehicle access to the site since the road washed out several years ago, which has reduced the potential for disturbance from recreational activities. Erosion caused by the northward shift of Connor

Creek has reduced the amount of habitat, but some suitable habitat remains at the end of the spit and the area has potential as a nesting site with habitat restoration and public education (U.S. Fish and Wildlife Service 2005, M. Jensen *in litt.* 2006).

Damon Point and Oyhut Wildlife Area (WA-2) lack western snowy plover records prior to 1971, but this is likely due to limited visitation rather than western snowy plover absence. Between 1971 and 1983, birders reported up to six western snowy plovers during infrequent visits-to Damon Point (Washington Department of Fish and Wildlife 1995). Western snowy plover research in 1985 and 1986 revealed up to 20 western snowy plovers and 8 nests at Damon Point (Anthony 1987). Although most of the locality is suitable habitat, increasing levels of public use have reduced the secure nesting areas to a small portion of the site that is difficult to access, and the breeding population has declined over the last two decades (M. Jensen *in litt.* 2006). From 1993 to 2006 the number of adults at Damon Point has ranged from 2 to 10 (Table 1). Only one nest was found in 2006 (Pearson *et al.* 2006).

Westport Spit (WA-3) held low numbers of western snowy plovers from before 1915 until at least 1968, and scientific collecting was concentrated there through 1934 (Washington Department of Fish and Wildlife 1995). A single nest, poorly documented, was reported in 1983 (Washington Department of Fish and Wildlife unpublished data). No other quantitative information on abundance or nesting is available for this site. Erosion of the site has rendered the beach too narrow to support successful nesting, and there is little opportunity for habitat restoration through beachgrass removal due to private ownership of upland dune habitat (M. Jensen *in litt.* 2006). Recreational use is also substantial. This location is no longer being surveyed due to lack of suitable habitat.

ii. Pacific County

Midway Beach (WA-4) and Cape Shoalwater once contained several hundred acres of suitable western snowy plover habitat, but the area lacks historical records of these birds except for specimens collected in 1914 and 1960 and labeled "Tokeland" (Washington Department of Fish and Wildlife 1995). In

recent years, Midway Beach has been accreting sand and creating high quality habitat. Recent nesting was first documented in 1998 (Richardson *et al.* 2000). Numbers of breeding adults have increased since 1998, and during 2003-2006 the numbers of adults during the breeding season have ranged from 23-33, with a peak number of 30 nests (M. Jensen *in litt.* 2006; Pearson *et al.* 2006). Approximately one third of the habitat is on State Park land with controlled access; on the privately owned land recreational disturbance is fairly high and contributes to high rates of nest failure.

In 2006, western snowy plovers were discovered nesting on Graveyard Spit in northern Willapa Bay, which is primarily on the Shoalwater Indian Reservation and State lands (M. Jensen *in litt.* 2006; Pearson *et al.* 2006). Three pairs of plovers used the spit in 2006 and produced three fledglings.

Leadbetter Point (WA-5) was rarely visited by western snowy plover observers prior to 1964. In the 1960s and 1970s, birders reported up to 35 western snowy plovers, with nesting confirmed in 1967 by the sighting of two chicks (Washington Department of Fish and Wildlife 1995). Western snowy plover numbers were estimated at up to 24 individuals and between 7 and 11 nests during surveys done between 1978 to 1997 (Widrig 1980, 1981; Willapa National Wildlife Refuge unpublished data; Williamson 1995, 1996, 1997). Numbers increased slightly from 1998-2006, with numbers ranging from 24 to 45 adults present (Table 1). The distribution of nesting by western snowy plovers has changed, however, with recent habitat loss from erosion on the tip of Leadbetter Point and shifting of nesting southwards. Since 2002 the refuge has cleared 25 hectares (63 acres) of non-native beachgrass and the habitat restoration site has been consistently used by nesting plovers. Western snowy plovers are also nesting in Leadbetter State Park and State-owned lands south of the Park. Use of predator exclosures at the refuge since 2004 has greatly improved hatching success in the habitat restoration area and outer beach. Gunpowder Sands Island became intertidal in 2001 and no longer is suitable for nesting western snowy plovers (K. Brennan *in litt.* 2006).

Table 1. Status of western snowy plovers at four nesting sites in Washington (Sundstrom-Bagley *et. al*. 2000; Jaques 2001; Sundstrom 2001, 2002*a*, 2002*b*, 2003, 2004, 2005; Brennan and Jaques 2002; Brennan 2003; Brennan and Fernandez 2004*a*, 2004*b*, 2006; Pearson *et al.* 2006).

Year	Estimated Number of Adults Present				
	Leadbetter Point	Midway Beach	Damon Point	Graveyard Spit	Total
1993	16	-	7	-	23
1994	13	-	6	-	19
1995	25	0	9	-	34
1996	19	0	4	-	23
1997	21	0	3	-	24
1998	45	6	5	-	56
1999	26	12	5	-	43
2000	25	21	4	-	50
2001	27	14	4	-	45
2002	32	23	4	-	59
2003	30	33	5	-	68
2004	24	19	10	-	53
2005	38	25	5	-	68
2006	39	23	2	6	70

b. Oregon Coast

In Oregon, western snowy plovers historically nested at over 20 sites on the coast. At present only seven core nesting sites are consistently used, with a few additional areas occupied during some years (Lauten *et al*. 2006*a*, 2006*b*). Annual window surveys of western snowy plovers in Oregon (Table 2), including both adults and young of the year, began in 1978, with counts ranging from a high of 139 at 13 sites (1981) to a low of 30 observed at 9 sites (1992). Populations reached a low from 1991 to 1993 with a mean of 33 individuals recorded annually. From 1994 to 2006 western snowy plover numbers have generally

Table 2. Number of adult western snowy plovers observed on window surveys of the Oregon coast during the breeding season (1978-2006). Window surveys record the number of birds seen during 1-day censuses in May to June (Lauten *et al.* 2006*a*, 2006*b*).

Year	Number	Year	Number
1978	93	1993	45
1979	100	1994	51
1980	80	1995	64
1981	139	1996	85
1982	78	1997	73
1983	52	1998	57
1984	46	1999	49
1985	48	2000	no surveys conducted
1986	73	2001	71
1987	61	2002	71
1988	53	2003	63
1989	58	2004	82
1990	59	2005	100
1991	35	2006	91
1992	30		

increased, with an average of 71 plovers observed. The increase in the numbers of plovers observed in recent years is believed to be related to intensive management that began at the time of Federal listing.

Since 1993, the population on the Oregon coast has been intensively monitored, with many of the adults and chicks being uniquely color-banded. The presence of marked birds has allowed for the development of two other means of estimating the population (Table 3, Lauten *et al.* 2006b). The number of western snowy plovers, as indicated by the three indices in Table 3, has increased between 1993 and 1997, declined in 1998/1999, then increased again through 2006. The trends

Table 3. Comparison of population estimates of adult western snowy plovers on the Oregon coast during the breeding season (1993 to 2005) based on three different measures of abundance (Lauten *et al.* 2006*a*, 2006*b*).

Year	Estimates		
	A	B	C
1993	45	55 to 61	72
1994	51	67	83
1995	64	94	120
1996	85	110 to 113	134 to 137
1997	73	106 to 110	141
1998	57	75	97
1999	45	77	95 to 96
2000	no survey	89	109
2001	71	79 to 80	111 to 113
2002	71	80	99 to 102
2003	63	93	102 to 107
2004	82	120	136 to 142
2005	100	104	153 to 158
2006	91	135	177 to 179

A = Window census.

B = Estimated number of breeding adults. This number is lower than those in column C because it is an estimate of the number of individual birds thought to be breeding birds.

C = Total number of individual adults present during breeding season (includes depredated adults).

for all three indices remained relatively consistent throughout that measurement period.

Management measures (Lauten *et al.* 2006a, 2006b) have included the use of exclosures to reduce predation, predator control measures, restoration of breeding habitat by removing European beachgrass (*Ammophila arenaria*), increased presence of law enforcement personnel, additional and improved signs, additional symbolic fencing (consisting of one or two strands of light-weight string or cable

tied between posts to delineate areas where pedestrians and vehicles should not enter), and increased efforts on public information and education.

c. California Coast

i. Coastwide Perspective

In California, there also has been a significant decline in breeding locations, especially in southern California. By the late 1970s, nesting western snowy plovers were absent from 33 of 53 locations with breeding records prior to 1970 (Page and Stenzel 1981). The first quantitative data on the abundance of western snowy plovers along the California coast came from window surveys conducted during the 1977 to 1980 breeding seasons by Point Reyes Bird Observatory (Page and Stenzel 1981). An estimated 1,593 adult western snowy plovers were seen on these pioneer surveys (Table 4). The surveys suggested that the western snowy plover had disappeared from significant parts of its coastal California breeding range by 1980. It no longer bred along the beach at Mission Bay or at Buena Vista Lagoon in San Diego County. In Orange County, the only remaining breeding location was the Bolsa Chica wetlands; historically, the western snowy plover was known to breed along the beach from Upper Newport Bay to Anaheim Bay. It was absent from Los Angeles County where it formerly nested along the shores of Santa Monica Bay. In Ventura County, it had ceased breeding on Ventura Beach (San Buenaventura Beach), and in Santa Barbara County on Carpinteria, Santa Barbara (East Beach), and Goleta Beaches. Nesting no longer occurred along the northernmost portion of Monterey Bay in Santa Cruz County or on Doran Beach at Bodega Harbor in Sonoma County.

Subsequent coast-wide surveys by Point Reyes Bird Observatory in 1989 and 1991 indicated a further decline in numbers of breeding adult western snowy plovers during the decade after the 1977 to 1980 survey. Along the mainland coast, including the shores of the Channel Islands, western snowy plover populations had declined by about 5 percent, and in San Francisco Bay by about 44 percent (Table 4).

Table 4. Number of adult western snowy plovers observed during breeding season window surveys of the California coast.

Location	1977/80	1989	1991	1995	2000	2002	2003	2004	2005	2006
Del Norte County	11	8	3	0	0	0	0	0	0	0
Humboldt County	54	32	30	19	39	49	38	37	32	49
Mendocino County	15	2	0	-	1	0	1	3	9	3
Sonoma County	0	10	9	3	0	0	0	0	5	0
Marin County	40	24	25	8	21	25	17	26	22	16
San Mateo County (incl. SF beaches)	4	8	1	-	4	3	4	17	3	7
Northern Santa Cruz County	25	19	22	26	19	9	2	2	3	4
Monterey Bay	146	146	119	125	120	270	279	331	297	317
Point Sur	3	4	-	-	8	5	6	5	7	13
Northern San Luis Obispo County			9	-	1	3	0	3	12	15
Morro Bay Area	80	126	87	85	113	150	172	268	259	167
Pismo Beach/Santa Maria River	45	123	246	124	81	170	137	167	200	211
Vandenberg AFB	119	115	242	213	106	179	256	420	259	245
Jalama Beach	0	1	1	0	0	0	0	0	0	0
Hollister Ranch			8	-	-	-	-	-	-	-
Coal Oil Point (Devereaux) vicinity			-	-	-	8	26	30	30	39
Oxnard Lowland	136	175	105	69	107	164	80	119	110	125
Channel Islands	(288)[1]	217	200	196	89	79	90	82	99	115
Orange County	19	21	5	9	27	38	31	31	66	62
Northern San Diego County	160	72	48	49	63	80	145	159	107	141
Mission Beach			-	-	-	-	-	1	0	-
San Diego Bay	60	36	31	33	73	61	76	76	30	81
Tijuana Estuary	37	21	4	10	8	16	12	14	6	14
Subtotal	**1,242**	**1,160**	**1,195**	**969**	**880**	**1,309**	**1,372**	**1,791**	**1,556**	**1,624**
S San Francisco Bay	351	216	176	-	96	78	72	113	124	99
Total	**1,593**	**1,376**	**1,371**	**-**	**976**	**1,387**	**1,444**	**1,904**	**1,680**	**1,723**

[1] 260 adults during the survey; 28 additional adults extrapolated for unsurveyed portions of Santa Rosa Island.

The more recent coast-wide surveys, during the summers of 1995, 2000, and 2002-2006, were accomplished through the collaboration of researchers studying western snowy plovers along the California coast. Between the 1977 to 1980 surveys and the 1995 survey, western snowy plovers apparently ceased nesting at Los Penasquitos, and Agua Hedionda Lagoons in northern San Diego County (A. Powell, pers. comm. 1998). Nesting has been absent or sporadic at San Elijo Lagoon; Año Nuevo State Beach and Pescadero State Beach in San Mateo County; Bolinas Lagoon in Marin County; the south and north spits of Humboldt Bay and Big Lagoon in Humboldt County; and the Lake Talawa region of Del Norte County (Point Reyes Bird Observatory, unpublished data).

By 2000 populations had declined further to 71 percent of the 1977-1980 levels along the California coast and 27 percent of the 1977-1980 levels in San Francisco Bay. However, since then populations have grown substantially, roughly doubling along the coast while fluctuating irregularly in San Francisco Bay (Table 4). Recent population increases along the coast have been associated with implementation of management actions for the benefit of western snowy plovers and California least terns, including predator management and protection and restoration of habitat.

ii. Regional Perspective

Del Norte, Humboldt, and Mendocino Counties - Numbers of western snowy plover breeding adults declined and then somewhat rebounded in this northern California region since the initial Point Reyes Bird Observatory survey in 1977. In this region where there were 80 adults counted in 1977, a low of 19 were found in 1995 and 52 in 2006. In 1996, breeding was documented on the gravel bars of the Eel River, Humboldt County, and this area has continued to be a successful nesting site for western snowy plover breeding (Colwell *et al.* 2002, 2005). Even with the nest success at the gravel bars there is still a reduction in western snowy plovers from 1977; Del Norte County has no breeding birds, and Mendocino County has very few.

San Francisco Bay - As indicated in Table 4, western snowy plover numbers in San Francisco Bay declined markedly between the initial survey in 1978 and

follow-up surveys. Western snowy plover numbers steadily declined over 26 years, reaching a low of 72 in 2003, followed by a moderate but irregular increase (124 in 2005 surveys; 99 in 2006).

Recent surveys in South San Francisco Bay (Strong and Dakin 2004, Strong *et al.* 2004, Tucci *et al.* 2006) indicate that the largest breeding populations are concentrated at Eden Landing Ecological Reserve/Baumberg North (CA-33), managed by California Department of Fish and Game. Other population centers occur at Oliver Salt Ponds (CA-31), managed by Hayward Area Recreation District and East Bay Regional Parks District; and at Dumbarton (CA-36), Warm Springs (CA-39), Alviso (CA-41), and Ravenswood (CA-44), managed by Don Edwards San Francisco Bay National Wildlife Refuge. Foraging and nesting activities are concentrated in specific salt ponds within these areas. Small numbers of western snowy plovers have been observed at Ponds 7 and 7A in Napa County (CA-25 and vicinity), the only currently known nesting site in the North Bay.

Sonoma, Marin, San Francisco, San Mateo, Santa Cruz, and Monterey Counties - Along the segment of coastline from Sonoma County to Monterey Bay, numbers of western snowy plover adults during window surveys declined from 215 in 1977 to 162 in 1995, and subsequently increased to a maximum of 376 in 2004. The numbers of adults breeding on the beaches and salt ponds of Monterey Bay, and the beaches of northern Santa Cruz County, has increased dramatically since management actions have been undertaken to increase nesting success (Neuman *et al.* 2004; G. Page *in litt.* 2004*b*)

San Luis Obispo, Santa Barbara, and Ventura Counties, including Channel Islands - There is no clear evidence of an overall decline in the number of breeding western snowy plovers for this region from 1978/1980 to the present. Numbers of adults fluctuated between a high of 1089 and a low of 497 between 1978 and 2006. While numbers for the region may not have changed overall, there have been definite changes at specific locations (Table 5). Most notable are the decline and loss of the population on San Miguel Island from 1978 /1980 to 2000, the decline at Santa Rosa Island from 1991 to 2006, and the sudden increase in numbers at Vandenberg Air Force Base between 2000 and 2004 and at Coal Oil Point Reserve between 2002 and 2006 (Table 4).

Table 5. Breeding season window surveys of western snowy plover adults at selected sites along the coast of San Luis Obispo, Santa Barbara, and Ventura Counties.

Location	Year												
	1978 -80	**1989**	**1991**	**1995**	**1996**	**1997**	**1998**	**2000**	**2002**	**2003**	**2004**	**2005**	**2006**
Atascadero Beach	0	17	2	38	28	23	26	5	19	23	21	21	24
Morro Bay Spit	80	94	69	34	40	39	55	87	93	114	203	205	120
Vandenberg AFB [1]	119	115	242	213	230	238	130	106	179	256	420	259	245
Ormond Beach	25	24	34	20	19	34	19	10	35	19	28	21	22
Naval Base Ventura County (Pt. Mugu)	82	81	59	40	49	26	47	81	85	51	75	83	79
Santa Rosa Island [2]	84	91	103	71	78	79	76	17	10	---	---	37	19
San Miguel Island [2]	133	36	19	9	3	5	1	0	0	0	0	---	0
San Nicolas Island [3]	71	90	78	116	104	91	90	72	69	90	79	62	96
Total	594	548	606	541	551	535	444	378	490	553	826	688	605

Unless footnoted, the source of all data is Point Reyes Bird Observatory.

[1] The source of this data is the U.S. Air Force (Phil Persons)

[2] The source of this data is the National Park Service

[3] The source of this data is the U.S. Navy

Los Angeles, Orange, and San Diego Counties - Western snowy plover numbers detected during window surveys declined from the 276 adults tallied during the 1978 Point Reyes Bird Observatory survey to 88 during the 1991 survey. Subsequently the population has increased to 298 in 2006.

2. Current Breeding Distribution

The current Pacific coast breeding range of the western snowy plover extends from Damon Point, Washington, to Bahia Magdelena, Baja California, Mexico. The population is sparse in Washington, Oregon, and northern California. In 2006, estimated populations were 70 adults along the Washington coast (Pearson *et al*. 2006), 177-179 adults along coastal Oregon (Lauten *et al.* 2006*b*), and 2,231 adults in coastal California and San Francisco Bay (window survey including correction factor: G. Page *in litt.* 2006, U.S. Fish and Wildlife Service 2006*a*). Approximately 7 percent of the California population was observed in San Francisco Bay, and 4 percent in northern California north of the Golden Gate bridge. Along the coast of Baja California, Mexico, most nesting western snowy plovers are associated with the largest wetlands, especially Bahia San Quintin, Laguna Ojo de Liebre, and Bahia Magdelena (Palacios *et al*. 1994). No recent quantitative data exist on the western snowy plover population in Baja California, but it is probably roughly similar in size to the U.S. Pacific coast population.

3. Habitat Carrying Capacity

There is no quantitative information on carrying capacity of beaches for western snowy plovers. Determining carrying capacity of beaches is confounded by human use that affects the numbers of snowy plovers using the beaches. Beaches vary substantially in their structure, width, vegetation, and level of human use, complicating such a measurement.

The maximum reported breeding density of western snowy plovers is associated with the Moss Landing Wildlife Area, where since 1995 Point Reyes Bird Observatory staff have conducted intensive management specifically for western snowy plovers. These measures include predator control, removal of excessive vegetation, and operation of water control structures to maintain desired water

levels. With extensive management of approximately 55 hectares (138 acres) of mostly dried ponds in the Moss Landing Wildlife Area, 25 active nests, 3 pairs within 5 days of initiating nests, and 10 broods have been documented simultaneously; thus a peak of 76 nesting adults was accommodated simultaneously by 55 hectares (138 acres) of playa, or 1.4 hectares (3.6 acres) per functional pair (some of the broods were only being cared for by males) (D. George, Point Reyes Bird Observatory, pers. comm.). However, the numbers of nesting western snowy plovers at the Moss Landing Wildlife Area cannot be applied to beach areas because of the physical differences between salt pond and beach habitats and because beach habitats are typically subject to much more human disturbance. Neither can these numbers necessarily be applied to other salt ponds (*e.g.*, San Francisco Bay) because habitat and management opportunities differ.

D. REASONS FOR DECLINE AND CONTINUING THREATS

Overall, western snowy plover numbers have declined on the U.S. Pacific coast over the past century (see Population Status and Trends section). The subspecies faces multiple threats throughout its Pacific coast range. The reasons for decline and degree of threats vary by geographic location; however, the primary threat is habitat destruction and degradation. Habitat loss and degradation can be primarily attributed to human disturbance, urban development, introduced beachgrass (*Ammophila* spp.), and expanding predator populations. Natural factors, such as inclement weather, have also affected the quality and quantity of western snowy plover habitat (U.S. Fish and Wildlife Service 1993*a*). The following discussion is organized according to the five listing criteria under section 4(a)(1) of the Endangered Species Act.

1. The Present or Threatened Destruction, Modification, or Curtailment of Habitat or Range

a. Shoreline Stabilization and Development

The wide, flat, sparsely-vegetated beach strands preferred by western snowy plovers are an unstable habitat, subject to the dynamic processes of accretion and erosion of sand, and dependent on natural forces for replenishment and renewal.

These habitats are highly susceptible to degradation by construction of seawalls, breakwaters, jetties, piers, homes, hotels, parking lots, access roads, trails, bike paths, day-use parks, marinas, ferry terminals, recreational facilities, and support services that may cause direct and indirect losses of breeding and wintering habitat for the western snowy plover.

Beach stabilization efforts may interfere with coastal dune formation and cause beach erosion and loss of western snowy plover nesting and wintering habitat. Shoreline stabilization features such as jetties and groins may cause significant habitat degradation by robbing sand from the downdrift shoreline (U.S. Fish and Wildlife Service 1996*a*). However, jetties also can redirect sand deposition, causing an increase in available habitat. Construction of homes, resorts, and parking lots on coastal sand dunes constitutes irrevocable loss of habitat for western snowy plovers. Urban development has permanently eliminated valuable nesting habitat on beaches in southern Washington (Brittell *et al.* 1976), Oregon (Oregon Department of Fish and Wildlife 1994), and California (Page and Stenzel 1981). In addition to causing direct loss of habitat, there are additional potential adverse impacts to western snowy plovers from urban development (Figure 5). Increased development increases human use of the beach, thereby increasing disturbance to nesting plovers. When urban areas interface with natural habitat areas, the value of breeding and wintering habitat to native species may be diminished by increased levels of illumination at night (*e.g.*, building and parking lot lights); increased sound and vibration levels; and pollution drift (*e.g.*, pesticides) (Kelly and Rotenberry 1996/1997). Beach raking removes habitat features for both plovers and their prey, and precludes nests from being established. Also, construction of residential development in or near western snowy plover habitat attracts predators, including domestic cats.

b. Resource Extraction

i. Sand Removal and Beach Nourishment

Sand is mined in coastal areas such as Monterey Bay. Mining sand from the coastal mid-dunes and surf zone can cause erosion and loss of western snowy plover breeding and wintering habitat. Sand removal by heavy machinery can disturb

Figure 5. New housing development next to beach at Monterey Bay, California (photo by Peter Baye, with permission).

incubating western snowy plovers, destroy their nests or chicks, and result in the loss of invertebrates and natural wave-cast kelp and other debris that western snowy plovers use for foraging. Mining of surface sand from the 1930s through the 1970s at Spanish Bay in Monterey County degraded a network of dunes by lowering the surface elevations, removing sand to granite bedrock in many locations, and creating impervious surfaces that supported little to no native vegetation (Guinon 1988).

Beach nourishment with sand can be beneficial for the western snowy plover if it results in an increase in habitat. However, unless beach nourishment projects are properly designed, they can result in changes to beach slope from redeposition of sediments by storm waves, and result in the loss of western snowy plover breeding and wintering habitat. For example, if an inappropriate size class of sand (*e.g.*, coarser-grained sand) and range of minerals are introduced that are different from the current composition of native sand on a beach, it can alter dune slope (making it steeper or narrower), affect mobility and color of sand, decrease the abundance of beach invertebrates, and facilitate establishment of invasive exotic plants that may

have a competitive advantage over native plants. Feeney and Maffei (1991) investigated the color hues of the ground surface within San Francisco Bay salt ponds used as western snowy plover nesting habitat. Predominant soils were silty clay with varying amounts of humus, salt crystals, and shell fragments. They found a strong similarity between the color of the substrate in habitat preferred by western snowy plovers and the color of western snowy plover mantles (upper parts).

ii. Dredging and Disposal of Dredged Materials

Dredging is detrimental to western snowy plovers when it eliminates habitat or alters natural patterns of beach erosion and deposition that maintain habitat. Disturbances associated with dredging, such as placement of pipes, disposal of dredged materials, or noise, also may negatively affect breeding and wintering western snowy plovers. Dredging also is detrimental when it promotes water-oriented developments that increase recreational access to western snowy plover habitat (*e.g.*, marinas, boat ramps, or other facilities to support water-based recreation). In some cases, however, dredged materials may provide important nesting habitat for western snowy plovers such as those at Coos Bay, Oregon (Wilson-Jacobs and Dorsey 1985). Western snowy plovers also have been observed using dredged material during the winter; however, these areas are not used nearly as often as the adjacent ocean beach (E.Y. Zielinski and R.W. Williams *in litt.* 1999).

iii. Driftwood Removal

Driftwood can be an important component of western snowy plover breeding and wintering habitat. Driftwood contributes to dune-building and adds organic matter to the sand as it decays (Washington Department of Fish and Wildlife 1995). Additionally, driftwood provides western snowy plovers with year-round protection from wind and blowing sand. Often, western snowy plovers build nests beside driftwood, so its removal may reduce the number of suitable nesting sites.

Driftwood removed for firewood or decorative items can result in destruction of nests and newly-hatched chicks that frequently crouch by driftwood to hide from predators and people. Chainsaw noise may disrupt nesting, and vehicles used to

haul wood may crush nests and chicks. Removal of driftwood has been documented as a source of nest destruction at Vandenberg Air Force Base where two nests were crushed beneath driftwood dragged to beach fire sites (Persons 1994). Also, driftwood beach structures built by visitors are used by avian predators of western snowy plover chicks such as loggerhead shrikes (*Lanius ludovicianus*) and American kestrels (*Falco sparverius*), and predators of adults such as merlins (*Falco columbarius*) and peregrine falcons (*Falco peregrinus*).

Although driftwood is an important component of western snowy plover habitat, too much driftwood on a beach, which may occur after frequent and prolonged storm events, can be detrimental if there is not sufficient open habitat to induce the birds to nest.

iv. Beach Fires and Camping

Beach fires and camping may be harmful to nesting western snowy plovers when valuable driftwood is destroyed, as described above. Camping near breeding locations can cause greater impacts due to the prolonged disturbance and increased chance for possible direct mortality from associated dogs and children (S. Richardson *in litt.* 2001). Nighttime collecting of wood increases the risk of stepping on nests and chicks, which are difficult to see even during daylight hours. Fires near a western snowy plover nest could cause nest abandonment due to disturbance from human activities, light, and smoke. Fires have the potential to attract large groups of people and result in an increase of garbage, which attracts scavengers such as gulls (*Larus* spp.) and predators such as coyotes (*Canis latrans*), American crows (*Corvus brachyrhynchos*), and common ravens (*Corvus corax*). Also, after fires are abandoned, predators such as coyotes may be attracted into the area by odors lingering from the fire, particularly if it was used for cooking. Occasionally fires escape into nearby driftwood; fire suppression activities may disturb and threaten western snowy plover nests and chicks.

v. Watercourse Diversion, Impoundment, or Stabilization

Water diversion and impoundment of creeks and rivers may negatively affect western snowy plover habitat by reducing sand delivery to beaches and degrading

water quality. Water diversions are a major threat to western snowy plovers when they impair hydrologic processes (such as migration of creek and river mouths) that maintain open habitat at river and creek mouths by retarding the spread of introduced beachgrass (*Ammophila* spp.) and other vegetation. Water diversion, impoundment, or stabilization activities could include construction of dams and irrigation, flood control, and municipal water development projects (Powell *et al.* 2002).

vi. Operation of Salt Ponds

Salt ponds of San Francisco Bay and San Diego Bay, which are filled and drained as part of the salt production process, provide breeding and wintering habitat for western snowy plovers. Dry salt ponds and unvegetated salt pond levees are used as western snowy plover nesting habitat. Ponds with shallow water provide important foraging habitat for western snowy plovers, with ponds of low and medium salinity providing the highest invertebrate densities. Ponds of high salinity have reduced invertebrate densities and therefore provide lower quality foraging habitat. Nesting western snowy plovers can be attracted to an area when ponds are drained during the breeding season, but flooding can then destroy the nests when the ponds are refilled. Also, human disturbance resulting from maintenance activities associated with the operation of commercial salt ponds can result in the loss of western snowy plovers and disturbance of their habitat. If conducted during the western snowy plover breeding season, reconstruction of salt pond levees could destroy western snowy plover nests. Maintenance activities that are conducted by vehicles, on foot, or through the use of dredging equipment could result in direct mortality or harassment of western snowy plovers (See Dredging, Pedestrian, and Motorized Vehicle sections).

c. Encroachment of Introduced Beachgrass and Other Nonnative Vegetation

One of the most significant causes of habitat loss for coastal breeding western snowy plovers has been the encroachment of introduced European beachgrass (*Ammophila arenaria*) and American beachgrass (*Ammophila breviligulata*). Foredunes dominated by introduced beachgrass have replaced the original low, rounded, open mounds formed by the native American dunegrass (*Leymus mollis*)

and other beach plants. Native dune plants do not bind sand like *Ammophila* spp., and thus allow for sand movement and regenerating open expanses of sand. However, *Ammophila* spp. forms a dense cover that excludes many native taxa. On beaches dominated by this invasive grass, species richness of vegetation is halved, in comparison with foredunes dominated by native dune grass (Barbour and Major 1990). Similarly, American beachgrass greatly depresses the diversity of native dune plant species (Seabloom and Wiedemann 1994).

European beachgrass was introduced to the west coast around 1898 to stabilize dunes (Wiedemann 1987). Since then, it has spread up and down the coast and now is found from British Columbia to Ventura County in southern California. This invasive species is a rhizomatous grass that sprouts from root segments, with a natural ability to spread rapidly. Its most vigorous growth occurs in areas of wind-blown sand, primarily just above the high-tide line, and it thrives on burial under shifting sand. In 1988, European beachgrass was considered a major dune plant at about 50 percent of western snowy plover breeding areas in California and all of those in Oregon and Washington (J. Myers *in litt.* 1988).

American beachgrass is native to the East coast and Great Lakes region of North America. The densest populations of American beachgrass on the Pacific coast are currently located between the mouth of the Columbia River and Westport, Washington. Like European beachgrass, American beachgrass is dominant on the mobile sands of the foredune and rapidly spreads through rhizome fragments. American beachgrass occurs along the entire coast of Washington, ranging from Shi Shi Beach, Washington, in the north, to Sand Lake, Oregon, in the south, although its frequency decreases markedly at the northern and southern limits of this range. Currently, American beachgrass is the dominant introduced beachgrass species in much of the western snowy plover range in the State of Washington (Seabloom and Wiedemann 1994).

Stabilizing sand dunes with introduced beachgrass has reduced the amount of unvegetated area above the tideline, decreased the width of the beach, and increased its slope (Wiedemann 1987). These changes have reduced the amount of potential western snowy plover nesting habitat on many beaches and may hamper brood movements. In Oregon, the beachgrass community may provide habitat for western

snowy plover predators (*e.g.*, skunks [*Mephitis* spp.], weasels [*Mustela* spp.], coyotes [*Canis latrans*], foxes [*Urocyon cinereoargenteus* and *Vulpes vulpes*.], raccoons [*Procyon lotor*], and feral cats [*Felis domesticus*]) that historically would have been largely precluded by the lack of cover in the dune community (Stern *et al.* 1991; K. Palermo, U.S. Forest Service, pers. comm. 1998).

In areas with European beachgrass, it has caused the development of a vegetated foredune that effectively blocks movement of sand inland and creates conditions favorable to the establishment of dense vegetation in the deflation plain, which occurs behind the foredunes (Wiedemann *et al.* 1969). In natural sand dunes, deflation plains consist of open sand ridges and flat plains at or near the water table. Thus, in areas with European beachgrass, the open features that characterize western snowy plover breeding habitat are destroyed. The establishment of European beachgrass has also caused sand spits at the mouths of small creeks and rivers to become more stable than those without vegetation because of the creation of an elevated beach profile. This elevated profile, in effect, reduces the scouring of spits during periods of high run-off and storms. A secondary effect of dune stabilization has been human development of beaches and surrounding areas (Oregon Department of Fish and Wildlife 1994). This development, in turn, has reduced available beach habitat and focused human activities on a smaller area that must be shared with western snowy plovers and other shorebirds.

On the Oregon coast, the establishment of European beachgrass has produced dramatic changes in the landscape (Oregon Department of Fish and Wildlife 1994). The spread of this nonnative species was greatly enhanced by aggressive stabilization programs in Oregon in the 1930s and 1940s (Wiedemann 1987). European beachgrass spread profusely along the Washington coast, and was well established by the 1950s (Washington Department of Fish and Wildlife 1995). In 1988, the spread of beachgrass was termed an "increasing threat" to traditional western snowy plover nesting areas at Leadbetter Point, Washington, having become established where absent only 4 years earlier (Willapa National Wildlife Refuge 1988).

In California, there are many beaches where European beachgrass has established a foothold. These beaches include the dunes at Lake Earl, Humboldt Bay (from

Trinidad to Centerville Beach), MacKerricher State Beach/Ten Mile Dunes Preserve, Manchester State Beach, Bodega Bay, Point Reyes National Seashore, Golden Gate National Recreation Area, Monterey Bay, Morro Bay Beach, Guadalupe-Nipomo Dunes, and Vandenberg Air Force Base (A. Pickart *in litt.* 1996). Chestnut (1997) studied the spread of European beachgrass at the Guadalupe-Nipomo Dunes in San Luis Obispo County. He documented an increase in beachgrass from approximately 8 to 109 hectares (20 to 270 acres) between 1969 and 1997, and found that its rapid spread through native vegetation posed a serious threat to nesting western snowy plovers and rare plants.

In addition to the loss of nesting habitat, introduced beachgrass also may adversely affect western snowy plover food sources. Slobodchikoff and Doyen (1977) found that beachgrass markedly depressed the diversity and abundance of sand-burrowing arthropods at coastal dune sites in central California. Because western snowy plovers often feed on insects well above the high-tide line, the presence of this invasive grass may also result in loss of food supplies for plovers (Stenzel *et al.* 1981).

In some areas of California, such as the Santa Margarita River in San Diego County, and the Santa Clara and Ventura Rivers in Ventura County, giant reed (*Arundo donax*) has become a problem along riparian zones. During winter storms, giant reed is washed downstream and deposited at the river mouths where western snowy plovers nest (Powell *et al.* 1997). Large piles of dead and sprouting giant reed eliminate nesting sites and increase the presence of predators, which use it as perches and prey on rodents in the piles of vegetation.

Other nonnative vegetation that has invaded coastal dunes, thereby reducing western snowy plover breeding habitat, includes Scotch broom (*Cytisus scoparius*), gorse (*Ulex europaeus*), South African iceplant (*Carpobrotus edulis*), pampas grass (*Cortaderia jubata* and *Cortaderia selloana*) and iceplant (*Mesembryanthemum* sp.); shore pine (*Pinus contorta*) is a native plant species that has invaded coastal dunes and resulted in similar impacts to western snowy plovers (Schwendiman 1975, California Native Plant Society 1996, Powell 1996). Many nonnative weed species also occur on and along San Francisco Bay salt pond levees, resulting in unsuitable nesting habitat for western snowy plovers (J. Albertson *in litt.* 1999).

d. Habitat Conversion for Other Special Status Species

It is not known whether western snowy plovers historically nested in San Francisco Bay prior to the construction of salt evaporator ponds beginning in 1860 (Ryan and Parkin 1998). However, western snowy plovers have wintered on the San Francisco Bay since at least the late 1800's, as indicated by a specimen dated November 8, 1889, in the California Museum of Vertebrate Zoology (Grinnell *et al.* 1918). It is possible that natural salt ponds in the vicinity of San Lorenzo once supported nesting birds, but insufficient data exist to assess this possibility (U.S. Fish and Wildlife Service 1992). Today, however, the San Francisco Bay recovery unit supports an important western snowy plover source population, representing approximately 5 to 10 percent of the total breeding population. Feeney and Maffei (1991) observed a sizable population of western snowy plovers at the Baumberg and Oliver salt ponds during the breeding and nonbreeding seasons, suggesting that these ponds are important to western snowy plovers throughout the year. They suspected that these ponds are used by western snowy plovers as both a pre-breeding and post-breeding staging area, based on the high numbers of plovers in mid-February and in late August/September, respectively.

As part of the Recovery Plan for Tidal Marsh Ecosystems of Northern and Central California (U.S. Fish and Wildlife Service, in preparation), extensive tidal marsh restoration is identified as a recovery action for listed and other sensitive species of tidal salt marshes including the California clapper rail (*Rallus longirostris obsoletus*) and salt marsh harvest mouse (*Reithrodontomys raviventris*). A large area of San Francisco Bay salt ponds, especially within the South Bay, are proposed for tidal marsh restoration for the benefit of federally listed tidal marsh species. Salt ponds are large, persistent hypersaline ponds that are intermittently flooded with South Bay water. Some of these ponds currently provide valuable breeding and wintering habitat for western snowy plovers. However, they occur within the historical areas of tidal salt marsh, which once dominated San Francisco Bay. Endangered tidal marsh species would benefit from conversion of these ponds back to salt marsh; however, western snowy plovers would lose suitable nesting and wintering areas.

The Recovery Plan for Tidal Marsh Ecosystems of Northern and Central California will focus primarily on management of tidal marsh species, but will also provide for some areas to be maintained as managed ponds that would provide habitat for western snowy plovers and California least terns (*Sternula antillarum browni*). The South Bay Salt Pond Restoration Project (Philip Williams & Associates *et al.* 2006) has identified sites on National Wildlife Refuge and California Department of Fish and Game lands with potential for salt marsh restoration and managed ponds under a range of alternatives; the projected area of managed ponds ranges from 647 to 3,035 hectares (1,600 to 7,500 acres). Six of the plover locations identified in Appendices B and L (CA-33, CA-34, CA-39, CA-40, CA-41, CA-44) occur within the South Bay Salt Pond Restoration Project area. These six locations comprise about 60 percent of the western snowy plover locations in San Francisco Bay by area, and currently support over 90 percent of the western snowy plover population in San Francisco Bay (Strong *et al.* 2004, Tucci *et al.* 2006). In particular, several salt ponds at Eden Landing (location CA-33 and vicinity) currently support the largest population of western snowy plovers in San Francisco Bay. Distribution of plover populations and nesting sites within San Francisco Bay can fluctuate with salt pond management and availability of appropriate habitat, such that some locations identified in Appendix L are not currently occupied and other locations not mapped in Appendix L may nonetheless support breeding birds as management practices change. Thus the boundaries of San Francisco Bay locations as mapped in Appendix L reflect current and historical conditions and should be considered as flexible in the context of planning for future tidal marsh restoration. Specific localities to be managed for plovers should be coordinated with tidal marsh restoration in an integrated fashion, and thus may not be identical with the current or historical localities identified in this recovery plan.

Thus intensive management of designated ponds within the South Bay Salt Pond Restoration Project area will be crucial to achieving success in meeting western snowy plover recovery goals in San Francisco Bay. However, establishing western snowy plover populations at a variety of sites in San Francisco Bay, both within and outside the South Bay Salt Pond Restoration Project area, is advisable to minimize their vulnerability to loss (L. Trulio *in litt.* 2007). Potential western snowy plover habitat in San Francisco Bay outside of the South Bay Salt Pond Restoration Project area includes several sites around Alameda, Napa County, Hayward Shoreline, and

Crissy Field. In addition, large salt pond tracts in the South Bay remain under the ownership of Cargill; certain areas are still managed for salt production and could incidentally provide habitat for western snowy plovers, while approximately 600 hectares (1,400 acres) of ponds near Redwood City are no longer in salt production and provide an opportunity for significantly increasing western snowy plover habitat through active management. If these locations can be managed to encourage western snowy plover nesting, they may contribute substantially to meeting the overall goal of 500 breeding birds in San Francisco Bay. Western snowy plover management targets for the South Bay Salt Pond Restoration Project should take into account the habitat quality and management potential of plover habitat elsewhere in San Francisco Bay to meet overall goals for the recovery unit.

Don Edwards San Francisco Bay National Wildlife Refuge is currently planning pilot studies to assess how best to manage salt ponds for high densities of breeding western snowy plovers. Special management for western snowy plover may include intensive control of avian predators (*e.g.*, California gull colonies, ravens); active management of water levels to control vegetation, maintain optimal salinity, and produce brine flies; timing of inundation to avoid flooding nests; and reconfiguration of shallow salt ponds with isolated islands and furrowed areas. Locations of managed salt ponds should be planned to minimize the proximity of western snowy plover populations to landfills, gull colonies, and areas with high predator densities. Intensive management of salt ponds for western snowy plovers generally appears feasible, and plovers have been observed to opportunistically disperse among sites and use habitat that becomes suitable (V. Bloom *in litt.* 2005), so we expect relocation of plover nesting concentrations away from tidal marsh restoration areas to be possible, but management success should be carefully evaluated. Those alternatives with greater acreages of tidal marsh restoration (e.g., Alternative C at 90 percent tidal habitat) would require correspondingly more intensive management and reconfiguration of the remaining salt ponds (Philip Williams & Associates *et al.* 2006), and should be implemented gradually in conjunction with evaluation of management effectiveness for western snowy plovers.

Thus, we believe tidal marsh restoration can be compatible with the recovery of western snowy plovers and should not preclude meeting a goal of 500 breeding

birds in San Francisco Bay. As described below under Recovery Action 2.6, occupied salt ponds should initially be conserved. Salt marsh restoration in occupied plover habitat, particularly at densely populated sites, should be phased in after intensive adaptive management of other compensating salt pond habitat has demonstrated success in increasing plover populations. Thus habitat quality should be continually assessed so that overall western snowy plover populations in San Francisco Bay are not adversely affected by the restoration project and can increase to meet the management goal for this recovery unit.

In southern California, unless carefully planned, conversion of western snowy plover habitat to tidal salt marsh may result in loss of western snowy plover habitat. The light-footed clapper rail (*Rallus longirostris levipes*) inhabits coastal tidal marshes from Santa Barbara County south to Baja California, Mexico. Several locations in Ventura, Orange, and San Diego Counties provide nesting and/or wintering habitat for western snowy plovers, but also provide high quality light-footed clapper rail habitat or represent high priority tidal marsh restoration sites in the recovery plan for the light-footed clapper rail (U.S. Fish and Wildlife Service 1985). These sites include Bolsa Chica, Agua Hedionda Lagoon, San Elijo Lagoon, San Dieguito Lagoon, and Los Penasquitos Lagoon. The Bolsa Chica wetlands were opened to tidal action in 2006, in a project combining tidal restoration work with construction of islands and sand flats for nesting of shorebirds and California least terns.

2. Overutilization for Commercial, Recreational, Scientific, or Education Purposes

Biologists and agency personnel monitor western snowy plovers to assess population status and evaluate management techniques. Additionally, nest searches at some sites allow for placement of predator exclosures that aid in hatching success. Measures to minimize disturbance from these activities include: time limits for surveys, exclosure construction and sign/rope maintenance; conducting walking surveys where feasible; and limited entries.

Egg collecting has been observed at several California nesting colonies (Stenzel *et al.* 1981, Warriner *et al.* 1986). Occasionally recreational birdwatchers also may

harass western snowy plovers. The significance of these factors to nesting success is uncertain but probably relatively minor.

Qualified individuals may obtain permits to conduct scientific research and population census activities on western snowy plovers under section 10(a)(1)(A) of the Endangered Species Act. Specific activities that may be authorized include: population censuses and presence/absence surveys; monitoring of nesting activity; capturing, handling, weighing, measuring, banding, and color-marking of young and adults on breeding and wintering grounds; radio-telemetry studies; translocation studies; genetic studies; contaminant studies; behavioral, ecological, and life history studies; and placing predator exclosures around active nests. Short-term impacts of these activities may include harassment and possible accidental injury or death of a limited number of individual western snowy plovers. The long-term impacts will be to contribute to recovery of the species by facilitating development of more precise scientific information on status, life history, and ecology (U.S. Fish and Wildlife Service 1993*b*).

Banding birds with metal and plastic bands to identify individuals and to monitor bird populations is a common practice. However, a number of leg injuries to western snowy plovers, possibly resulting from banding, have been reported (G. Page *in litt.* 2005*b*). These injuries include swelling and abrasion of legs possibly from sand or other particles becoming lodged between the bands and the leg. Some banding injuries appear to have resulted in foot loss and in a few instances, death of the bird. Similar injuries have been observed in piping plovers (*Charadrius melodus*) banded on the Atlantic coast and interior U.S., and resulted in a moratorium on banding of that species (Lingle *et. al.* 1999, U.S. Fish and Wildlife Service 1996*a*, U.S. Fish and Wildlife Service 2002). Despite leg injuries, several piping plovers were observed to successfully breed and fledge young (Lingle *et. al.* 1999). However, these injuries may contribute directly or indirectly to mortalities or reduce breeding performance. It should be noted that incidents of foot loss in Pacific coast western snowy plovers usually appear to result from fine fibers wrapping around the bird's ankle, and have occurred in unbanded as well as banded individuals (J. Watkins, pers. comm. 2006). Despite risk of injuries, banding remains the best technique to study population traits such as survival, recruitment, and dispersal, and may be the most effective way to monitor populations of the

western snowy plover to determine effectiveness of management strategies. Currently the percentage of banded birds range-wide that become injured from banding and the impacts of banding injuries on populations of the western snowy plover are unknown; a study was initiated in 2005 by Point Reyes Bird Observatory to assess the effectiveness of alternative banding techniques in reducing injuries and band loss (G. Page *in litt.* 2005*b*).

Concerns that color bands increase the vulnerability of western snowy plovers to predation by reducing effectiveness of camouflage do not appear to be supported by existing evidence. Because western snowy plovers crouch and flatten to the sand at the approach of avian predators, color bands are typically hidden from sight; terrestrial predators are evaded by running or taking flight at their approach (J. Watkins, pers. comm. 2006).

3. Disease or Predation

West Nile virus, a mosquito-borne disease which can infect birds, reptiles, and mammals, has spread rapidly across the United States from the initial introduction in New England (National Audubon Society 2006). The disease has killed birds of various species in all coastal California counties since its arrival in the state in 2003 (U.S. Geological Survey 2006). In 2004 to 2006 the disease was reported from two coastal counties (Lane and Lincoln) in Oregon but has not been reported from any coastal counties in Washington (U.S. Geological Survey 2006). The deadliness of the disease varies by species; however, the virus has been identified in dead piping plovers (*Charadrius melodus*) and killdeer (*C. vociferus*), both closely related to the western snowy plover (Center for Disease Control 2004).

Since 2004 numerous western snowy plovers in southern California have been found dead or exhibited neurological signs consistent with avian botulism (M. Long *in litt.* 2006). Confirmation of disease diagnosis is currently pending availability of specimens for autopsy. We are currently coordinating with the USGS National Wildlife Health Center to better understand the causes of these mortalities and to develop a program for treatment of ill birds diagnosed with botulism. Additionally, 32 western snowy plovers died in 2006 from unknown causes in San Diego County (U.S. Navy *in litt.* 2007).

Predator density is a significant factor affecting the quality of western snowy plover nesting habitat (Stenzel *et al.* 1994). Predation can result in the loss of adults, chicks, or eggs; separation of chicks from adults is also caused by the presence of predators. Powell *et al.* (2002) found that predation accounted for most nest failures in 1994, 1996, and 1997, in San Diego County, California. Western snowy plovers generally cannot defend themselves or their nests against predation but must rely on antipredator adaptation, including (1) pale coloration of adults, eggs, and young, which acts as camouflage against detection by predators; (2) a skulking retreat from the nest at a predator's approach; (3) extreme mobility and elusiveness of precocial young and; (4) maintenance of low nesting density (Page *et al.* 1983). In natural ecosystems, there is a co-evolution of the predator-prey relationship, where prey species slowly evolve with evading behavior as predator species slowly evolve effective prey-capturing behavior. However, when exotic predators are introduced into the ecosystem and thrive there, they frequently occur in much higher densities and possess more effective strategies than native predators and, hence, usually have a more severe effect.

Predation, by both native and nonnative species, has been identified as a major factor limiting western snowy plover reproductive success at many Pacific coast sites. Known mammalian and avian predators of western snowy plover eggs, chicks, or adults include the following native species: gray foxes (*Urocyon cinereoargenteus*), Santa Rosa Island foxes (*Urocyon littoralis santarosae*), coyotes, striped skunks (*Mephitis mephitis*), spotted skunks (*Spilogale putorius*), raccoons, California ground squirrels (*Citellus beecheyi*), long-tailed weasels (*Mustela frenata*), American crows, common ravens (*Corvus corax*), ring-billed gulls (*Larus delawarensis*), California gulls (*Larus californicus*), western gulls (*Larus occidentalis*), glaucous-winged gulls (*Larus glaucescens*), gull-billed tern (*Gelochelidon nilotica*), American kestrels (*Falco sparverius*), peregrine falcons (*Falco peregrinus*), northern harriers (*Circus cyaneus*), loggerhead shrikes, merlins (*Falco columbarius*), great horned owls (*Bubo virginianus*), burrowing owls (*Speotyto cunicularia*), great blue herons (*Ardea herodias*); and the following nonnative species: eastern red foxes (*Vulpes vulpes regalis*), Norway rats (*Rattus norvegicus*), Virginia opossums (*Didelphis marsupialis*), domestic and feral dogs (*Canis familiaris*), and cats (*Felis domesticus*). Loss or abandonment of eggs due

to predation by fire ants and Argentine ants (*Iridomyrmex humilis*) has also been observed (Fancher *et al.* 2002, Powell *et al.* 2002).

In Oregon, nest predation by corvids (common ravens and American crows) is the major cause of nest failures. Of 63 unexclosed nests in 2005, corvid predation accounted for 22 nest failures, by comparison with 14 failures due to mammalian or unknown predators and 10 due to abandonment (Lauten *et al.* 2006*a*). Exclosures were effective in protecting nests against this threat (0 of 83 exclosed nests failed due to nest predation).

American crows have been consistently documented as a major predator on western snowy plover nests along the California and Oregon coasts (Page 1990; Persons and Applegate 1997; T. Applegate, Bioresources, pers. comm. 1999; M. Stern, The Nature Conservancy, pers. comm. 1999). At Coal Oil Point, American crows were the most frequent predator on western snowy plover nests and experimentally placed quail eggs (Lafferty *et al.* 2006). Populations of American crows have increased in the San Francisco Bay and central California coast over the past several decades, and are positively associated with human population density (Leibezet and George 2002).

Common ravens are known predators of western snowy plover eggs (Wilson-Jacobs and Dorsey 1985, Point Reyes Bird Observatory unpublished data, George 1997, Stein 1993, Point Reyes Bird Observatory unpublished data, J. Albertson *in litt.* 1999, Point Reyes Bird Observatory unpubl. data, Stern *et al.* 1991). Ravens have consistently been the most significant nest predator at Point Reyes, accounting for 69 percent of all predation events over 5 years and destroying approximately 50 percent of nests (Hickey *et al.* 1995). Hatching success at Point Reyes National Seashore increased after exclosures were used to protect western snowy plover nests from ravens in 1996. Approximately 12 percent of nests in San Diego County were destroyed by ravens (Powell *et al.* 1996, Powell *et al.* 1997). Raven populations in coastal California have significantly increased in recent decades (Leibezet and George 2002), and as their range expands they are becoming increasingly significant as a nest predator on western snowy plovers; ravens were observed to destroy nests in Monterey Bay for the first time in 2002 and 2003 (G. Page *in litt.* 2004*b*). In northern California ravens are the single most limiting

factor on western snowy plover reproduction (Colwell *et al.* 2006). Ravens also prey on western snowy plover chicks, but not nearly to the extent that they do on eggs. However, at Point Reyes raven predation primarily affected chicks after exclosures were erected to protect snowy plover eggs (S. Allen *in litt.* 2004).

Gulls pose a special threat to breeding western snowy plovers because they not only depredate nests and chicks, but also usurp and trample western snowy plover nesting habitat and crush eggs (Persons and Applegate 1997, Point Reyes Bird Observatory unpublished data, Widrig 1980, J. Albertson *in litt.* 1999, Page *et al.* 1983).

The first time a gull-billed tern was found in San Diego County, California, was in 1985. Two years later they were nesting in south San Diego Bay (Unitt 2004). Since then, the nest colony has steadily increased with an estimated 52 pairs in 2006 (Patton 2006a). Gull-billed terns have become a concern to managers of beach-nesting birds in the region. Gull-billed terns were first documented taking California least terns (presumably chicks) in south San Diego Bay in 1992 (Caffrey 1993). Patton (2006a) summarizes recent incidents of gull-billed tern predation on both terns and western snowy plovers. He notes roughly 20 to 60 California least terns and 1 to 4 western snowy plover depredations by gull-billed terns and a greater number was suspected. Although the documented number of gull-billed tern depredations on western snow plovers is considerably lower than on California least terns, it is difficult to know the full extent of gull-billed tern impacts (Patton 2006b), especially for the plovers whose nests are more dispersed and less easily monitored.

Unlike management of other avian predators, management of gull-billed terns is problematic. The local subspecies of gull-billed tern, *G. n. vanrossemi*, is limited to western North America (Molina and Erwin 2006, but see Unitt 2004). The subspecies nests in scattered, localized colonies and "[i]n 2003 and 2005, the entire North American population of *vanrossemi* gull-billed terns ranged from about 533 to 810 pairs" (Molina and Erwin 2006). This means that this predator is considerably rarer than the listed bird species upon which it preys (California least terns and western snowy plovers), which poses a conundrum for managers of western snowy plovers and California least terns (Unitt 2004). Because of the gull-

billed tern's status, lethal predator control has not been used on this species since 1999 (Unitt 2004). Gull-billed terns will likely become a greater source of management concem as the local population of this species grows. Gull-billed terns have been observed at other locations of beach-nesting birds farther north from San Diego Bay, including Camp Pendleton, San Diego County (Foster 2005); Bolsa Chica, Orange County (Hamilton and Willick 1996), and Venice Beach, Los Angeles County (McCaskie and Garrett 2005).

Loggerhead shrikes are not known to take western snowy plover eggs, but do prey upon chicks and locally can have substantial effects on fledging success (Warriner *et al.* 1986, D. George *in litt.* 2001, Page *et al.* 1997, George 1997, Page 1988, Feeney and Maffei 1991).

Although not known to be predators of western snowy plover eggs, American kestrels are predators of chicks and possibly adults (D. George, pers. comm. 1998). Fledging success increased from 9 to 64 percent after a kestrel unexpectedly disappeared from a western snowy plover nest site in Moss Landing Wildlife Area (Page *et al.* 1998). In 1997, a merlin was suspected of taking 13 banded adults within the period of a few days at Salinas River National Wildlife Refuge. Also, western snowy plover chicks and adults are among the avian prey of the peregrine falcon (B. Walton, University of California Santa Cruz, pers. comm. 1998; D. George, pers. comm. 1998; Feeney and Maffei 1991). Northern harriers are effective predators of western snowy plover chicks and adults. In 1987, a harrier was observed hunting on the islands in the Salinas River where only approximately one third of the hatched chicks reached fledging age (Point Reyes Bird Observatory unpubl. data). At the Moss Landing Wildlife Area, fledging success dropped from 61 to 23 percent after a harrier began foraging there (Page *et al.* 1997). A northern harrier was seen capturing 2 to 4 western snowy plover chicks at Moss Landing salt ponds in 2000 (D. George *in litt.* 2001).

In recent decades, alien eastern red foxes have become a serious new predator of endangered and threatened animals in coastal habitats (Jurek 1992, Golightly *et al.* 1994, Lewis *et al.* 1993). Nonnative red foxes were imported into the southern Sacramento Valley, primarily for hunting and fur farming purposes, as early as the 1870s and experienced explosive spread in the 1970s and 1980s (Jurek 1992, Lewis

et al. 1993, 1995). The red fox now occurs throughout a significant portion of coastal California, including Marin, San Mateo, Santa Cruz, Monterey, San Luis Obispo, Santa Barbara, Ventura, Orange, and Los Angeles Counties (California Department of Fish and Game 1994). It also occurs at Monterey Bay (G. Page *in litt.* 1988) and San Francisco Bay (Harding *et al.* 1998), including the additional San Francisco Bay area counties of Napa, Solano, Contra Costa, Alameda, and Santa Clara (California Department of Fish and Game 1994). Red foxes also are present in some areas of coastal Oregon where western snowy plovers breed (D. George *in litt.* 2001, Lauten *et al.* 2006*b*).

Red foxes have been identified as a significant predator of western snowy plover eggs in the Monterey Bay area, where they are suspected of also preying on adults and chicks. On Monterey Bay beaches, red fox depredation of western snowy plover eggs resulted in a decline in clutch hatching rate of 30 percent from 1984 to 1990. After exclosures and mammalian predator control came into use to protect nests around Monterey Bay, annual clutch hatching rates have climbed from 43 to 68 percent (Neuman *et al.* 2004).

Predation of western snowy plover nests and chicks by red fox have been documented at Bandon Beach, New River and other portions of OR-15 on the Oregon coast. Biologists have documented red fox tracks around western snowy plover nest exclosures and have followed fox tracks back to dens located within western snowy plover nest areas. As part of the emergency response to the New Carissa oil spill in February 1999, a predator program was implemented. Animal and Plant Health Inspection Service (APHIS) Wildlife Services Division personnel removed 17 red fox from the New River area over a 3 month period (S. Richardson *in litt.* 2001). Ongoing predator management since 2002 has removed an average of 15 foxes per year from Bandon Beach/New River (Lauten *et al.* 2006*b*).

The U.S. Department of Agriculture, Wildlife Services Branch, has been involved in predator damage management for protection of threatened and endangered species for over 10 years in California. The management of nonnative red foxes has become a controversial issue in many areas of California, particularly in coastal habitats near urban areas (California Department of Fish and Game 1994). In November 1998, California voters approved Proposition 4, which banned the use of

leghold traps in California. In February 1999, the U.S. District Court issued a Preliminary Declaratory Relief Order, which allows the use of padded leghold traps on Federal and non-Federal lands for the purpose of protecting threatened or endangered species. Trapping of nonnative and native predators of western snowy plovers will therefore not be affected by Proposition 4 (J. Albertson *in litt.* 1999).

Coyotes are known predators of western snowy plover eggs in the Pismo Beach/Santa Maria River area of San Luis Obispo County (T. Applegate, pers. comm. 1996). They are the main nest predator of eggs on Vandenberg Air Force Base where they were the cause of 43 percent of all clutch losses attributed to predators from 1994 to 1997 (Persons and Applegate 1997). At Vandenberg Air Force Base, coyotes may be attracted to marine mammal carcasses on the beach early in the western snowy plover nesting season (Page and Persons 1995). Coyotes also have been identified as predators of western snowy plover nests at Mono Lake, California (Page *et al.* 1983).

Striped skunks have been recorded as predators of western snowy plover eggs (Hickey *et al.* 1995, George 1997, Page *et al.* 1997, Hutchinson *et al.* 1987, Stein 1993, Stern *et al.* 1991). Skunks were believed to be the main cause of nest loss on Morro Bay Spit in 1987, the only year that the reproductive success of western snowy plovers has been monitored at that location (Hutchinson *et al.* 1987). Persons and Ellison (2001) reported that the striped skunk was the predominant predator of nests at Morro spit, destroying 87 percent of depredated nests in 2000.

Domestic and feral cats are widespread predators. The threat of predation of western snowy plovers by cats increases when housing is constructed near western snowy plover breeding habitat. As natural-appearing beaches continue to be surrounded by urban areas, western snowy plovers will increasingly be subjected to this predator in the future. Predation by cats is difficult to measure because of the difficulty in finding evidence of bird remains, but they are known to take western snowy plover adults and eggs (B. Farner, pers. comm. *in* Powell and Collier 1994; Page 1988; D. George *in litt.* 2001).

Predation, while predominantly a natural phenomenon, is exacerbated through the introduction of nonnative predators and unintentional human encouragement of

larger populations of native predators. Elevated predation pressures result from landscape-level alterations in coastal dune habitats which, in turn, now support increased predator populations within the immediate vicinity of nesting habitat for western snowy plovers. Urbanization benefits red fox population growth by eliminating coyotes, which are the red fox's most common native predator and competitor; by providing ready sources of food, water and denning sites; and by aiding dispersion of foxes into new areas. Red foxes disperse readily in urban areas because there are no predators besides the domestic dog. Red foxes traverse most urban habitats, and readily cross busy highways and travel long distances underground through culverts (Lewis *et al.* 1993). Other predators, such as corvids, attracted by the presence of human activities (*e.g.*, improper disposal of trash), may frequent beaches in increasing numbers. Gulls have greatly expanded their range and numbers, especially along the United States portion of the Pacific coast, as a result of human-supplied food sources (trash, fish offal, and dumps). Thousands of California gulls now breed in the southern part of San Francisco Bay, where only a few were present in the early 1980s (J. Albertson *in litt.* 1999). This population growth is attributed largely to the increase in landfills along the Bay within the last 20 years. Also, crows and ravens forage at landfills. Buick and Paton (1989) found that losses of hooded plover (*Charadrius rubricollis*) nests with human footprints around them were higher than at those without footprints, suggesting "that scavenging predators may use human footprints as a visual cue in locating food." Beach litter and garbage also attract predators such as skunks and coyotes (*e.g.*, N. Read *in litt.* 1998). Unnatural habitat features such as landscaped vegetation (*e.g.*, palm trees), telephone poles, transmission towers, fences, buildings, and landfills near western snowy plover nesting areas attract predators and provide them with breeding areas (*e.g.*, J. Buffa *in litt.* 2004). These alterations all combine to make the coastal environment more conducive to various native and nonnative predators that adversely affect western snowy plovers.

Substantial evidence exists that human activities are affecting numbers and activity patterns of predators on western snowy plovers. For example, increased depredation of westem snowy plover nests by ravens at the Oliver Brothers salt pond, California, may be an indirect adverse impact of nearby installation of light structures by the California Department of Transportation and high-tension power lines by the Pacific Gas and Electric Company, thereby creating corvid nesting sites

(G. Page, Point Reyes Bird Observatory, pers. comm. 1997). Raven nests have also been discovered by National Wildlife Refuge biologists in transmission towers near other snowy plover nesting areas managed by the Don Edwards San Francisco Bay National Wildlife Refuge in Warm Springs, Alviso, and Mountain View (J. Buffa *in litt.* 2004). On the Oregon coast, predation risk by mammals has increased as a result of the spread of European beachgrass, Scotch broom, and shore pine, which has transformed vast areas of open sand into dense grass-shrub habitat, providing excellent habitat for native and nonnative mammalian predators, such as skunks, raccoons, foxes, and feral cats (Stern *et al.* 1991). At Vandenberg Air Force Base, coyote predation can be exacerbated by human presence when trash or debris is left behind (N. Read *in litt.* 1998).

Signing and fencing of restricted areas on the beach may provide perches for avian predators of western snowy plover adults or chicks (Hallett *et al.* 1995). Although signs and fences are important conservation tools in many areas, land managers need to be aware that modifications to them may be necessary to deter predators in some circumstances.

4. The Inadequacy of Existing Regulatory Mechanisms

The western snowy plover is protected by the Federal Migratory Bird Treaty Act (16 U.S.C. 703 *et seq.*) and, in each state, by State law as a nongame species. The western snowy plover's breeding habitat, however, receives only limited protection from these laws (*e.g.*, the Migratory Bird Treaty Act prohibition against taking "nests"). Listing of the western snowy plover under State endangered species laws generally provides some protection against direct take of birds, and may require State agencies to consult on their actions, but may not adequately protect habitat. State regulations, policies, and goals include mandates both for protection of beach and dune habitat and for public recreational uses of coastal areas; consequently they may conflict with protection of western snowy plovers in some cases. Section 404 of the Clean Water Act (33 U.S.C. 1251 et seq.) and section 10 of the Rivers and Harbors Act (33 U.S.C. 403) are the primary Federal laws that could provide some protection of nesting and wintering habitat of the western snowy plover that is determined by the U.S. Army Corps of Engineers (Corps) to be wetlands or historic navigable waters of the United States. These laws, however, would apply to only a

small fraction of the nesting and wintering areas of the western snowy plover on the Pacific coast. Aside from the Migratory Bird Treaty Act, western snowy plovers have no protection status in Mexico.

To effectively recover the western snowy plover, it is necessary to develop participation plans among cooperating agencies, landowners, and conservation organizations to assure protection and appropriate management of breeding, wintering, and migration areas. Since listing of the western snowy plover in 1993, several local working groups have been developed and local governments and State and Federal agencies have cooperated extensively to implement a wide variety of western snowy plover conservation actions. These partners continue to work to implement appropriate management of coastal areas for recovery of the western snowy plover. These conservation efforts and the environmental policies of State and Federal agencies are described in greater detail in the Conservation Efforts section, below.

For additional discussion of regulatory mechanisms and management actions taken by California State Parks and other entities, see U.S. Fish and Wildlife Service (2006a).

5. Other Natural or Manmade Factors Affecting Their Continued Existence

a. Natural Events

Western snowy plover breeding and wintering habitat is subject to constant change from weather conditions. Stenzel *et al.* (1994) reported that the quality and extent of western snowy plover nesting habitat is variable in both the short- and long-term. Coastal beaches increase in width and elevation during the summer through sand deposition, making marginal beaches more suitable for nesting later in the season. Over the longer term, an increase or decrease in habitat quality may occur after several years of winter storms. Based on the amount of flooding, the availability of dry flats at the edges of coastal ponds, lagoons, and man-made salt evaporators also varies within and between seasons. Therefore, the number of western snowy plovers breeding in some areas may change annually or even over one breeding season in response to natural alterations in habitat availability (Stenzel *et al.* 1981).

Because most western snowy plover nesting areas occur on unstable sandy substrates, nest losses caused by weather-related natural phenomena commonly occur. High tides and strong winds cause many nest losses. Events such as extreme high tides (Wilson 1980, Stenzel *et al.* 1981), river flooding (Stenzel *et al.* 1981), and heavy rain (Wilson 1980, Warriner *et al.* 1986, Page 1988) have been reported to destroy or wash away nests. The annual percentage of total nest losses attributed to weather-related phenomenon has reached 15 to 38 percent at some locations (Wilson 1980, Warriner *et al.* 1986, Page 1988).

Stormy winters can adversely affect the western snowy plover. It is suspected that the severe storms occurring during the El Niño atmospheric and oceanic phenomenon of the winter of 1997/1998 caused a 10 to 30 percent decline in the 1998 western snowy plover breeding population, depending on the coastal region. In all monitored recovery units, the number of breeding birds in 1998 was lower than in the 1997 nesting season. Additionally, a very wet spring resulted in a later than normal breeding initiation and fewer nesting attempts.

The western snowy plover population naturally varies, both spatially and temporally, because of natural changes in weather and habitat conditions from year to year. However, as described above, human influences over the past century (*e.g.*, habitat destruction, invasion of introduced beachgrass, and elevated predation levels) have reduced the western snowy plover's ability to respond to these natural perturbations.

b. Disturbance of Breeding Plovers by Humans and Domestic Animals

The coastal zone of the United States, including both open coastal areas and inland portions of coastal watersheds, is home to over one-third of the U.S. human population, and that proportion is increasing (U.S. Fish and Wildlife Service 1995*a*). The southern California coastal area, which constitutes the central portion of the western snowy plover's coastal breeding range, attracts large crowds on a regular basis (Figure 6). The increasing level of human recreation was cited as a major threat to the breeding success of the Pacific coast population of the western snowy plover at the time of listing (U.S. Fish and Wildlife Service 1993*a*).

Figure 6. Recreationists at Salt Creek Beach, California (photo by Ruth Pratt, with permission).

i. Pedestrians

Pedestrians (*e.g.*, beach walkers and joggers) can cause both direct mortality and harassment of western snowy plovers. Pedestrians on beaches may crush eggs or chicks and chase westem snowy plovers off their nests. Separation of western snowy plover adults from their nests and broods can cause mortality through exposure of vulnerable eggs or chicks to heat, cold, blowing sand, and/or predators. Pedestrians have been known to inadvertently step on eggs and chicks, deliberately take eggs from nests, and remove chicks from beaches, erroneously thinking they have been abandoned. People also may cause broods of western snowy plovers to run away from favored feeding areas. These effects are described in more detail below. Trash left on the beach by pedestrians also attracts predators. In addition to public pedestrians, military personnel using the beach for maneuvers, boat launches, and landings have the potential to similarly cause adverse impacts to western snowy plovers.

Beach-related recreational activities that are concentrated in one location (*e.g.*, sunbathing, picnicking, sandcastle building, birding, and photography) can negatively affect incubating adult western snowy plovers when these activities occur too close to their nests. Recreational activities that occur in the wet sand area (*e.g.*, sand sailing) can adversely affect western snowy plovers when they disturb plover adults or broods, which feed at the edge of the surf along the wrack line. Recreational activities that occur in or over deep water (such as the beach- and water-oriented activities of surfing, kayaking, wind surfing, jet skiing, and boating, and the coastal-related recreational activity of hang gliding) may not directly affect western snowy plovers; however, they can potentially be detrimental to western snowy plovers when recreationists use the beach to take a break from these activities, or as access, exit, or landing points.

Concentrations of people may deter western snowy plovers and other shorebirds from using otherwise suitable habitats. Anthony (1985) found that intensive human activity at Damon Point had a "bracketing effect" on the distribution of nesting western snowy plovers, confining their breeding activity to a section of the spit and precluding their regular use of otherwise suitable habitat. Fox (1990) also found that western snowy plovers avoided humans at Damon Point, and the presence of fishermen and beachcombers kept them hundreds of yards away from potential habitat. Because early-nesting western snowy plovers have narrower beaches from which to select nest locations, recreational use may be more concentrated in the limited habitat available. Also, repeated intrusions by people into western snowy plover nesting areas also may cause birds to move into marginal habitats where their chances of reproductive success are reduced. Studies of the Atlantic coast population of the piping plover (*Charadrius melodus*), an eastern species with habitat requirements very similar to the snowy plover, indicate that some piping plovers that nest early in the season are forced to move elsewhere when human use becomes too intense (Cairns and McLaren 1980). These authors concluded that piping plovers that nest early, before beaches become heavily used for recreation, "cannot predict and avoid reproductive failure in habitats that otherwise appear suitable to them." Burger (1993) observed that piping plovers, in response to human disturbance, spent more energy on vigilance and avoidance behavior at the expense of foraging activity, and sometimes abandoned preferred foraging habitat.

Page *et al.* (1977) observed western snowy plovers' response to human disturbance at two coastal beaches where normal beach use ranged from light to heavy. The study included 156 hours of observation at 15 western snowy plover nests. At Point Reyes, they found that pedestrians disrupt incubation of nests. When humans approached western snowy plovers, adults left their nests 78 percent of the time when people were within 50 meters (164 feet) and 34 percent of the time when people were over 100 meters (328 feet). They also found that western snowy plovers' reaction to disturbance by humans varied, ranging from one bird remaining off the nest for less than 1 minute when a person walked within 1 meter (3 feet) of the nest on a heavily-used beach to another western snowy plover leaving the nest when three people were 200 meters (656 feet) away on a less-used beach. They noted that "birds exposed to prolonged human activity near the nest seemed to become accustomed to it." It has been speculated that predators of western snowy plovers may benefit from a decline in wariness by western snowy plovers nesting on beaches that are subject to ongoing high levels of human disturbance (Persons and Applegate 1997).

Lafferty (2001) observed western snowy plovers' response to people, pet dogs, equestrians, crows and other birds. Observations were made at Devereux Slough in Santa Barbara County, Santa Rosa Island, San Nicolas Island, and Naval Base Ventura County (Point Mugu). This study found that western snowy plover are most frequently disturbed when approached closely (within 30 meters) by people and animals. The most intense disturbance (causing the western snowy plover to fly away) were in response to crows, followed by horses, dogs, humans, and other birds. Lafferty (2001) created a management model based on his findings and estimated flight response disturbances under different scenarios. The model predicted a reduced disturbance response for buffer zones of 20 to 30 meters.

Fahy and Woodhouse (1995) quantified the levels of recreational disturbance, their effect on western snowy plovers, and the effectiveness of the Linear Restriction Program at Ocean Beach, Vandenberg Air Force Base in 1995. Under this program signs directed visitors not to cross from the outer beach into the Linear Restriction area (inland of mean high tide mark, in dune habitat used by western snowy plovers). Seventy percent of all disturbances were in compliance with restriction warning signs. The disturbance types that were most and least frequently in

compliance with the boundary were joggers or walkers and stationary visitors, respectively. The closer the disturbance occurred to the plover, the more severe the plover response. All-terrain vehicles caused the most significant alert and flight behaviors by western snowy plovers, even though they were in compliance with the Linear Restriction. The disturbance types that caused incubating western snowy plovers to flush from their nests most frequently were joggers and walkers, followed by joggers or walkers with dogs off leash, and stationary visitors. The disturbance types that kept incubating western snowy plovers off their nests for the longest period of time were stationary visitors and surf fishermen, probably because of the duration of these stationary disturbances that occurred close to nests. Weekends accounted for 60 percent of all disturbances. The enforcement personnel appeared to have a limited presence; their presence was documented during only 14 percent of all identified disturbances.

Hoopes *et al.* (1992) quantified human use and disturbance to piping plovers in Massachusetts during the 1988 and 1989 nesting seasons. They found pedestrians caused piping plovers to flush or move at an average distance of 23 meters (75 feet). Pedestrians within 50 meters (164 feet) of the birds caused piping plovers to stop feeding 31 percent of the time.

Point Reyes Bird Observatory found that management actions that included exclusion zones around nesting areas, seasonal closure to dogs, and active weekend docent programs reduced mortality of chicks and eggs during the weekend such that the weekend and weekday mortality was the same (Peterlein and Roth 2003).

At the Pajaro River mouth in California, at least 14 percent of western snowy plover clutches were destroyed by being driven over, stepped on, or deliberately taken by people (Warriner *et al.* 1986). Since exclosures have been used to protect nests at the Pajaro River mouth and other locations at Monterey Bay, a few nests have still been deliberately destroyed by vandals in most years (Point Reyes Bird Observatory unpublished data). At South Beach, Oregon, the number of western snowy plovers declined from 25 in 1969 to 0 in 1981 when a new park was constructed next to the beach and the adjacent habitat became more accessible to vehicles and people (Hoffman 1972 *in* Oregon Department of Fish and Wildlife 1994).

At Vandenberg Air Force Base, western snowy plover monitoring during 1993 at South Beach (where recreational use was high) and North Beach (where recreational use was low) found the rate of nest loss caused by humans differed markedly: 24.3 percent of South Beach nests were lost compared to only 3.0 percent of North Beach nests (Persons 1994). Persons and Applegate (1997) reported that "rates of reproductive success, combined for 1994 through 1997, were substantially higher on North Beach than on South Beach." This difference occurred despite the fact that nesting habitat was posted as off-limits during the nesting season in 1994. However, at that time restrictions were new and not strictly enforced (R. Dyste *in litt.* 2004). Since 2000, public access has been restricted and fully enforced by Vandenberg Air Force Base personnel. Additionally, Santa Barbara County-supported volunteer docents were present at Surf Station (within Vandenberg Air Force Base) during the 2001-2003 plover breeding seasons when the beach was open for public access. In 2003, plover monitors did not document the loss of any nests within Surf Station Beach as a result of trampling by humans (R. Dyste *in litt.* 2004).

Loss of western snowy plover chicks also may occur because of human activities. The number of young produced per nesting attempt increased from 0.75 in disturbed habitat to 2.0 for nests free of disturbance at Willapa National Wildlife Refuge, Washington (Saul 1982). At Vandenberg Air Force Base, the 1997 fledging success of western snowy plovers was 33 to 34 percent on North Beach where recreational activity is restricted and only 12 percent on South Beach where recreational use is high (Persons and Applegate 1997). In 1999 and 2000, Ruhlen *et al.* (2003) found that increased human activities on Point Reyes beaches had a negative effect on western snowy plover chick survival. In both 1999 and 2000, western snowy plover chick loss was about three times greater on weekends and holidays than on weekdays. In most coastal areas, beach visitation in summer months is much higher on weekends and holidays than on weekdays.

Flemming *et al.* (1988) measured the effects of human disturbance on reproductive success and behavior of piping plovers in Nova Scotia. To assess human disturbance, they recorded positions of people, pedestrian tracks, and vehicle tracks, then defined classes based on visits per week. They found significantly fewer young survived in areas of high versus low disturbance; humans elicited a

significantly higher response level from adult piping plovers than did predators or nonpredatory species; chicks fed less and were brooded less when humans were within 160 meters (525 feet); and chick peck rate during feeding was lower when humans were present. They speculated that because chicks shifted from feeding and energy conservation activities to vigilance and cryptic predator avoidance behaviors, their energy reserves would be depleted, making them more susceptible to predators and inclement weather. They postulated that a decline in piping plover abundance in Nova Scotia could be caused by human disturbance altering chick behavior. Fewer chicks survived to 17 days in areas heavily disturbed by humans.

Schultz and Stock (1993) studied the effects of tourism on colonization, distribution, and hatching success of Kentish plovers (*Charadrius alexandrinus alexandrinus*), a Eurasian subspecies of the snowy plover, at the Wadden Sea in Germany. They measured disturbance intensity by counting and mapping tourists on 50 days from April to July, during times of peak human activity (1500 to 1600 hours) and in intervals of 30 minutes throughout other days. An index of person-hours per area per day was calculated. They found that Kentish plovers did not colonize heavily-disturbed areas and that resting and sunbathing people were apparently more disruptive than walking people because the latter generally followed the high-tide line. Clutch losses were lowest in areas with little disturbance and highest in areas with heavy disturbance. They indicated that hatching success in highly disturbed areas, even with optimal habitat, is as low as in poor habitat with a low level of disturbance.

ii. Dogs

Dogs on beaches can pose a serious threat to western snowy plovers during both the breeding and nonbreeding seasons. Unleashed pets, primarily dogs, sometimes chase western snowy plovers and destroy nests. Repeated disturbances by dogs can interrupt brooding, incubating, and foraging behavior of adult western snowy plovers and cause chicks to become separated from their parents. Pet owners frequently allow their dogs to run off-leash even on beaches where it is clearly signed that dogs are not permitted or are only permitted if on a leash. Enforcement of pet regulations on beaches by the managing agencies is often lax or nonexistent.

A number of examples of disruptive ways that dogs affect western snowy plovers have been noted at beaches in Monterey County (Marina State Beach), Santa Cruz County (Laguna, Scott Creek, and Seabright Beaches) and San Mateo County (Half Moon Bay and Pacifica Beaches) (D. George, pers. comm. 1997). Incubating birds have been flushed from nests by dogs, including nests located inside areas protected by symbolic fencing. Dogs also have displaced adults from nests with newly-hatched chicks. Roosting and feeding flocks, as well as individual birds, have been deliberately and persistently pursued by dogs. At Laguna Creek Beach, Zmudowski State Beach, and Salinas River State Beach, dogs partially or entirely destroyed western snowy plover nests which were in several cases, protected with symbolic fencing (D. George, pers. comm. 1997; Point Reyes Bird Observatory unpublished data; G. Page, pers. comm. 1998). Feral dogs are suspected to have disturbed western snowy plover nests and chicks on San Francisco Bay salt ponds (J. Albertson *in litt.* 1999).

Even when not deliberately chasing birds, dogs on a beach may disturb western snowy plovers and other shorebirds that are roosting or feeding. Page *et al.* (1977) found that western snowy plovers flushed more frequently and remained off their nests longer when a person was accompanied by a dog than when alone. They collected data during 156 hours of observation at 15 nests at Point Reyes, California, and found the following distances at which western snowy plovers flushed from their nests as a result of disturbance by people with dogs. Within 50 meters (164 feet), people with dogs caused flushing 100 percent of the time. At a distance of over 100 meters (328 feet), people with dogs caused flushing 52 percent of the time (Page *et al*. 1977). Fahy and Woodhouse (1995) found that joggers or walkers with off-leash dogs caused a significantly greater number of avoidance responses from western snowy plovers than other types of disturbances at Ocean Beach, Vandenberg Air Force Base, California. Lafferty's (2001) management model predicted that intense disturbances could be dramatically reduced by removing dogs.

At wintering sites such as Ocean Beach in San Francisco, California, off-leash dogs have caused frequent disturbance and flushing of western snowy plovers and other shorebirds. Off-leash dogs chase wintering western snowy plovers at this beach and have been observed to regularly disturb and harass birds (P. Baye, U.S. Fish

and Wildlife Service, pers. comm. 1997). Observations by National Park Service volunteers suggest that unleashed pets represent the most significant recreational threat to wintering western snowy plovers and migratory shorebirds at Ocean Beach, because of the prolonged and repeated disturbance created when they chase birds (Hatch 1997). In 1995 and 1996, during 45 hour-long observations of wintering flocks of western snowy plovers at Ocean Beach, western snowy plovers responded by moving in 73 percent of 74 instances when dogs with or without people approached to within 15 meters (50 feet) (Golden Gate National Recreation Area unpublished data). When shorebirds are flushed, they must spend more energy on vigilance and avoidance behaviors at the expense of foraging and resting activity (Burger 1993, Hatch 1997). Disruption of foraging and roosting may result in decreased accumulation of energy reserves necessary for shorebirds to complete the migration cycle and successfully breed (Burger 1986, Pfister *et al.* 1992). Dog disturbance at wintering and staging sites, therefore, may adversely affect individual survivorship and fecundity, thereby affecting the species at the population level.

iii. Motorized Vehides

Unrestricted use of motorized vehicles on beaches is a threat to western snowy plovers and their habitat. Motorized vehicles may affect remote stretches of beach where human disturbance would be slight if access were limited to pedestrians. The magnitude of this threat is variable, depending on level of use and type of terrain covered. Use of motor vehicles on coastal dunes may also be destructive to dune vegetation, especially sensitive native dune plants.

Driving vehicles in breeding habitat may cause destruction of eggs, chicks, and adults, abandonment of nests, and considerable stress and harassment to western snowy plover family groups (G. Page, pers. comm. 1997; J. Myers *in litt.* 1988; J. Price *in litt.* 1992; Stern *et al.* 1990; Casler *et al.* 1993; S. Richardson, pers. comm. 1998; Widrig 1980). In addition to recreational vehicles, vehicles used for military activities have also caused western snowy plover mortality (Powell *et al.* 1995, 1997; Persons 1994).

Driving motor vehicles at night seems to be particularly hazardous to western snowy plovers. Drivers of all-terrain vehicles at night have run over and killed western snowy plover adults at Vandenberg Air Force Base, and State park ranger patrol vehicles have crushed western snowy plover chicks at Oceano Dunes State Vehicular Recreation Area during night patrols (R. Mesta *in litt.* 1998).

On the Eel River gravel bars, vehicle use (including motorcycles, ATVs, and full-size 4x4s) has resulted in the crushing of nests and disturbance to nesting plovers (Colwell *et al.* 2006).

Western snowy plover adults and chicks have been observed using tire tracks and human footprints for loafing at Camp Pendleton and Naval Amphibious Base Coronado (Powell and Collier 1994). This behavior increases their chances of being run over. Western snowy plover chicks also may have difficulty getting out of tire ruts, thereby increasing their likelihood of being run over. Their cryptic coloring and habit of crouching in depressions like tire tracks makes western snowy plover chicks especially vulnerable to vehicular traffic. In Massachusetts, between 1989 and 1997, a total of 25 piping plover chicks and 2 adults were found dead in off-road vehicle tire ruts on the upper beach between the mean high tide line and the foredune (U.S. District Court of Massachusetts 1998).

Hoopes *et al.* (1992) found off-road vehicles caused piping plovers to flush or move at an average distance of 40 meters (131 feet). Off-road vehicles within 50 meters (164 feet) of the birds caused piping plovers to stop feeding 77 percent of the time. While most responses by piping plovers to off-road vehicles resulted in movement by the birds, they observed three instances where the plovers "froze" in response to the off-road vehicles. Both types of responses have a negative impact on plovers through either disturbance, interruption of feeding behavior, or increasing the risk that piping plovers will be hit or crushed by vehicles.

At wintering sites, disturbance from motorized vehicles may harass western snowy plovers and disrupt their foraging and roosting activities, thereby decreasing energy reserves needed for migration and reproduction. When motorcycles, most of which were in the wet sand zone, were driven at high speed along Ocean Beach in San

Francisco, Hatch (1997) observed that western snowy plovers and other shorebirds were continually disturbed and often took flight.

iv. Beach Cleaning

Removal of human-created trash on the beach is desirable to reduce predation threats by eliminating food for predators of western snowy plovers; however, the indiscriminate nature of mechanized beach-cleaning adversely affects western snowy plovers and their habitat. Mechanized beach cleaning can be dangerous to western snowy plovers by crushing their clutches and chicks or causing prolonged disturbance from the machine's noise. Also, this method of beach cleaning removes the birds' natural wrackline (area of beach containing seaweed and other natural wave-cast organic debris) feeding habitat, reducing the availability of food. Kelp and driftwood, with their associated invertebrates, are regularly removed and the upper layer of sand is disturbed. Beach grooming also alters beach topography, removes objects associated with western snowy plover nesting, and prevents the establishment of native beach vegetation (J. Watkins *in litt.* 1999). In all of Los Angeles County and parts of Ventura, Santa Barbara, and Orange Counties, California, entire beaches are raked on a daily to weekly basis. Large rakes, with tines 5 to 15 centimeters (2 to 6 inches) apart, are dragged behind motorized vehicles from the waterline to pavement or to the low retaining wall bordering the beaches (Stenzel *et al.* 1981). Even if human activity was low on these beaches, grooming activities completely preclude the possibility of successful western snowy plover nesting (Powell 1996).

v. Equestrian Traffic

Most equestrian use on beaches is directed to wet-sand areas. However, during high tide periods, horseback riders on the beach sometimes enter coastal dunes or upper beach areas (Figure 7), where they may crush clutches or disturb western snowy plovers (Point Reyes Bird Observatory unpublished data, Page 1988, Persons 1995, Craig *et al.* 1992, Woolington 1985).

Figure 7. Equestrians on beach (photo by U.S. Forest Service, with permission).

vi. Fishing

Impacts on western snowy plover nesting may be associated with surf fishing and shellfish harvesting in and near western snowy plover habitat. The improper disposal of offal (waste parts of fish), bait, and other litter attracts crows, ravens, and gulls, which are predators of western snowy plover eggs and chicks. Also, western snowy plovers may become entangled in discarded fishing lines (G. Page, pers. comm. 1998).

Surf fishing is a commercial enterprise in many coastal locations, including the ocean smelt fishery in northern California (C. Moulton *in litt.* 1997). Recreational surf fishing occurs throughout the California coast. In Humboldt County, California, Redwood National and State Parks have proposed allowing beach vehicle use, by annual permit, for commercial fishing and tribal fishing/gathering on Gold Bluffs Beach, Freshwater Spit, and Crescent Beach (J. Watkins *in litt.* 1999). In the State of Washington, the most popular season for surf fishing is April through July (Washington Department of Fish and Wildlife 1995). At present,

demand for surf perch fishing is relatively low in Oregon. However, the Oregon Department of Fish and Wildlife is promoting a surf perch fishery to lessen the demand for anadromous fishing. This fishery would increase vehicle driving to remote and relatively undisturbed sites used by western snowy plovers (K. Palermo *in litt.* 1998*a*).

Because the earliest western snowy plover clutches in Washington are laid between mid-April and mid-May, harvesting of razor clams during the mid-March to mid-May clamming season may have adverse impacts on prospecting or nesting western snowy plovers. Clammers near nesting areas may disturb adults and chicks; human activity in feeding areas may restrict western snowy plover foraging activity, and increased motorized traffic may increase the risk of nest and chick loss (Washington Department of Fish and Wildlife 1995). However, observations of western snowy plover and human activities during the spring 1995 razor clam season showed clamming had no visible impact on western snowy plovers where clamming intensity was low (Kloempken and Richardson 1995). Instances of trespassing into the western snowy plover protection area were noted; however, movement of the western snowy plover protection area boundary about 327 meters (1,073 feet) west of its previous location seemed to benefit the birds by providing more space between them and pedestrian and vehicular disturbances.

vii. Fireworks

Fireworks are highly disturbing to western snowy plovers. All western snowy plovers flushed from Coal Oil Point Reserve during a nearby July 4, 2005, fireworks display (C. Sandoval, University of California Santa Barbara, pers. comm. 2005). At Del Monte Beach, California, a western snowy plover chick hatched on July 4, 1996, within an area demarcated by symbolic fencing, and was abandoned by its parents after a fireworks display. Disturbance from the noise of the pyrotechnics is exacerbated by disturbance caused by large crowds attracted to fireworks events. California Department of Parks and Recreation staff estimated that 6,000 people visited Del Monte Beach on that day. Because of the extensive disturbance, the adult western snowy plovers left the nest site with two chicks, abandoned the third chick, and were not seen again (K. Neuman, California Department of Parks and Recreation, pers. comm. 1997). During July 4, 1992,

observations of piping plovers that nest on the Breezy Point Cooperative and adjacent beaches of Gateway National Recreation Area in Queens, New York, the birds were disturbed by fireworks displays (Howard *et al.* 1993). Management recommendations for this area included prohibition of fireworks in or near the fenced and posted nesting and brood-rearing areas.

viii. Kite Flying and Model Airplanes

Biologists believe plovers perceive kites as potential avian predators (Hoopes *et al.* 1992, Hatch 1997). The reaction of western snowy plovers to kites at Ocean Beach in San Francisco, California, "ranged from increased vigilance while roosting in close proximity to the kite flying, to walking or running approximately 10 to 25 meters (33 to 82 feet) away and resting again while remaining alert" (Hatch 1997). It is expected that stunt-kites would cause a greater response from western snowy plovers than traditional, more stationary kites. Stunt kites include soaring-type, two-string kites with noisy, fluttering tails, which often exhibit rapid, erratic movements.

Hoopes *et al.* (1992) found that piping plovers are intolerant of kites. Compared to other human disturbances (i.e., pedestrian, off-road vehicle, and dog/pet), kites caused piping plovers to flush or move at a greater distance from the disturbance, to move the longest distance away from the disturbance, and to move for the longest duration. Piping plovers responded to kites at an average distance of 85 meters (279 feet); moved an average distance of over 100 meters (328 feet); and the average duration of the response was 70 seconds.

It is expected that model airplanes may also have a detrimental impact to western snowy plovers because western snowy plovers may perceive them as potential predators (Hatch 1997).

ix. Aircraft Overflights

Low-flying aircraft (*e.g.*, within 152 meters (500 feet) of the ground) can cause disturbances to breeding and wintering western snowy plovers. Hatch (1997) found that all types of low-flying aircraft potentially may be perceived by western snowy

plovers as predators. She also found that the general response of roosting western snowy plovers to low-flying aircraft at Ocean Beach, San Francisco, California, was to increase vigilance and crouch in depressions on the beach, whereas foraging western snowy plovers frequently took flight. Plovers may, however, become acclimated to aircraft overflights in some instances, since at Naval Air Station North Island they chose to nest repeatedly within military airfield boundaries on runway ovals next to busy military runways (S. Vissman, U.S. Fish and Wildlife Service, pers. comm. 1997). Federal Aviation Regulations, Part 91, General Operating and Flight Rules, require that over open water, aircraft may not be operated closer than 152 meters (500 feet) to any person, vessel, vehicle, or structure. Emergency operations, including those by Coast Guard helicopters, are exempted from these rules. However, helicopters may be operated at less than 152 meters (500 feet) if the operation is conducted without hazard to people or property on the surface (U.S. Federal Aviation Administration 1997). Helicopters can cause excessive noise, which can also disturb western snowy plovers, even at an altitude of 152 meters (500 feet) (Howard *et al.* 1993; J. Watkins *in litt.* 1999; D. Stadtlander, pers. comm. 1999). At Marine Corps Base Camp Pendleton, California, where military training can require aircraft (especially helicopters) to fly at very low elevations, the Marine Corps minimizes impacts to western snowy plovers and California least terns by requiring aircraft to stay at least 91 meters (300 feet) above the ground over tern and plover nesting areas during the nesting season (U.S. Marine Corps 2006).

x. Special Events

Special events which attract large crowds, such as media events, sporting events, and beach clean-ups, have a potential for significant adverse impacts when held in or near western snowy plover habitat. An example is the National Marine Debris Monitoring Program, implemented by the U.S. Environmental Protection Agency in conjunction with the National Oceanic and Atmospheric Administration, National Park Service, and the U.S. Coast Guard. This year-round program uses volunteers (including high school students) to document and collect trash and marine debris on coastal transects within western snowy plover nesting and wintering habitat. Potential threats from crowds of people attracted to special events are similar to

those previously identified for pedestrians, including direct mortality and harassment of western snowy plovers.

xi. Coastal Access

Expanding public access to the coast (*e.g.*, State Coastal Trails) for recreation (*e.g.*, walking, hiking, biking) may adversely affect western snowy plovers and their breeding or wintering habitat. Expanded coastal access brings significantly greater numbers of people to the beach and other coastal habitats, exacerbating potential conflicts between human recreational activities and western snowy plover habitat needs (see Pedestrian section). Expanded coastal access may exceed the threshold of beach visitors that public resource agencies (*e.g.*, State Parks and National Park Service) can effectively manage while also meeting their responsibilities to protect natural resources.

Bicycles are known to adversely affect western snowy plovers nesting on levees and roads near San Francisco Bay salt ponds within the Don Edwards San Francisco Bay National Wildlife Refuge. Many of these levees are closed to human access, but some bicyclists trespass onto closed levees. In 1998, one western snowy plover nest, located on the main access road to the Refuge, was run over by a bicycle as biologists were putting up a barrier to protect it (J. Albertson *in litt.* 1999).

xii. Livestock Grazing

Western snowy plover nests have been trampled by cattle, causing both direct mortality of eggs and flushing of adults from the nests (U.S. Fish and Wildlife Service *in litt.* 1995). Additionally, feral pigs (*Sus scrofa*) may trample westem snowy plover habitat and disturb nesting western snowy plovers (R. Klinger, The Nature Conservancy, pers comm. 1998, D. George *in litt.* 2001). Cow and horse manure can introduce seeds of non-native plants into the dunes.

c. Oil Spills

The Pacific Coast population of the western snowy plover is vulnerable to oil spills. Western snowy plovers forage along the shoreline and in sea wrack (seaweed and other natural wave-cast organic debris) at the high-tide line and are thus at risk of direct exposure to oil during spills. The loss of thermal insulation is considered to be the primary cause of mortality in oiled birds (National Research Council 1985, Leighton 1991). Oiled feathers lose their ability to keep body heat in and cold water out, causing reduced insulation, increased metabolic rate, and hypothermia. Ingestion of oil may lead to physiological changes in birds, including pathological effects on the alimentary tract, blood, adrenal glands, kidneys, liver, and other organs (Fry and Lowenstine 1985, Khan and Ryan 1991, Burger and Fry 1993). Exposure of adult birds to oil also may impair reproduction, including reductions in egg laying and hatchability (Ainley *et al.* 1981, Fry *et al.* 1986) and reductions in survival and growth of chicks (Trivelpiece *et al.* 1984). Oil transferred to eggs from plumage or feet of incubating birds can kill embryos (Albers 1977, Albers and Szaro 1978, King and Lefever 1979). Oiled shorebirds may spend more time preening and less time feeding than unoiled birds, such that their body condition and ability to migrate to breeding grounds and reproduce may be impaired (Evans and Keijl 1993, Burger 1997).

Oil spills may result in contamination or depletion of western snowy plover food sources. Elevated concentrations of total petroleum hydrocarbons have been found in the sand crab (*Emerita analoga*), a potential western snowy plover food item, following a southern California oil spill (J.E. Dugan, unpublished data). Oil or other chemicals washed onto mudflats or sand beaches may result in reduction in the availability of invertebrate prey (Kindinger 1981). Elimination of shorebird food resources on intertidal flats of the Saudi Arabian Gulf coast as a result of the large oil spills associated with the 1991 Gulf War led to drastic reductions in the number of shorebirds supported by this habitat (Evans *et al.* 1993). Disturbance and other adverse impacts to western snowy plovers also may occur during oil clean-up activities if response teams are not careful when driving heavy equipment and vehicles or traversing on foot through western snowy plover habitat.

During the 1990s, at least six oil spill incidents in California and one in Oregon resulted in adverse impacts to western snowy plovers. The U.S. Coast Guard and various other State and Federal agencies and the responsible parties responded to these spills. One of these incidents occurred between 1984 and 1998 at Unocal's Guadalupe Oil Field in San Luis Obispo, California contaminated western snowy plover habitat with toxic hydrocarbons. In 1993, oil spilled from a ruptured oil transfer line into McGrath Lake, Ventura County, California and then flowed into the Pacific Ocean. Western snowy plover habitat and prey were contaminated with oil and wintering western snowy plovers were displaced during the cleanup activities (S. Henry *in litt.* 1998, McGrath Oil Spill Restoration Scoping Document 1995). In 1996, the SS Cape Mohican discharged fuel oil into the San Francisco Drydock Shipyard, California, where it spread throughout the central bay and into the Pacific Ocean, oiling western snowy plovers and their beach habitat (Cape Mohican Trustee Council 2002, Point Reyes Bird Observatory unpublished data). In 1997, a pipeline extending between an offshore oil platform (Platform Irene) and the mainland ruptured near Pedernales Point, Santa Barbara County, California, oiling western snowy plovers and wrack where western snowy plovers were seen feeding (Applegate 1998, Ford 1998, Lockyer *et al.* 2002). In 1997 and 1998, large numbers of tarballs became stranded on beaches at Point Reyes National Seashore and resulted in oiling of snowy plovers and their habitat. Subsequent tarball incidents in 2001 and 2002 resulted in identification of the source of the tarballs as the SS Jacob Luckenbach, an oil tanker that sank in 1953 (Carter and Golightly 2003, Point Reyes Bird Observatory unpublished data, Hughes 2003). In 1999, the dredge M/V Stuyvesant spilled fuel oil into the Pacific Ocean off Humboldt Bay, California (U.S. Coast Guard 2001), resulting in oiling of western snowy plovers and their habitat (LeValley *et al.* 2001).

In February 1999, the freighter New Carissa went aground near the North Jetty of Coos Bay, Oregon, breaking apart and spilling 25,000 to 70,000 or more gallons of oil into coastal water. (U.S. Bureau of Land Management 2001). The incident oiled approximately 52 snowy plovers, representing at least 60 percent of the Oregon wintering population of western snowy plover (Stern *et al.* 2000). In Washington, the 1988 Nestucca oil spill and the 1991 Tenyo Maru oil spill may also have affected western snowy plovers or their habitats, although impacts are not as well documented as in the above cases (Larsen and Richardson 1990).

In addition to catastrophic spills like those described above, chronic oil pollution may affect western snowy plovers. Surveys of beached birds have shown that small-volume, chronic oil pollution is an ongoing source of avian mortality in coastal regions (Burger and Fry 1993). Dead oiled birds and tarballs are found regularly on Pacific coast beaches in the absence of reported oil spills (Roletto *et al.* 2000). Potential sources of chronic oiling include natural seeps, bilge water pumping, sunken vessels, urban runoff, and small or unreported spills from vessels, tankers, pipelines, and offshore oil platforms. Elevated concentrations of total petroleum hydrocarbons have been found in the sand crab *(Emerita analoga*), a potential western snowy plover food item, in the vicinity of natural oil seeps (Dugan *et al.* 1997).

Intensive oil spill cleanup operations, including use of vehicles to deploy beach booms, move personnel, and remove debris, cause disturbance to nesting and foraging activities of western snowy plovers. These temporary impacts are offset by restoration of habitat and cleaning affected birds.

d. Contaminants

The most likely route of exposure of western snowy plovers to contaminants other than spilled oil is through the diet. Western snowy plovers feed on aquatic and terrestrial insects, and the bioaccumulation of environmental contaminants on western snowy plover nesting and wintering grounds may adversely affect their health and reproduction. Organochlorines are known to have caused reduced avian egg production, aberrant incubation behavior, delayed ovulation, embryotoxicosis, and mortality of chicks and adults (Blus 1982). Selenium has caused decreased hatchability of avian eggs, developmental abnormalities, altered nesting behavior, and embryotoxicosis in birds in field and laboratory studies (Ohlendorf *et al.* 1986, Heintz *et al.* 1987). Mercury can cause decreased hatchability of avian eggs (Connors *et al.* 1975), boron has been shown to reduce hatchability of waterfowl eggs in laboratory experiments (Smith and Anders 1989), and arsenic may also adversely affect avian reproduction (Stanley *et al.* 1994).

Hothem and Powell (2000) analyzed 23 western snowy plover eggs collected from 5 sites (Camp Pendleton Marine Corps Base, Batiquitos Lagoon, Naval Amphibious

Base Coronado, Sweetwater Marsh National Wildlife Refuge, and Tijuana Estuary) in southern California from 1994 to 1996 for metals and trace elements, and 20 eggs for organochlorine pesticides and metabolites. All eggs were either abandoned or failed to hatch. Organochlorines, including dieldrin, o,p'-DDD, o,p'-DDE, o,p'-DDT, p,p'-DDD, p,p'-DDE, p,p'-DDT, oxychlordane, and trans-nonachlor were found above the detection limits in western snowy plover eggs. Median DDE and PCB concentrations were less than those normally associated with eggshell thinning. deformities, or other detrimental effects on birds. Twelve metals and trace elements (arsenic, boron, chromium, copper, iron, magnesium, manganese, mercury, nickel, selenium, strontium and zinc) were detected in at least 90 percent of the samples, but generally at background levels. Mean concentrations of all contaminants were below those that would adversely affect reproduction.

Concentrations of mercury in western snowy plover eggs that failed to hatch at Point Reyes National Seashore were five to ten times higher than the mercury concentrations in the five Southern California locations studied by Hothem and Powell (Schwarzbach *et al.* 2003). The mean mercury concentration of 1.07 micrograms/gram (1.07 parts per million), wet weight, in western snowy plover eggs from Point Reyes National Seashore is probably high enough to account for egg failure through direct toxic effects to western snowy plover embryos (Schwarzbach *et al.* 2003). Because only failed and abandoned eggs were taken rather than randomly collected eggs, the extent of mercury contamination of the entire breeding western snowy plover population at Point Reyes can not be reliably assessed from these data; however, the data from the 2000 field season would suggest that about one fifth of the nests appeared to be at risk from adverse effects of mercury (Schwarzbach *et al.* 2003).

e. Litter, Garbage, and Debris

Placement of litter, garbage, and debris in the coastal ecosystem can result in direct harm to western snowy plovers and degradation of their habitats. Litter and garbage feed predators and encourage their habitation at higher levels than would otherwise occur along the coast, making predators a greater threat to western snowy plovers. For example, as noted previously, the California gull (*Larus californicus*) has become far more prevalent in the South San Francisco Bay area. Currently, the

estimated 25,000 California gulls in this area feed in landfills and forage in salt marshes using habitat that once supported the western snowy plover (J. Albertson, pers. comm. 2005).

Marine debris and contaminated materials on the beach also adversely affect western snowy plovers. Marine debris is attributed to both ocean and shoreline sources. Ocean sources of marine debris and contamination include fishing boats, ships, and cruise lines. Cruise line debris may include small plastic shampoo, conditioner, hand lotion, and shoe polish containers, plastic cups, and balloons (Center for Marine Conservation 1995). Shoreline debris is usually from land sources. Western snowy plovers may become entangled in discarded fishing line, fishing nets, plastic rings that hold together six-packs of canned drinks, and other materials on the beach. Containers of contaminated materials (*e.g.*, motor oil, cleaning fluid, and syringes) can introduce toxic chemicals to the beach. The National Marine Debris Monitoring Program, headed by the U.S. Environmental Protection Agency, was established to clean and track sources of marine debris in coastal areas. This monitoring program, while beneficial to western snowy plovers in the long-term, could potentially adversely affect nesting western snowy plovers since the program is conducted year-round. Similarly, the annual spring SOLV beach cleanup held on the Oregon Coast in late March and the annual Coastal Cleanup Day held on the California coast in September are two organized beach events that are poorly timed with respect to prospecting and nesting western snowy plovers. These programs could greatly improve western snowy plover habitat if timed appropriately.

f. Water Quality and Urban Run-off

Many coastal beaches used as habitat by western snowy plovers contain channelized streams or outfalls receiving run-off from urban, industrial, and agricultural areas. Nonpoint sources of water pollution (including hydrocarbons, heavy metals, and household chemicals) could end up at coastal beaches used as western snowy plover foraging areas. In 1995, three dead male western snowy plovers (all banded and local breeders) were found in an area containing local outfalls, including an outfall connected to a sewage treatment plant at Monterey Bay. By the beginning of the next breeding season, it was discovered that another

male western snowy plover from this area disappeared and possibly died. Factors unrelated to the outfall have not been ruled out in the disappearance of this bird. One of the birds was analyzed through necropsy and found to have an enlarged liver, but it could not be determined whether there was a relationship between the mortality and the outfall (Point Reyes Bird Observatory unpublished data).

g. Management for Other Special Status Species

In several instances fencing used to enclose California least tern colonies has caused mortality of western snowy plover chicks that have become entangled within the fence mesh (Powell and Collier 1995, Powell *et al.* 1995), or prevented western snowy plover chicks from following their parents to feeding areas by blocking their movement (Powell *et al.* 1996). These issues have largely been resolved by utilizing fencing with a mesh size of less than 0.64 centimeter (0.25 inch), tightening gaps in fencing seams, and installing "gates" in tern fencing (Foster 2005). Monitoring and minimization measures to avoid these impacts continue to be implemented in coordination with the appropriate Fish and Wildlife Offices. Increasing density and abundance of California least terns within colonies may also result in western snowy plovers being displaced a short distance, but the benefits of tern management for western snowy plovers appear to outweigh such conflicts.

At the Channel Islands and other lands managed by the National Park Service and the Department of the Navy, a decline of western snowy plovers may be caused by disturbance and habitat loss resulting from the large increase in numbers of marine mammals on beaches (U.S. Fish and Wildlife Service *in litt.* 1995, U.S. Department of the Navy *in litt.* 2001). Breeding pinnipeds, including northern elephant seals (*Mirounga angustirostris*), northern fur seals (*Callorhinus ursinus*) and California sea lions (*Zalophus californianus*) at San Miguel Island and San Nicolas Island, have occupied western snowy plover nesting habitat. Beach-cast dead whales have, on occasion, posed threats to nesting western snowy plovers. At Point Reyes beaches, large, whole carcasses have washed ashore and other agencies such as the National Marine Fisheries Service have sought to collect them for scientific purposes. They also attract people who are curious about whales. These activities

could potentially cause direct mortality and disturbance to western snowy plovers. In addition, mammal carcasses attract scavengers such as gulls, ravens, crows, and coyotes that are potential predators to western snowy plovers.

E. IMPLICATIONS FOR THE COASTAL BEACH-DUNE ECOSYSTEM

The western snowy plover lives in an ecosystem that has been significantly degraded. Environmental stressors (*i.e.*, development, human recreation, degraded water quality, etc.) have adversely affected the biological diversity of the coastal dune ecosystem. Many of the characteristics that attract people to coastal areas make these areas prime habitat for fish and wildlife resources. Although they comprise less than 10 percent of the Nation, coastal ecosystems are home to over one-third of the United States human population, nearly two-thirds of the Nation's fisheries, half of the migratory songbirds, and one-third of our wetlands and wintering waterfowl (U.S. Fish and Wildlife Service 1995*a*). The coasts also provide habitat for 45 percent of all threatened and endangered species, including three-fourths of the federally-listed birds and mammals (U.S. Fish and Wildlife Service 1995*a*). Proper stewardship of this unique ecosystem is needed to maintain its ecological integrity while meeting its human demands.

1. Description of Coastal Beach-Dune Ecosystem

The coastal beach-dune ecosystem may include several features such as beaches, foredunes, deflation plains, blow-outs, and reardunes. The beach includes the expanse of sandy substrate between the tide line and the foredune or, in the absence of a foredune, to the furthest inland reach of storm waves. Beach steepness, height, and width are affected by wave height, tidal range, sand grain size, and sand supply. The beach has high exposure to salt spray and sand blast and contains a shifting, sandy substrate with low water-holding capacity and low organic matter content. Dunes include sandy, open habitat, extending from the foredune to typically inland vegetation on stabilized substrate. Major differences occur between beach and dune in salt spray, soil salinity, and air and soil temperatures (Barbour and Major 1990).

Coastal dunes generally consist of three primary zones (Powell 1981). The foredunes are the line of dunes paralleling the beach behind the high tide line. Foredunes are characterized by unstabilized sand and a simple community of low-growing native dune plant species, such as American dunegrass (*Leymus mollis*). Foredunes also support a rich community of sand-burrowing insects (Powell 1981). Behind the foredunes is the deflation plain, which is at or near the water table and is characterized by a mixture of water tolerant plants and dune species. Deflation plains are also called dune hollows and can be invaded by hydrophilic (having a strong affinity for water) trees, shrubs, or herbs (*e.g.*, species of *Carex, Juncus, Salix, Scirpus*) (Barbour and Major 1990). The inner zone of coastal dunes consists of stabilized dunes, which are dominated by woody perennial plants (Powell 1981). Beach flora can also colonize inland dune areas, where the sand is actively moving (Barbour and Major 1990).

Barren dunes, receiving sand from the beach and losing it to wind erosion, are mobile. Older, more inland dunes are stabilized by a nearly continuous plant cover; these dunes are referred to as stable dunes or fixed dunes. Localized openings in the plant cover, which permit wind erosion, are called blowouts, but they are not deep enough to allow invasion by mesophytes (plants growing in moderately moist environments). The innermost ridge of sand is generally high and is called a precipitation ridge; sand is blown over the ridge and down the slipface, continuing the process of dune advance (Barbour and Major 1990). The conditions necessary for dune growth at the coast are partly climatic, but more important is the occurrence of strong onshore winds, abundant sand supply, and vegetation that traps sand. Low, near-shore slopes with a large tidal range providing wide expanses of sand that dries at low tide are ideal for dune growth (Pethick 1984).

Very few coastal dunes are "natural," because they have been extensively altered over time by humans for agriculture, mineral extraction, military training, and recreation (Carter 1988). Before the introduction of European beachgrass, foredunes were low and rose gradually, and a large number of native species shared this habitat. They were composed of a series of dunes alternating with swales oriented perpendicular to the coast and aligned with prevailing onshore winds. Since the introduction of European beachgrass, most systems have been replaced by

a steep foredune that gives way inland to a series of dunes and swales oriented parallel to the coast (Barbour and Major 1990).

Western snowy plovers use the beach and mobile dunes as nesting habitat. Other habitat features that occur within or adjacent to the coastal beach-dune ecosystem, and serve as important foraging habitat for the western snowy plover, include river, stream, and creek mouths, river bars, lagoons, and tidal and brackish-water wetlands.

2. Sensitive Species of the Coastal Beach-Dune Ecosystem

Along with the western snowy plover, many other sensitive species inhabit the coastal beach-dune ecosystem and adjacent habitats. Appendix E contains a list of, and brief species accounts for, sensitive species associated with this ecosystem and adjacent habitats. We recognize these fish and wildlife species as endangered, threatened, candidate species, or species of concern. This list includes a number of sensitive species recognized by the states of California, Oregon, and Washington. This appendix also describes several marine mammals associated with the coastal beach-dune ecosystem and protected under the Marine Mammal Protection Act of 1972 (16 U.S.C. 1361 *et. seq.*), as amended.

Some of these sensitive species have many threats in common with the western snowy plover. Habitat loss and degradation from shoreline development and beach stabilization, invasion of exotic species, and crushing by off-road vehicles are cited as major factors contributing to the status and listing of these species. European beachgrass is a current or potential threat to six federally-listed endangered plants that occur in coastal dunes of California: beach layia (*Layia carnosa*), Howell's spineflower (*Chorizanthe howellii*), Monterey spineflower (*Chorizanthe pungens* var. *pungens*), Menzies' wallflower (*Erysimum menziesii*), Monterey gilia *(Gilia tenuiflora* ssp. *arenaria*), and Tidestrom's lupine (*Lupinus tidestromii*) (Pickart 1997). European beachgrass is also a current and potential threat to native and sensitive plants in Washington and Oregon, including the pink sand-verbena (*Abronia umbellata* ssp. *breviflora*), which is classified as endangered in the State

of Oregon. Equestrian use has also been identified as a threat to several endangered plant species, including the endangered Howell's spineflower, Menzies' wallflower, Monterey gilia, and the coastal dunes milk vetch (*Astragalus tener* var. *titi*). Off-road vehicles are cited as threats to several sensitive plant and animal species, including the endangered beach layia, Menzies' wallflower, Monterey gilia, Tidestrom's lupine, Hoffman's slender-flowered gilia (*Gilia tenuiflora* var. *hoffmanii*), and Smith's blue butterfly (*Euphilotes enoptes smithi*); the federally endangered La Graciosa thistle (*Cirsium longholepis*), and the following species considered to be of Federal concern: beach spectacle pod (*Dithyrea maritima*) and Morro blue butterfly (*Icaricia icarioides morroensis*).

The precarious status of these species is a symptom of a highly stressed ecosystem. Remedial efforts aimed at restoration of the natural processes that maintain this ecosystem, rather than single-species "fixes," are likely to have the greatest and most successful long-term benefits. Important components of ecologically-sound coastal beach-dune ecosystem management include (1) removal of exotic, invasive vegetation; (2) management of human recreation to prevent or minimize adverse impacts on dune formation, vegetation, invertebrate and vertebrate fauna; and (3) efforts to counter the effects of human-induced changes in the types, distribution, numbers, and activity patterns of predators. Implementation of more ecosystem-oriented approaches to western snowy plover protection would provide important benefits to other sensitive species within the coastal dune ecosystem and merits serious consideration.

Some western snowy plover recovery efforts implemented to date (*e.g.*, removal of European beachgrass) support the natural functions of the coastal dune ecosystem. Furthermore, many protection efforts for western snowy plovers should benefit other sensitive beach species, such as California least terns, and vice versa. Many of the same predators that take western snowy plover eggs also prey on California least tern eggs. The relatively low rate of predation of western snowy plover nests in San Diego County has been attributed to predator control programs to benefit California least terns and other species, funded primarily by the Department of Defense and National Wildlife Refuge System (Powell *et al.* 1995). These programs are implemented under contract with the U.S. Department of Agriculture, Wildlife Services branch. Control of ants at California least tern colonies probably

also benefits western snowy plovers nesting nearby. Opportunities also may exist for reestablishment of special status plant species that occur in coastal dunes, including Menzies' wallflower, beach spectacle pod, Tidestrom's lupine, beach layia, and pink sand verbena.

Some conflicts have occurred in management of western snowy plovers and California least terns in southern California, including harm to western snowy plover chicks due to entanglement in the mesh of California least tern fencing as described above. These problems have now largely been minimized with the use of new methods and materials, however such management measures should continue to be coordinated to meet the habitat needs of both western snowy plovers and California least terns.

Potential conflicts also exist between native dune restoration and western snowy plover habitat. Revegetation efforts could result in too much cover, thereby reducing the amount of suitable breeding habitat available for western snowy plovers.

Conflicting habitat requirements for western snowy plovers and pinnipeds have also occurred on lands where marine mammals haul out or breed on beaches that would otherwise be suitable for nesting western snowy plovers (U.S. Fish and Wildlife Service *in litt.* 1995, U.S. Department of the Navy *in litt.* 2001). Where this conflict continues to occur, coordination with land management agencies and NOAA's National Marine Fisheries (NMFS) may be helpful to identify methods for modifying or discouraging use by breeding pinnipeds during the western snowy plover nesting season.

Although some management measures may benefit a broad array of sensitive species within the coastal dune ecosystem (*i.e.*, control of *Ammophila*, access restrictions, and integrated predator management programs), some single-species protection measures for the western snowy plover, such as exclosures, are needed. Although exclosures can be risky to nesting western snowy plovers in some situations (see Lauten *et al.* 2006), they can be an effective way to protect nests against heavy recreational use and predation, especially where reductions in

predator numbers would otherwise be temporary and difficult to achieve or would have adverse ecological effects.

F. CONSERVATION EFFORTS

Western snowy plover recovery efforts have accelerated since this population was federally listed as a threatened species in 1993. Current breeding and wintering site protection efforts are documented in Appendix C (Summary of Current and Additional Needed Management Activities). The most common management strategies include protection of nests with predator exclosures; signing and symbolic fencing of nesting areas; restrictions on motorized vehicles in the vicinity of western snowy plover nests and broods; restrictions on dogs (even though enforcement of dogs on-leash has been problematic); and public information and outreach. These strategies are effective means of improving western snowy plover reproductive success.

1. Conservation Planning on Federal and State Lands

The direction of land management on Federal lands is often outlined in management plans or agency regulations that provide objectives and guidelines for western snowy plovers. These plans include the Naval Base Coronado Integrated Natural Resources Management Plan (U.S. Navy 2001), Camp Pendleton Integrated Natural Resources Management Plan (U.S. Marine Corps 2006), San Diego Bay National Wildlife Refuge Comprehensive Conservation Plan (U.S. Fish and Wildlife Service 2006*c*), Oregon Dunes National Recreation Area Management Plan (U.S. Forest Service 1994), the Coos Bay Shorelands Final Management Plan (U.S. Bureau of Land Management 1995*a*), the New River Area of Critical Concern Management Plan (U.S. Bureau of Land Management 1995*b*), the Draft Snowy Plover Management Plan for Ocean Beach, Golden Gate National Recreation Area (Hatch 1997), and the Western Snowy Plover Management Plan for the Point Reyes National Seashore (White and Allen 1999).

Wildlife protection, especially the preservation, restoration, and enhancement of threatened and endangered species and migratory birds, is the primary goal of national wildlife refuges, as stated in the National Wildlife Refuge System

Administration Act of 1997 (16 U.S.C. 668dd *et. seq.*). Western snowy plover habitat on national wildlife refuges has been accorded intensive protection, including (1) integrated predator management and (2) closures during the nesting season where appropriate, to minimize adverse effects of disturbance. Consistent with requirements of the National Wildlife Refuge System Administration Act and the Refuge Recreation Act of 1962, as amended (16 U.S.C. 460k *et. seq.*) regarding compatibility of refuge activities, western snowy plover nesting areas within some national wildlife refuges are closed to public use during the breeding season. Western snowy plover use areas within some national wildlife refuges (such as Salinas River National Wildlife Refuge) are closed to public use year-round.

Additionally, the Department of Defense manages for western snowy plovers on military installations through actions associated with section 7 of the Endangered Species Act and through conservation planning efforts (*e.g.*, Programmatic Activities and Conservation Plans in Riparian and Estuarine/Beach Ecosystems on Marine Corps Base Camp Pendleton, 1995; see also Federal Regulatory Program, below). This includes avoidance and minimization measures, which have resulted in individual military installations placing limits on or otherwise restricting military activities and implementing management actions to specifically benefit western snowy plovers, such as monitoring, predator control, habitat improvement, and research. This management, in conjunction with other factors such as habitat availability and restricted public access, has allowed certain Department of Defense lands to significantly contribute to regional western snowy plover populations.

The *Washington State Recovery Plan for the Western Snowy Plover* recommends strategies to recover this species, including protection of the population, evaluation, and management of habitat, and initiation of research and education programs (Washington Department of Fish and Wildlife 1995).

The State of Oregon's *Conservation Program for the Coastal Population of the Western Snowy Plover,* required by the Oregon Endangered Species Act and adopted by the Oregon Fish and Wildlife Commission (Oregon Revised Statutes 496.171 through 496.192), requires a variety of actions to protect this subspecies. These actions include: (a) protecting all existing western snowy plover sites from negative impacts; (b) monitoring impacts and responding to damaging activities

(*e.g.*, urban development and recreation disturbance) to minimize or eliminate their effects to western snowy plovers; (c) maintaining a long-term monitoring program to track numbers, distribution, and nesting success; (d) habitat management, such as local control of European beachgrass and maintaining predator protection measures to maximize breeding success for as long as deemed necessary; (e) conducting additional research to maintain and recover western snowy plovers; and (f) enhancing information availability, education, and awareness of western snowy plovers and their requirements for survival and recovery (Oregon Department of Fish and Wildlife 1994).

The California Public Resources Code (Section 5019.71) allows designation of natural preserves, the most protective designation given to a part of any California State Park system unit. The purpose of natural preserves is to preserve such features as rare or endangered plant and animal species and their supporting ecosystems, and representative examples of plant or animal communities existing in California prior to the impact of civilization. The Pajaro Rivermouth Natural Preserve, Wilder Creek Natural Preserve, and Salinas Rivermouth Natural Preserve were designated by the California State Park and Recreation Commission in recognition of the need to protect western snowy plovers. In addition, Section 5019.62 of the California Resources Code allows the designation of State seashores to preserve the outstanding values of the California coastline and provide for public enjoyment of those values. Within the state of California, the following California State seashores containing western snowy plover habitats have been established: Del Norte State Seashore; Clem Miller State Seashore; Sonoma Coast State Seashore; Año Nuevo State Seashore; Monterey Bay State Seashore; San Luis Obispo State Seashore; Point Mugu State Seashore; Capistrano Coast State Seashore; and San Diego Coast State Seashore. Under the California Public Resources Code, the California Department of Parks and Recreation has the authority to identify additional lands appropriate for inclusion in California State seashores and recommend land acquisition for these purposes.

Special management actions for western snowy plovers are conducted within the portions of California State Seashores that are owned by the California Department of Parks and Recreation. An example is the Monterey State Seashore, where the California Department of Parks and Recreation has conducted intensive

management activities for western snowy plovers since 1991. Strategies include resource management, interpretation, law enforcement, and park operations. Resource management actions include monitoring, predator trapping, and use of exclosures, symbolic fences, and signage, and consideration of snowy plovers during planning recreational access and trails in San Francisco Bay. Interpretative efforts include informational signage at nesting areas, information brochures, small handout cards with photographs and information on western snowy plovers, several annual public outreach programs (*e.g.*, slide programs and field trips), and actions to engage community support for the western snowy plover guardian program (*i.e.*, recruitment, training, and scheduling for volunteer presence in sensitive habitat). Enforcement actions include verbal warnings, written warnings, citations, and arrests as necessary. Key enforcement concerns include dogs off-leash and off-road vehicles, which are prohibited on all beaches. Operational management includes a permit process that screens special events to avoid the nesting season in sensitive areas, and regulation of recreational use of beaches to avoid sensitive areas (*i.e.*, kite flying, hang gliding, fishing, etc.). Other management actions on California Department of Parks and Recreation property within some other State seashores are shown in Appendix C.

2. Conservation Efforts on Federal and State Lands

a. Exclosures, Symbolic Fencing, and Signs

Since 1991, one of the primary techniques to protect nesting western snowy plovers has been the use of exclosures (Appendix F). Exclosures are small, circular, square, or triangular metal fences that can be quickly assembled and are designed to keep predators out of nests and/or prevent people from trampling nests (Figure 8). Exclosure designs are described in Appendix F; modifications to exclosure design in response to site specific predator conditions may be appropriate on a case by case basis but should be coordinated in advance with the Fish and Wildlife Service.

Nests protected from predators by exclosures have consistently had increased nest success (White and Hickey 1997, Stern *et al.* 1991, Craig *et al.* 1992, Mabee and Estelle 2000, U.S. Fish and Wildlife Service 2002, Lauten *et al.* 2006). At some locations in Oregon and California, exclosures are designed with tops consisting of

parallel lengths of nylon seine lines spaced approximately 15 centimeters (6 inches) apart -or- mesh netting with a minimum spacing of approximately 10 centimeters (4 inches), designed to discourage entry by avian predators. At Eden Landing State Ecological Reserve in San Francisco Bay, nest predation decreased from 32 percent in 2000 to 3 percent in 2001, largely due to a switch from string tops to net tops on exclosures (Marriott 2001).

Figure 8. Erecting western snowy plover exclosure (photo by Sue Powell, with permission).

Although exclosures are contributing to improved productivity and population increases in some portions of the western snowy plover's Pacific coast range, problems have been noted in some localities. Potential risks associated with exclosures include vandalism, disturbance of the birds by curiosity seekers, and use of exclosures as predator perches. Over time, exclosures may provide a visual cue to predators, making it easier for them to target adults, chicks, and eggs, and requiring predator management. On several occasions depredations of adult western snowy plovers have been documented in or near exclosures, and efforts have been made to establish exclosures later in the season after the peak migration

of raptors (Brennan and Fernandez 2004, Lauten *et al.* 2006). Also, predator exclosures may be impractical where western snowy plovers nest within California least tern colonies or other instances where such exclosures may conflict with the needs of other threatened or endangered species.

Symbolic fencing also is used to passively protect western snowy plover nests, eggs, and chicks during nesting season. This fencing consists of one or two strands of light-weight cord or cable strung between posts to delineate areas where humans (*e.g.*, pedestrians and vehicles) should not enter (Figure 9). It is placed around areas where there are nests or unfledged chicks, and is intended to prevent accidental crushing of eggs, flushing of incubating adults, and, if large enough, to provide an area where chicks can rest and seek shelter when large numbers of people are on the beach. Directional signs (regarding closed areas, nesting sites, etc.) also are used within western snowy plover habitats and near protective fencing to alert the public and other beach users of the sensitivity of western snowy plover nesting and wintering areas. Installation of symbolic fencing at Coal Oil Point Reserve (CA-88) in conjunction with a docent program has allowed management of

Figure 9. Symbolic fencing on beach at Monterey Bay, California (photo by Ruth Pratt, with permission).

recreational use and resulted in successful re-establishment of a breeding population of western snowy plovers at the site (Lafferty *et al.* 2006).

Additionally, land managers may prevent or restrict access to areas used by nesting western snowy plovers. For example, military installations often curtail or redirect training activities near western snowy plover nesting areas and some State parklands and recreation areas restrict public access in certain areas during the breeding season.

b. Law Enforcement

Management agencies recognize that law enforcement is needed for protection measures to be effective. Though a majority of beach visitors respect restrictions to protect western snowy plovers, there will always be a certain percentage who do not. Enforcement of western snowy plover area restrictions shows that managers are serious about compliance. In Oregon, biologists have established a working relationship with a variety of law enforcement agencies who have jurisdiction in western snowy plover habitat. Their goal is to increase awareness, gain advice, increase communication and coordination to alleviate jurisdictional conflicts, and train officers on how to minimize disturbance while patrolling western snowy

plover habitat. Conflicting priorities and personnel turnover require perseverance to maintain effective working relationships across law enforcement jurisdictions.

c. Predator Control

Lethal and nonlethal means of predator control have been used with mixed success to protect western snowy plovers on Pacific beaches. Nonlethal methods include litter control at campgrounds (to reduce available food sources), exclosures and fencing, and trapping and relocation. Lethal methods include reducing local populations of avian predators by addling (*i.e.* killing the developing chick within the egg) of raptor and corvid eggs, trapping and euthanizing nonnative mammalian predators, and killing individual predators upon which nonlethal methods have proven ineffective.

On the Oregon Coast, snowy plover predator control has historically been in the form of nest exclosures and site specific lethal control. The use of nest exclosures, adaptively modified in response to predator behavior, has been very successful in increasing hatching success. However, because in some cases predation on adults has been linked to the presence of exclosures, their use is presently targeted to specific instances where it appears most beneficial, and the program is working toward elimination of exclosure use (Lauten *et al.* 2006a, 2006b).

In 2002, Federal and State agencies approved an integrated predator management program to improve western snowy plover nesting and fledging success in Oregon. The decision followed public review and comment on an analysis of the effects of the proposed predator control methods and alternatives to protect the western snowy plover in Oregon (U.S. Department of Agriculture 2002). To date lethal predator control has been implemented at selected plover breeding sites along the Oregon Coast at Coos Bay North Spit, Bandon Beach, New River, Siltcoos, Overlook, Tahkenitch, and Tenmile, resulting in an overall positive effect on western snowy plover productivity (Lauten *et al.* 2006*a*, 2006*b*).

Another form of predator control is fencing, which is used on the south spoils area of Coos Bay, North Spit, where the U.S. Bureau of Land Management, U.S. Army Corps of Engineers, and Oregon Department of Fish and Wildlife have fenced 8

hectares (20 acres) of western snowy plover nesting habitat. This wire mesh fence was installed to exclude mammalian predators, especially skunks, and to discourage human disturbance from off-highway vehicle use. The original fence, constructed in 1991, suffered from the effects of weathering and although it continued to deter vehicles, it was no longer an effective barrier to predators. In 1998, the U.S. Army Corps of Engineers and U.S. Bureau of Land Management jointly constructed a new fence and removed the old fence. The new fence matched the design of the 1991 fence (5-centimeter by 5-centimeter (2-inch by 2-inch) mesh fence material with an effective fence height of about 1.2 meters (4 feet) after burial of the bottom). However, the new fence has increased the protected area from 8 hectares (20 acres) to 28 hectares (71 acres), and includes both the south spoils area and the 1994 Habitat Restoration Area (E.Y. Zielinski and R.W. Williams *in litt.* 1999).

At the Don Edwards San Francisco Bay National Wildlife Refuge, fences are sometimes constructed across salt pond levees to block access by terrestrial predators (J. Albertson *in litt.* 1999). However, fences are not feasible in many areas, and do not restrict aerial predators.

Exclosures are much more effective when used in conjunction with an integrated predator management program that includes selective removal of non-native predators and other individual problem predators. Otherwise, exclosures may promote better hatching success, but not fledging success if predators such as red fox (*Vulpes vulpes*) focus on adults protecting the nest or newly-hatched chicks that leave the exclosure to feed. These measures are also much more effective where combined with other access restrictions to increase survival of clutches and broods. Trapping the nonnative red fox has been credited with substantially increased western snowy plover abundance and productivity at Salinas River National Wildlife Refuge (E. Fernandez, U.S. Fish and Wildlife Service, pers. comm. 1998). At the Don Edwards San Francisco Bay National Wildlife Refuge, predation on western snowy plovers and California clapper rails by red foxes prompted the initiation of a predator management program targeting red foxes, feral cats, skunks, and raccoons, in conjunction with use of western snowy plover nest exclosures (J. Albertson *in litt.* 1999, Strong *et al.* 2004). This ongoing program has resulted in improved nest success. Use of exclosures has subsequently been discontinued due to the success of the trapping program and incidents of nest abandonment at exclosures. At Eden

Landing Ecological Reserve selective removal of problem corvids and their nests has also been practiced by USDA Wildlife Services since 2004 (Tucci *et al.* 2006).

The U.S. Air Force has used electric fencing around the California least tern colony at Purisima Point, Vandenberg Air Force Base, California, where western snowy plovers also nest and winter. The electrified portion of this fence is approximately 273 meters (300 yards) long and 1.2 meters (4 feet) high. The electric fence contains six strands of electrified wire placed approximately 10.2 centimeters (4 inches) apart. This fence is generally effective at keeping out mammalian predators of California least terns. It has also incidentally protected a small population of western snowy plovers by deterring western snowy plover predators.

Proposals have been developed to test a conditioned taste aversion technique on predators of piping plovers (*i.e.*, red fox) by using quail eggs treated with the chemical emetine (McIvor 1991). The purpose of this technique is to condition foxes to avoid eating plover eggs, expecting that if foxes eat treated quail eggs prior to the nesting season and become sick, they might develop a conditioned aversion to eating plover eggs. This technique requires that the predator consumes the needed dose that will produce short-term illness but no mortality. Due to uncertainty in effectiveness, at this point in time we do not advocate this taste aversion technique. Proposals to test conditioned taste aversion techniques on predators of piping plovers on the east coast have not been implemented due to difficulties obtaining permission to field test emetine (A. Hecht, U.S. Fish and Wildlife Service, pers. comm. 1996). Avery *et al.* (1995) found that deployment of quail eggs treated with the chemical methiocarb might be a useful means of reducing predation of California least terns by ravens and crows. However, subsequent tests of aversion methods have proven to be unsuccessful (E. Copper and B. Foster *in litt.* 2001).

With proper research, techniques that have been used to deter predators of other wildlife species may prove beneficial to western snowy plovers. Strategic placement of crow and gull carcasses around the perimeter of a California least tern colony has been used at Vandenberg Air Force Base (Persons and Applegate 1996), however, this method may not be effective for more loosely colonial species such as snowy plover (J. Buffa *in litt.* 2004). Moreover, the presence of gull carcasses could prove

counterproductive by attracting mammalian predators (N. Read, U.S. Air Force, pers. comm. 1998).

In 1999 Vandenberg Air Force Base initiated studies of coyote ecology and movements, with the goal of developing non-lethal alternatives for reducing coyote predation on western snowy plover. Although results are preliminary, in 2001 beach access restrictions and regular pick-up of trash, in combination with availability of alternative prey such as rabbits, may have contributed to the lowest incidence of coyote predation ever recorded at Vandenberg Air Force Base, even though evidence of coyote presence continued to be observed on a daily basis.

For top-level predators such as coyotes, westem snowy plover nests are not a primary food source. Vandenberg Air Force Base has avoided large-scale coyote removal to prevent exacerbated predation on listed species from mesopredators such as racoons, and to prevent expansion of non-native predators such as feral cats and red foxes into western snowy plover nesting areas (N. Read Francine *in litt.* 2001).

d. European Beachgrass Control

Experiments to find cost-effective methods to control or eradicate European beachgrass are ongoing. Control methods employed in various situations have included foredune grading and foredune breaching with front-end loaders and bulldozers, subsoiling with a winged subsoiler (essentially a heavy duty three-point plow), discing with a standard farm tractor and disk, burning, saltwater irrigation, spraying of herbicide, and hand-pulling. Herbicide treatment is not always possible, however, when rare or federally-listed plants are present. In these cases hand-pulling or other mechanical removal may need to be employed. At Point Reyes National Seashore mechanical and hand-removal were used to remove non-native beach grass on 12 hectares (30 acres) with immediate beneficial response by nesting snowy plovers (Peterlein and Roth 2003). Some control methods are only suitable for the inland sites. Areas containing heavy growth of European beachgrass and woody vegetation are prescribed-burned prior to using heavy equipment. Areas are leveled to allow discing for maintenance. In some areas, oyster shell hash provided by a local oyster grower has been distributed after vegetation has been removed. Effectiveness of the various control methods varies, though some form of

maintenance may always be required. Maintenance is critical and achieved through multiple treatments over a succession of years. Discing requires maintenance twice per year to keep beachgrass from reestablishing. Comparatively, yearly maintenance in portions of some restoration sites may not be needed after employing several years of bull-dozing, herbicides, or hand-pulling following initial mechanical removal.

Since 1994, multiple projects have been conducted in Oregon to control beachgrass on existing nest sites and to clear and maintain additional areas. These Habitat Restoration Areas (HRAs) are essential for the recovery of the western snowy plover. Three significant HRAs established on the Oregon Coast between 1994 and 2002 include the Dunes Overlook (Oregon Dunes National Recreation Area), Coos Bay North Spit, and New River. Other habitat restoration areas have recently been established or are planned at Baker Beach (140 acres), Tenmile Creek (200 acres) and Bandon Beach State Natural Area (30 acres). HRAs accounted for 34 percent of nests (Table 6) and 43 percent of fledglings (Table 7) found on the Oregon Coast between 1999 and 2004.

The Oregon Dunes National Recreation Area contains about 2,428 hectares (6,000 acres) of European beach grass and now has few remaining examples of intact native plant communities (Pickart 1997). Habitat restoration was initiated in the summer of 1998 and by 2002, the U.S. Forest Service had treated 24 hectares (60 acres) of the 208 hectares (516 acres) of habitat planned for restoration. Prior to 1999, no western snowy plovers were found at the Overlook site, but after habitat was restored, western snowy plovers began nesting there successfully (Table 6, Table 7).

The U.S. Forest Service employs a combination of mechanical, manual, and herbicide treatments to control European beachgrass. Mechanical treatment consists of scalping off the top 1 meter (3 feet) of beachgrass and then burying it in an adjacent trench with a minimum covering of l meter (3 feet) of sand. Moderate to heavy resprouting occurs with this method, requiring manual or chemical follow-up treatment. Other mechanical treatments have consisted of placement of dredged material on the beachgrass and scalping the top half of foredunes to remove beachgrass and allow for inland sand movement and tidal action to maintain open dunes (K. Palermo *in litt.* 1998*b*).

Herbicide treatments have been conducted as a primary control method and as follow-up to mechanical control. In recent years, from 2 to 26 hectares (5 to 65 acres) of beachgrass were sprayed with an herbicide treatment of 8 percent Rodeo and nonionic surfactant (spray-to-wet) at three locations. Employees found that a follow-up application within 2 weeks of the first application was critical to obtain optimum coverage and initial die-off rates (90 percent). Additionally, herbicide treatments were most effective when conducted consecutively over 2 to 3 years depending on density. Beachgrass control at the Oregon Dunes is still considered experimental. Preliminary results suggest that maintenance will always be necessary (K. Palermo *in litt.* 1998*b*).

Table 6. Total number of nests at habitat restoration areas on the Oregon Coast 1994-2004 (J. Heaney, pers. comm. 2003; C. Burns, pers. comm.; M. VanderHeyden, pers. comm.; Castelein *et. al.* 2002; Lauten *et al.* 2006).

Site Name	1994	1995	1996	1997	1998	1999	2000	2001	2002	2003	2004	**Total Nests**
Baker Beach									0	1	0	1
Dunes Overlook						2	8	15	8	9	14	56
Coos Bay North Spit	4	3	2	3	7	12	22	13	15	11	16	108
Bandon State NRA										4	17	21
New River						2	4	10	7	5	6	34

Table 7. Total number of fledged young at habitat restoration areas on the Oregon Coast 1994-2004. Includes fledglings from broods from undiscovered nests (J. Heaney, pers. comm. 2003; C. Burns, pers. comm; M. VanderHeyden pers. comm.; Castelein *et. al.* 2002; Lauten *et al.* 2006).

Site Name	1994	1995	1996	1997	1998	1999	2000	2001	2002	2003	2004	**Total Nests**
Baker Beach									0	0	0	0
Dunes Overlook						3	5	2	2	3	6	21
Coos Bay North Spit	7	2	1	1	1	23	6	6	8	14	22	91
Bandon State NRA										4	15	19
New River						2	1	3	3	7	5	21

On Coos Bay North Spit, the Bureau of Land Management has cleared and maintained approximately 67 hectares (166 acres) of vegetation dominated by European beachgrass, shore pine, Sitka spruce, and Scotch broom. The objective is to remove predator cover, remove encroaching beachgrass, and expand the existing habitat. The goal is to create an area for western snowy plovers to nest that is large enough to lessen possible detection of nests and chicks by predators. Nest sites used by western snowy plovers on the North Spit include both beach habitat and inland areas of previous dredged material deposition. Many of the cleared areas were used almost immediately by nesting western snowy plovers or for brood rearing activities. Prior to 1994, western snowy plovers were not nesting in these areas, but after 1994, the Coos Bay North Spit became the most productive western snowy plover nesting sites on the Oregon Coast (Table 6, Table 7) (M. VanderHeyden, Bureau of Land Management, pers. comm.).

At the Coos Bay North Spit, an inmate crew from the Shutter Correctional Facility, hired by the U.S. Bureau of Land Management, hand pulled European beachgrass on approximately 6 hectares (15 acres) of the south spoil area. The 4-month project cost $11,500; most of these costs covered the crew supervisor's salary and transport vehicle charges. Another European beachgrass removal project around the south spoil areas of the Coos Bay North Spit, included burning European beachgrass, followed by scarification using a bulldozer in March 1994. By August, most of the area had resprouted (Oregon Department of Fish and Wildlife 1996). New beachgrass sprouts are relatively easy to remove. However, initial and maintenance work can be costly and labor intensive. At the Coos Bay North Spit, eradication of European beachgrass using 91.4 centimeters (36 inches) of sprayed seawater was attempted in 1996. The saltwater application was not effective because desiccated sand layers did not allow seawater penetration to the grass's root zone. Future experimentation using wetting agents to achieve water penetration on small-scale applications could demonstrate potential applicability of this technique (G. Dorsey, U.S. Army Corps of Engineers, pers. comm. 1997).

The New River Spit is another key nesting area for the western snowy plover that is managed by the Coos Bay U.S. Bureau of Land Management. Each year since 1998, the U.S. Bureau of Land Management has used heavy equipment (i.e., front-end loader, bulldozer) to remove European beachgrass from in and around a target

restoration site. Typically, the bulldozer is used to push the beachgrass into depressions and bury it under several feet of sand, or to push sand and beachgrass out into the surf zone. Just over two miles of foredune have been lowered and select areas along the foredune have been removed to allow ocean surf to overwash into interior portions of the spit. The overwashing aids in scouring vegetation and appears to self-maintain portions of the overwashes throughout the restoration area. By 2002, approximately 48 hectares (120 acres) of foredune and overwash were cleared of beachgrass (Jim Heaney, Bureau of Land Management, pers. comm. 2003).

Work at Lanphere-Christensen Dune Preserve in Humboldt County, California, showed that hand pulling can eliminate European beachgrass, but 3 years of multiple maintenance treatments were required (Pickart and Sawyer 1998). Use of heavy equipment (*e.g.*, "V" ripper) and herbicides may be more cost-effective; however, resprouting of the grass occurs, necessitating follow-up, manual pulling for long-term beachgrass removal (A. Pickart, The Nature Conservancy, pers. comm. 1997).

The effective strategy used by the California Department of Parks and Recreation to remove beachgrass at Marina Dunes and Salinas River State Beaches, Monterey Bay, included multiple herbicide applications of 10 percent Round-Up. Approximately 25 patches of beachgrass covering a total of approximately 0.5 hectare (1.3 acres) have been treated along a 6.4-kilometer (4-mile) section of beach. Each patch of beachgrass was sprayed every 3 months over a 3-year period. All treated sites were marked so that they could be easily located and monitored for regrowth and spread. Current plans include beachgrass removal on approximately 30 hectares (75 acres) at Zmudowski State Beach at the Pajaro River mouth (D. Dixon *in litt.* 1998).

Western snowy plover habitat restoration efforts at the Leadbetter Point Unit of the Willapa National Wildlife Refuge began in 2002 and continue. American beachgrass and some European beachgrass have been mechanically removed, clearing approximately 25 hectares (63 acres) as of 2006. In addition, cuts have been made through the foredune and oystershell placed to cover 11 hectares (28 acres) within the restored area (K. Brennan *in litt.* 2006).

Pickart (1997) suggested that chemical treatment of European beachgrass is likely to be the most cost-effective method used to date. Herbicides that have been used for

this purpose are glyphosates (trade names Rodeo and Round-Up). The most effective period for herbicide treatment of beachgrass is during its flowering stage (Wiedemann 1987); plants should be treated during periods of active growth (Pickart 1997). However, potential adverse biological impacts to other native plants and animals must be considered when using herbicides, and selective spraying may be difficult in some areas. Chemical treatment in active western snowy plover nesting areas may need to be limited to the period outside the breeding season in certain areas to avoid disturbing nesting western snowy plovers.

Additional management options for beach and dune erosion control are needed. Beachgrass continues to be used because it has been tried successfully in the past, nursery stock is available, and field planting technology is well known. However, negative aspects of its monoculture are recognized. Proper planting and management of a mixture of native vegetation, together with the provision of walkways for pedestrian traffic and the elimination of horse traffic, cattle grazing, and off-road vehicles, may result in stabilization as effective as beachgrass, yet there has been minimal experimentation with this technique (Barbour and Major 1990).

e. Off-Road Vehicle Restrictions and Management

Management strategies to reduce off-road and other vehicle impacts have been implemented at some western snowy plover breeding areas. At Pismo/Oceano Dunes State Vehicular Recreation Area, California, management strategies include fenced-off nesting areas; placement of exclosures around nests; restrictions on vehicle speed and access areas; and requirements that car campers remove all trash. At Pismo/Oceano Dunes State Vehicle Recreation Area, the California Department of Parks and Recreation, Off-Road Vehicle Division, has developed an interim management plan, which is adapted annually in coordination with us to address what effects current management measures have on hatching rates and fledging success, as well as recruitment into the western snowy plover population (California Department of Parks and Recreation 2005). The Off-Road Vehicle Division of the California Department of Parks and Recreation is now funding the development of a habitat conservation plan (in anticipation of applying for a section 10(a)(1)(B) permit under the Endangered Species Act) for the Pismo/Oceano Dunes State Vehicular Recreation

Area and other State parks within the San Luis Obispo Coast District of the California Department of Parks and Recreation.

The conservation issues for western snowy plovers and California least terns at the Pismo/Oceano Dunes State Vehicular Recreation Area are directing the development of the habitat conservation plan, but other species also will be covered. This plan will evaluate the effects that recreation and park management activities are having on the covered species.

On Camp Pendleton, the Marine Corps conducts its vehicle operations in and near nesting areas in ways that minimize impacts to western snowy plovers. Under the Marine Corps' Base Regulations all training activities, including vehicle training, are prohibited within 300 meters of fenced nesting areas during the breeding season (1 March to 15 September). Further, amphibious vehicles are directed to transit adjacent to nesting areas with tracks in the ocean whenever possible (U.S. Marine Corps 2006).

On the Don Edwards San Francisco Bay National Wildlife Refuge, part of the main access road (Marshlands Road) is closed to motorized vehicles from April 1 to August 31, to protect western snowy plovers nesting near the roadway. Highway traffic cones and ribbons are installed to discourage vehicle access to nesting areas on roads and levees (J. Albertson *in litt.* 1999).

In 1995, after the Oregon Dunes National Recreation Area completed its management plan, the U.S. Forest Service petitioned the Oregon Parks and Recreation Department to close several kilometers of beach that had been open to vehicles. Resulting closures reduced conflicts between off-highway vehicles and nonmotorized recreationists, western snowy plovers, and other wildlife (E.Y. Zielinski and R.W. Williams *in litt.* 1999).

Leadbetter State Park (immediately to the south of Willapa National Wildlife Refuge) is closed to beach driving from April 15 to the day after Labor Day. The entire beach along Willapa National Wildlife Refuge is closed to driving year round, except during razor clam openers (K. Brennan *in litt.* 2006). Diligent surveillance and

enforcement by applicable agencies is extremely important due to the potential for violations.

f. Population Monitoring

Western snowy plover researchers in Washington, Oregon and California conduct intensive population monitoring programs. Tasks include some or all of the following: (1) conducting winter and breeding season window surveys; (2) banding adults and chicks; (3) determining nest success; (4) determining fledging success, (5) monitoring and documenting brood movements; and (6) collecting general observational data on predators.

The Point Reyes Bird Observatory has been monitoring the distribution and breeding success of western snowy plovers since 1977. Monitoring at Vandenberg Air Force Base has been conducted by Point Reyes Bird Observatory and SRS Technologies. Additionally, Santa Barbara County-supported volunteer docents stationed at Surf Station, within Vandenberg Air Force Base, keep tallies of numbers of visitors, violations prevented, and predators seen (R. Dyste *in litt.* 2004). The U.S. Geological Survey Biological Resources Division monitored western snowy plovers in San Diego County from 1994 to 1998. Teams led by Elizabeth Copper, Robert Patton, Shauna Wolf, and Brian Foster have monitored western snowy plovers in San Diego County since 1999 for military installations. The Oregon Natural Heritage Program and The Nature Conservancy have conducted western snowy plover monitoring since 1990 in Oregon. The Point Reyes Bird Observatory, Oregon Natural Heritage Program, and U.S. Geological Survey, Biological Resources Division, also band western snowy plovers at some locations (Figure 10). The California Department of Parks and Recreation conducts annual monitoring throughout the state and at the Pismo/Oceano Dunes State Vehicular Recreation Area (J. Didion *in litt.* 1999). Mad River Biologists and Humboldt State University are currently conducting intensive population monitoring in northern California. Department of Defense installations continue to maintain long-term programs for monitoring and management of western snowy plover populations and predators in San Diego and Ventura Counties, including programs at Camp Pendleton, Naval Amphibious Base Coronado, Naval Radio Receiving Facility Imperial Beach, North Island, and San Clemente Island.

Figure 10. Banding a western snowy plover chick (photo by Bonnie Peterson with permission)

g. Salt Pond Management

Intensive management at the Moss Landing Wildlife Area has made a major contribution to western snowy plover breeding success in the Monterey Bay area. Management by Point Reyes Bird Observatory staff, in coordination with the California Department of Fish and Game, has been ongoing since 1995. Management activities include draw-down of water levels in part of the salt ponds at the beginning of the nesting season to provide dry sites for nests, and flooding of remnant wet areas twice per month through the nesting season to maintain foraging habitat for adults and their young. Predator control is conducted by the U.S. Department of Agriculture, Wildlife Services Branch.

The Don Edwards San Francisco Bay National Wildlife Refuge manages a former salt pond called the "Crescent Pond" (within location CA-36, mapped in Appendix L) for

western snowy plovers by reducing the water levels prior to the breeding season. In the early 1990s, this pond was mostly unvegetated salt flat, but since then native pickleweed (*Salicornia virginica*) has slowly increased on the site, making the areas less valuable for western snowy plover nesting habitat. The Refuge has begun to conduct winter flooding in the Crescent Pond to reduce vegetative cover and improve western snowy plover nesting habitat.

The 2003 acquisition of Cargill's West Bay, Alviso, and Baumberg Salt Ponds in the South Bay by California Department of Fish and Game and Don Edwards San Francisco Bay National Wildlife Refuge will greatly further the goal of achieving 810 hectares (2,000 acres) of ponds managed for western snowy plover habitat (see Recovery Action 2.6). The Refuge's long-term management plans for these areas will include management that is compatible with western snowy plover and will coordinate with the recovery goals of this Recovery Plan (J. Albertson, pers. comm. 2005). Many of the salt ponds are currently used for breeding and wintering by western snowy plovers. San Francisco Bay Bird Observatory is assisting the Refuge with salt marsh management and western snowy plover monitoring**.**

h. Habitat Acquisition

Acquisition and management of key sites is an important conservation effort. In October 1998, The Nature Conservancy transferred the approximately 193-hectare (483-acre) Lanphere-Christensen Dunes Preserve (part of Mad River Mouth and Beach, California, CA-7) to us for conservation purposes. The area will be managed by the Humboldt Bay National Wildlife Refuge for natural resources, including the western snowy plover. In October 1998, the Port of San Diego announced an agreement enabling approximately 560 hectares (1,400 acres) of Western Salt Company land (CA-131) to be managed by the San Diego National Wildlife Refuge. The salt ponds are a western snowy plover nesting and wintering area. As noted above, Cargill's transfer of the West Bay, Alviso, and Baumberg salt ponds, including 6,110 hectares (15,100 acres), to California Department of Fish and Game and Don Edwards San Francisco Bay National Wildlife Refuge was completed in 2003; portions of this area will be managed as western snowy plover habitat.

i. Use of Volunteers

Volunteers contribute to the conservation of western snowy plovers and their habitat at many beach locations, including Morro Bay and Oceano Dunes State Vehicular Recreation Area, Point Reyes National Seashore, and Golden Gate National Recreation Area. Volunteers and docents assist public land managers in many ways (Appendix K), including informing park visitors about threats to the western snowy plover, reducing human and pet disturbances, and assisting with direct habitat enhancement (*e.g.*, manual removal of European beachgrass; Figure 11). In 1998, the Western Snowy Plover Guardian Program was developed to assist the conservation and recovery of western snowy plovers in Monterey Bay. This program is mainly a volunteer effort by local citizens who assist in protecting western snowy plovers through monitoring, reporting, and educational activities (D. Dixon *in litt.* 1998).

Figure 11. High school students removing European beachgrass (photo by Kerrie Palermo, with permission).

j. Public Outreach and Education

Public land managers and private conservation organizations have produced public educational materials, including brochures, posters, flyers, and informational/interpretative signs regarding western snowy plovers (Appendix K). Environmental education/interpretation is recognized by land management agencies as an important tool that supports their mission of resource stewardship. Increased understanding and appreciation of natural resources (specifically threatened and endangered species) often results in increased public support. This support is not easily measured and when the audience is children, results may not be seen until they reach adulthood. However, those agencies conducting western snowy plover education to date have found a positive response by individuals. In Oregon, on-site monitors of the U.S. Forest Service (Oregon Dunes National Recreation Area) and U.S. Bureau of Land Management report a willingness of the majority of contacted individuals to comply with restrictions after better understanding the reasons for them.

The La Purisima Audubon Society, Santa Barbara County, produced an educational video about the western snowy plover and the California least tern in 1999. It was distributed to public schools and museums within Santa Barbara County in 2000.

k. Section 6 Cooperative Agreements

Section 6 of the Endangered Species Act allows us to enter into cooperative agreements with states that establish and maintain active programs for the conservation of listed species. Through funding under section 6, those states assist the recovery of endangered and threatened species and monitor their status. Between 2000 and 2006, traditional section 6 funds have been used for creation of a docent program at Silver Strand State Beach in California ($8,300); development of a water management plan at Moss Landing Wildlife Area, California ($4,886); surveillance and protection of snowy plover nests on California beaches ($92,000); and surveys, nest monitoring, protecting nests with exclosures, collecting data on human uses of beaches, and encouraging beach uses compatible with snowy plovers in Oregon ($64,386) and Washington ($48,677). HCP Planning grants were used for development of a habitat conservation plan to address management of beach use by the Oregon Parks and Recreation Department ($103,950) and development of an Environmental Impact Statement for this Habitat Conservation Plan ($200,000). A Recovery Land Acquisition grant ($307,000) supported purchase of a conservation easement on 89 hectares (220 acres) of western snowy plover habitat along 3.7 kilometers (2.3 miles) of the Elk River Spit.

3. Conservation Efforts on Private Lands

Private landowners interested in conservation efforts for western snowy plovers and coastal dune habitats have made important contributions to recovery efforts for coastal dune species. At Ormond Beach, California, Southern California Edison has enhanced approximately 60 hectares (150 acres) of degraded wetlands and coastal dune habitat for several special status species, including the western snowy plover and California least tern (D. Pearson, Southern California Edison, pers. comm. 1996).

4. Federal Regulatory Program

a. Critical Habitat

On March 2, 1995, we published a proposed rule to designate critical habitat for western snowy plover at 28 areas along the coast of California, Oregon, and Washington (U.S. Fish and Wildlife Service 1995*b*). At that time, critical habitat was proposed to fulfill an outstanding requirement under section 4 of the Endangered Species Act to highlight important habitat areas on which activities that require Federal actions need to be evaluated under section 7 of the Endangered Species Act. A funding moratorium by the U.S. Department of the Interior for listing actions was in place during the period April 1995 to April 1996. We subsequently acknowledged a serious backlog of listing actions and the need to prioritize them (U.S. Fish and Wildlife Service 1996*b*). Hence, we developed guidance for assigning relative priorities to listing actions conducted under section 4 of the Endangered Species Act during fiscal years 1998 and 1999 (U.S. Fish and Wildlife Service 1998). Designation of critical habitat was placed in the lowest priority (Tier 3). Under this guidance, we placed higher priority on listing imperiled species that currently have limited or no protection under the Endangered Species Act than on devoting limited resources to the process of designating critical habitat for currently-listed species. In addition, we found that because the protection afforded by critical habitat designation applies only to Federal actions, such designation provides little or no additional protection beyond the "jeopardy" prohibition of section 7 of the Endangered Species Act, which also applies only to Federal actions (U.S. Fish and Wildlife Service 1998).

In December 1995, legal challenges by the Environmental Defense Center, Santa Barbara, California, against the U.S. Department of the Interior to finalize designation of critical habitat for the western snowy plover were overruled by the California District Court (U.S. District Court, Central District of California 1995). At that time, the Court's order was based on its decision that lack of funding prevented the Secretary of the Interior from taking final action on proposals for designating critical habitat. However, on November 10, 1998, the U.S. District Court for the Central District of California ruled that the Secretary of the Interior must publish a final designation of critical habitat for the western snowy plover before December 1, 1999 (U.S. District Court, Central District of California 1998).

A final rule designating critical habitat was published on December 7, 1999 (U.S. Fish and Wildlife Service 1999). In May of 2002 the Coos County Board of County Commissioners, Friends of Oceano Dunes, and Concerned Citizens for western Lane County filed a complaint asking for invalidation of the rule. The United States moved for voluntary remand to reconsider the economic analysis and for partial vacatur of the existing designation. On July 19, 2003, the District Court for the District of Oregon granted the United States' motion, ordering the Service on remand to consider the economic impact analysis and ensure that the new rule is based on the best scientific evidence available. This Order was converted to Judgment on July 2, 2003. Based on the potential for harm to the population, at the Service's request the court left most of the established units in place during the redesignation process, but vacated two units in southern California and two units in Washington.

On December 17, 2004, we published a new proposal to designate critical habitat for the Pacific coast distinct population segment of the western snowy plover (U.S. Fish and Wildlife Service 2004*b*). The final rule to designate critical habitat was published on September 29, 2005 (U.S. Fish and Wildlife Service 2005). This rule designated critical habitat in 32 units, compared to 28 units in the 1999 critical habitat final rule, but covers only 4,921 hectares (12,145 acres) compared to 7,881 hectares (19,474 acres) in the 1999 rule. Of the 32 units, 23 are in California, 5 are in Oregon, and 3 are in Washington. Of the total acreage, 1,002 hectares (2,478.5 acres), or 20 percent, are on Federal lands; 2620.5 hectares (6,474 acres), or 53 percent, are on land owned by States or local agencies; and 1294.5 hectares (3,191 acres), or 26 percent, are privately-owned.

It is important to understand what critical habitat means and how it differs from this recovery plan. Section 3 of the Endangered Species Act defines critical habitat to mean: (i) the specific areas within the geographical area occupied by the species at the time it is listed on which are found those physical or biological features (I) essential to the conservation of the species and (II) which may require special management considerations or protection; and (ii) specific areas outside the geographical area occupied by the species at the time it is listed, upon determination that such areas are essential for the conservation of the species. The term "conservation" is defined in section 3 as "the use of all methods and procedures which are necessary to bring any endangered species or threatened species to the

point at which the measures provided pursuant to this Act are no longer necessary." Therefore, critical habitat is to include biologically suitable areas necessary to recovery of the species.

Section 7 of the Endangered Species Act requires Federal agencies to consult with us to evaluate the effects that any activities they fund, authorize, or carry out may have on designated critical habitat. Agencies are required to ensure that such activities are not likely to adversely modify (*e.g.*, damage or destroy) critical habitat. Because the issuance of permits under section 10(a)(1)(B) of the Endangered Species Act constitutes a Federal action or connection and is subject to an internal section 7 consultation, habitat conservation plans developed for actions on private lands must also analyze the potential for adverse modification of critical habitat. Accordingly, where Federal activities may affect western snowy plover critical habitat, we will consult with Federal agencies under section 7 to ensure that these actions do not adversely modify critical habitat.

Critical habitat designation does not create a wilderness area, preserve, or wildlife refuge, nor does it close an area to human access or use. It applies only to activities sponsored at least in part by Federal agencies. Such federally-permitted land uses as grazing and recreation may take place if they do not adversely modify critical habitat. Designation of critical habitat does not constitute a land management plan, nor does it signal any intent of the government to acquire or control the land. Therefore, if there is no Federal involvement (*e.g.*, Federal permit, funding, or license), activities of a private landowner, such as farming, grazing, or constructing a home, generally are not affected by a critical habitat designation, even if the landowner's property is within the geographical boundaries of critical habitat (U.S. Fish and Wildlife Service 1993*c*). Without a Federal connection to a proposed action, designation of critical habitat does not require that landowners of State or other non-Federal lands do anything more than they would otherwise do to avoid take of listed species under provisions of section 9 of the Endangered Species Act.

By comparison, a recovery plan delineates site-specific management actions that we believe are required to recover and/or protect listed species, establishes objective, measurable criteria for downlisting or delisting the species, and estimates time and cost required to carry out these actions. A recovery plan is not a regulatory document

and does not obligate cooperating or other parties to undertake specific tasks or expend funds.

Critical habitat designation is not necessarily intended to encompass a species' entire current range. Recovery plans, however, address all areas determined to be important for recovery of listed species and identify needed management measures to achieve recovery. Because critical habitat designations may exclude areas based on factors such as economic cost, approved or pending management plans, or encouragement of cooperative conservation partnerships with landowners, the areas identified in recovery plans as important for recovery of the species may not be identical to designated critical habitat. The recovery units described in this recovery plan include but are not restricted to the 32 areas designated as critical habitat: Damon Point, Midway Beach, Leadbetter Point, Bayocean Spit, Baker/Sutton Beaches, Siltcoos to Tenmile, Coos Bay North Spit, and Bandon to Floras Creek in Recovery Unit 1; Lake Earl, Big Lagoon, McKinleyville area, Eel River area, MacKerricher Beach, and Manchester Beach in Recovery Unit 2; Point Reyes Beach, Limantour Spit, Half Moon Bay, Santa Cruz Coast, Monterey Bay Beaches, and Point Sur Beach in Recovery Unit 4; San Simeon Beach, Estero Bay, Devereaux Beach, Oxnard Lowlands in Recovery Unit 5; and Zuma Beach, Santa Monica Bay, Bolsa Chica area, Santa Ana River Mouth, San Onofre Beach, Batiquitos Lagoon, Los Penasquitos, and South San Diego in Recovery Unit 6. Implementation of the recovery actions in this recovery plan (*e.g.*, monitoring, habitat improvement, nest protection, recreation management) may not be limited to designated critical habitat areas.

b. Section 9 Take Prohibitions

Section 9 of the Endangered Species Act of 1973, as amended, prohibits any person subject to the jurisdiction of the United States from taking (i.e., harassing, harming, pursuing, hunting, shooting, wounding, killing, trapping, capturing, or collecting) listed wildlife species. It is also unlawful to attempt such acts, solicit another to commit such acts, or cause such acts to be committed. Regulations implementing the Endangered Species Act (50 CFR 17.3) further define "harm" to include significant habitat modification or degradation that results in the killing or injury of wildlife by significantly impairing essential behavioral patterns including breeding, feeding, or

sheltering. "Harass" means an intentional or negligent act or omission that creates the likelihood of injury to wildlife by annoying it to such an extent as to significantly disrupt normal behavioral patterns, which include, but are not limited to, breeding, feeding, or sheltering.

As an example under the authority of section 9 of the Endangered Species Act, on May 15, 1998, we received preliminary injunctive relief against the Town of Plymouth, Massachusetts, because their beach management failed to prevent take (killing) of a piping plover chick by an off-road vehicle (U.S. District Court for Massachusetts 1998). The judge's order prohibited off-road vehicle traffic through the piping plover's nesting season unless the town implemented specific management measures to preclude take, including twice-daily monitoring of nests and a 400-meter (1,148-foot) buffer of protected habitat for newly-hatched chicks.

The proposed special rule under section 4(d) of the Endangered Species Act (U.S. Fish and Wildlife Service 2006b) would exempt most recreational and commercial activities within a county from section 9 prohibitions on take of western snowy plovers, if documentation of conservation actions was provided and populations within the county met targets based on the Management Goal Breeding Numbers in Appendix B of the recovery plan. Research and monitoring actions would continue to require recovery permits under section 10(a)(1)(A) of the Endangered Species Act.

c. Section 10 Permits

Section 10 of the Endangered Species Act and related regulations provide for permits that may be granted to authorize activities otherwise prohibited under section 9, for scientific purposes or to enhance the propagation or survival of a listed species (i.e., section 10(a)(1)(A) permits). These permits have been granted to certain biologists of conservation organizations (*e.g.*, Point Reyes Bird Observatory and Oregon Natural Heritage Program) and Federal and State agencies to conduct western snowy plover population monitoring and banding studies and construct predator exclosures. It is also legal for employees or designated agents of certain Federal or State agencies to take listed species without a permit if the action is necessary to aid sick, injured, or orphaned animals or to salvage or dispose of a dead specimen.

Section 10(a)(1)(B) of the Endangered Species Act also allows permits to be issued for take of endangered and threatened species that is "incidental to, and not the purpose of, carrying out an otherwise lawful activity" if we determine that certain conditions have been met. An applicant for an incidental take permit must prepare a habitat conservation plan that specifies the impacts of the take, the steps the applicant will take to minimize and mitigate the impacts, funding that will be available to implement these steps, alternative actions to the take that the applicant considered, and the reasons why such alternatives are not being utilized. Conditions that we must meet include a determination: (1) whether the taking will be incidental, (2) whether the applicant will minimize and mitigate the impacts of such taking to the maximum extent possible, (3) that adequate funding for the recovery will be provided, (4) that the taking will not appreciably reduce the likelihood of the survival and recovery of the species in the wild, and (5) of any other measures that we may require as being necessary or appropriate for the recovery plan. Section 10(a)(1)(B) of the Endangered Species Act provides for permits that have the potential to contribute to conservation of listed species. Such permits are intended to reduce conflicts between the conservation of listed species and economic activities, and to develop partnerships between the public and private sectors.

d. Section 7 Requirements and Consultations

Section 7(a)(1) of the Endangered Species Act requires all Federal agencies to "utilize their authorities in furtherance of the purposes of [the] Act by carrying out programs for the conservation of endangered species and threatened species". Hence, Federal agencies have a greater obligation than do other parties, and are required to be pro-active in the conservation of listed species regardless of their requirements under section 7(a)(2) of the Act. Section 7(a)(2) of the Endangered Species Act requires Federal agencies to consult with us prior to authorizing, funding, or carrying out activities that may affect listed species. Section 7 obligations have caused Federal land management agencies to implement western snowy plover protection measures that go beyond those required to avoid take; for example, eradicating European beachgrass and conducting research on threats to western snowy plovers. Other examples of Federal activities that may affect western snowy plovers along the Pacific coast, thereby triggering a section 7 consultation, include permits for sand management activities or major restoration projects that affect coastal processes or

that are targeted to protect other species on Federal lands such as dune plants (National Park Service, U.S. Department of the Interior); disposal of dredged materials (U.S. Army Corps of Engineers); military training (U.S. Department of Defense); and funding to public agencies for projects to repair beach facilities, such as public access paths (Federal Emergency Management Agency).

e. Other Federal Regulations, Executive Orders, and Agreements

Section 404 of the Clean Water Act, as amended, and section 10 of the Rivers and Harbors Act of 1899 are the primary Federal laws that could provide some protection of nesting and wintering habitat of the western snowy plover that is determined by the U.S. Army Corps of Engineers (Corps) to be wetlands or historic navigable waters of the United States. Excavation or placement of any fill material (including sand) below the high tide line, as defined under 33 CFR, Section 328.3(d), Definition of Waters of the United States, also requires a permit from the U.S. Army Corps of Engineers.

Executive Order 11644, Use of Off-Road Vehicles on Public Lands, and Executive Order 11989, Off-Road Vehicles on Public Lands, pertain to lands under custody of the Secretaries of Agriculture, Defense, and Interior (except for Native American Tribal lands). Executive Order 11644 requires administrative designation of areas and trails where off-road vehicles may be permitted. Executive Order 11989 states that "... the respective agency head shall, whenever he determines that the use of off-road vehicles will cause or is causing considerable adverse effects on the soil, vegetation, wildlife, wildlife habitat ... immediately close such areas or trails to the type of off-road vehicles causing such effects, until such time as he determines that such effects have been eliminated and that measures have been implemented to prevent future recurrence". Compliance with this executive order would promote prohibitions or restrictions on off-road vehicles so that they are not allowed to adversely affect sensitive habitats used by western snowy plovers.

Executive Order 11988, Floodplain Management, and Executive Order 11990, Protection of Wetlands, provide protective policies that apply to western snowy plover habitats. Executive Order 11988 mandates that all Federal agencies avoid direct or indirect support of floodplain development wherever there is a practicable

alternative. Executive Order 11990 mandates that all Federal agencies shall "provide leadership and shall take action to minimize the destruction, loss or degradation of wetlands, and to preserve and enhance the natural and beneficial values of wetlands..." Compliance with Executive Order 11988 would promote protection of beach and dune habitats through restrictions on development within floodplains. Application of Executive Order 11990 would promote protection of wetland habitats used by western snowy plovers.

Executive Order 13112, Invasive Species, directs Federal agencies to prevent the introduction of invasive species; control their populations in a cost-effective and environmentally sound manner; monitor invasive species; restore native species and habitat conditions in ecosystems that have been invaded; conduct research and develop technologies to prevent their introduction; and promote public education on invasive species and the means to address them. This executive order also requires that a Federal agency "not authorize, fund, or carry out actions that it believes are likely to cause or promote the introduction or spread of invasive species..." Compliance with this executive order would enhance western snowy plover habitats through (1) avoidance of use, approval, or funding the planting of invasive species like European beachgrass; and (2) active programs to remove this invasive species and restore coastal dune habitats with native plant species.

The Fish and Wildlife Coordination Act (16 U.S.C. 661-667e), as amended, requires that whenever a proposed public or private water development project is subject to Federal permit, funding, or license, the conservation of fish and wildlife resources shall be given equal consideration. This Act also requires that project proponents shall consult with us and the State agency responsible for fish and wildlife resources. Compliance with the Fish and Wildlife Coordination Act highlights the importance of considering and providing for the habitat needs of fish and wildlife resources when reviewing projects that would adversely affect these resources.

The National Environmental Policy Act of 1969, (42 U.S.C. 4321-4347), as amended, requires that each Federal agency prepare an environmental impact statement on the potential environmental consequences of major actions under their jurisdiction. Environmental impact statements must include the impacts on ecological systems, any direct or indirect consequences that may result from the action, less

environmentally damaging alternatives, cumulative long-term effects of the proposed action, and any irreversible or irretrievable commitment of resources that might result from the action. Compliance with the National Environmental Policy Act highlights the need to disclose, minimize, and mitigate impacts to biological resources, including western snowy plovers.

The Coastal Zone Management Act of 1972 (16 U.S.C. 1451-1464), as amended, established a program for states to voluntarily develop comprehensive programs to protect and manage coastal resources. To receive Federal approval and funding under this Act, states must demonstrate that they have programs and enforceable policies that are sufficiently comprehensive and specific to regulate land uses, water uses, and coastal development, and must have authorities to implement enforceable policies. Local coastal plans, local comprehensive plans, and implementing measures by coastal planning jurisdictions pursuant to the Coastal Zone Management Act should be developed, updated, and implemented with protective measures for western snowy plovers.

Western snowy plovers are protected under the Migratory Bird Treaty Act of 1918 (16 U.S.C. 703-712), as amended. Under the Migratory Bird Treaty Act, prohibited acts include pursuing, hunting, shooting, wounding, killing, trapping, capturing, or collecting any migratory bird, nest, or eggs without a permit from the U.S. Fish and Wildlife Service.

5. State Regulatory Protection, Policies, and Agreements

In Washington, Oregon, and California, each state holds title to, and has regulatory jurisdiction over, the coastal intertidal zone. In Washington, the area between mean high tide to extreme low tide is the seashore conservation area under the authority of the Washington State Parks and Recreation Commission. In California, the California State Lands Commission has regulatory authority to the mean high tide line along the California coast.

In Oregon, the Oregon Parks and Recreation Department administers the State beach for the ocean shore recreation area, which is defined as the area between the line of extreme low water and the statutory vegetation line, which is a line surveyed to the

approximate line of vegetation that existed in 1969 (Oregon Revised Statutes 390.770). The Oregon Division of State Lands also has jurisdiction over waters of the state along the Pacific coast to the line of highest tide or the line of established vegetation, whichever is higher. Therefore, the Oregon Parks and Recreation Department has direct jurisdiction, authority, and responsibility for management of western snowy plover habitats in the State of Oregon, which owns not only to the mean high tide line, which is western snowy plover foraging habitat, but also into the vegetation line, which is essentially the dry sand area used by western snowy plovers for nesting.

State coastal planning and regulatory agencies, such as the California Coastal Commission, require preparation of local coastal zone management plans by local coastal municipalities. These local coastal zone management plans must comply with the Coastal Zone Management Act of 1972 regarding protection of coastal resources, including natural resources. Under the California Coastal Management Program, coastal resources are managed and cumulative impacts addressed through: (1) coastal permits and appeals; (2) planning and implementation of local coastal programs; and (3) Federal consistency review. However, effective management of cumulative impacts is difficult under the existing management framework because multiple jurisdictions have varying policies and standards in different geographic areas (California Coastal Commission 1995). Through the Coastal Commission's regional cumulative assessment program, cumulative impacts to coastal resources can be addressed through the periodic review of local coastal programs. In California, most local coastal programs and general plans were completed prior to 1993 (when we listed the western snowy plover as a threatened species); therefore, many do not reflect protective measures specifically for the western snowy plover.

The Oregon Department of Land Conservation and Development is the designated coastal zone management agency for the State of Oregon. The State of Oregon's land use planning system has several elements that are related to conservation of western snowy plovers and their habitats. In Oregon, local jurisdictions (cities and counties), service districts, and State agencies are required to develop Local Comprehensive Plans and Implementing Measures, such as zoning and land division ordinances, to effect these plans. Each plan must satisfy a set of 19 goals established through Oregon land use law and policy. Plans must be reviewed by the Land Conservation

and Development Commission for consistency with these goals before they can be put into effect. Several of the planning goals have application to, or should be considered during, planning for western snowy plover conservation and recovery. These goals include: Goal 5 - Open Spaces, Scenic and Historic Areas, and Natural Resources; Goal 7 - Areas Subject to Natural Disasters and Hazards; Goal 8 - Recreational Needs; Goal 16 - Estuarine Resources; Goal 17 - Coastal Shorelands; and Goal 18 - Beaches and Dunes.

Taken in aggregate, the elements of these goals that can contribute to western snowy plover recovery include:

- several requirements for protection of wildlife habitat;
- requiring protection of estuarine ecosystems including habitats, diversity, and other natural values;
- establishing that uses of beaches and dunes shall be based on factors including the need to protect areas of critical environmental concern and significant wildlife habitat;
- requiring that coastal plans provide for uses of beaches and dunes that are consistent with their ecological values and natural limitations;
- requiring an evaluation of the beneficial effects to natural resources from allowing continuation of natural events that are hazardous to human developments (such as erosion and ocean flooding);
- establishing a preference for nonstructural solutions to erosion and flooding of coastal shorelands over structural approaches (such as seawalls and rip-rap);
- requiring that development of destination resorts be compatible with adjacent land uses and maintain important natural features such as threatened and endangered species habitats;
- encouraging coordination among State, Federal, and local governmental agencies while developing recreation plans, and discouraging development of recreation plans that exceed the carrying capacity of the landscape;
- encouraging planning for Open Space, Scenic and Historic Areas, and Natural Resources (Goal 5), Recreational Needs (Goal 8), and Coastal Shorelands (Goal 17) in close coordination; and
- allowing dune stabilization programs only when in conformance with the overall comprehensive plan and after assessment of the potential impacts.

Some aspects of these planning goals could be interpreted to be contrary to western snowy plover conservation and recovery when viewed in isolation. However, when viewed in the context of the entire goal or all the planning goals, these elements should be compatible with western snowy plover conservation and carefully-planned habitat restoration activities. Two such elements are the directive to increase recreational access to coastal shorelands and the restrictions placed on dune grading and removal of vegetation. Goal 17 - Coastal Shorelands directs local governments and the Oregon Parks and Recreation Department to develop a program to increase public access. In many areas, recreational use of western snowy plover habitat during the nesting season is detrimental to or incompatible with western snowy plover conservation. However, this goal also recognizes that many shorelands have unique or exceptional natural area values, includes the objective of reducing adverse impacts to fish and wildlife habitat associated with use of coastal shorelands, clearly establishes that significant wildlife habitat shall be protected, establishes that uses of such habitat areas shall be consistent with protection of natural values, and directs recreation plans to provide for "appropriate" public access and recreational use. Goal 18 - Beaches and Dunes directs local governments and State and Federal agencies to regulate actions in beach and dune areas to minimize any resulting erosion and only allows foredune breaching to replenish interdune areas or in the case of an emergency. Western snowy plover habitat restoration efforts in areas that have been overtaken by European beachgrass (*Ammophila arenaria*) may involve foredune breaching, vegetation removal, dune grading, and other actions that will remove the European beachgrass and restore the natural beach and dune processes of sand movement, including erosion and deposition. However, this goal also recognizes the need to protect areas of critical environmental concern, areas of biological importance, and areas with significant habitat value, specifically identifies removal of "desirable" vegetation as an action requiring minimization of erosion, and requires that any foredune breaching be consistent with sound principles of conservation.

The Washington State Parks and Recreation Commission administers the Seashore Conservation Act of 1988 in accordance with the Revised Code of Washington and the Washington Administrative Code. The Seashore Conservation Area (Revised Code of Washington 43.51) emphasizes the importance of beaches to the public for recreational activities. In designating beach areas to be reserved for pedestrian use, it

considers natural resources, including protection of shorebird and marine mammal habitats, preservation of native beach vegetation, and protection of sand dune topography. Chapter 352-37 (Ocean Beaches) of the Washington Administrative Code requires local governments within the Seashore Conservation Area to prepare recreation management plans that designate at least 40 percent of the ocean beach for use by pedestrians and nonmotorized vehicles from April 15 to the day after Labor Day. These regulations also identify restrictions on certain uses within ocean beaches, including motor vehicles, equestrian traffic, speed limits, aircraft, wind/sand sailers, parasails, hovercraft, group recreation events, and beach parking and camping. In 1989, an interagency agreement was signed by the Washington Department of Natural Resources, Washington State Parks and Recreation Commission, Washington Department of Wildlife, and City of Ocean Shores regarding management of mixed uses at Damon Point. The intent of the agreement was to protect western snowy plovers while allowing recreation.

State regulations, policies, and goals for the States of California, Oregon, and Washington provide many protective measures for western snowy plovers. However, because they frequently emphasize public uses of beach habitat, there is potential for conflicts between human uses of the coastal zone and needed management measures for recovery of the western snowy plover.

The California Department of Parks and Recreation has written management guidelines for the western snowy plover which are meant to be used in conjunction with the recovery plan. Management actions will be implemented from the guidelines and may result in changes in how coastal units are operated. Increased emphasis will be required for monitoring, nest area protection, prohibition of certain activities in important nesting areas, and public education.

6. Consultations, Habitat Conservation Plans, and Other Regulatory Actions

Through consultations with Federal agencies under section 7 of the Endangered Species Act and through the development of habitat conservation plans with non-Federal agencies developed under section 10 of the Endangered Species Act, we provide nondiscretionary terms and conditions that minimize (sections 7 and 10) and mitigate (section 10) the impacts of covered activities on listed species and their

habitat. Several major consultations and habitat conservation planning efforts to benefit the western snowy plover have been completed or are currently under way.

In 1995 our Sacramento Fish and Wildlife Office completed formal consultation with the National Park Service, Golden Gate National Recreation Area, on the effects of their management of Ocean Beach, San Francisco on the western snowy plover. Ocean Beach experiences tremendous visitor use year-round because of its proximity to San Francisco, yet it supports high numbers of nonbreeding western snowy plovers, which may be present from May through July. The consultation covered actions and policies the National Park Service had taken that resulted in unnecessary harassment of nonbreeding western snowy plovers. Most significant of these measures was their policy not to enforce regulations requiring pets to be leashed and under control by their owners on all National Park Service lands. Data collected by the National Park Service clearly identified that unleashed dogs were the most significant disturbance factor of the many sources of disturbance to western snowy plovers on Ocean Beach. As a result of the consultation, the National Park Service began to enforce their "leash law" along 3.2 kilometers (2 miles) of beach utilized by western snowy plovers. The National Park Service implemented this policy despite vocal and persistent opposition by the San Francisco Society for the Prevention of Cruelty to Animals and other local advocacy groups, including the "Rovers for Plovers", which organized themselves to challenge the National Park Service's leash law. These groups were successful in advocating their position in numerous television news stories and articles in local newspapers. At the height of this discourse, the local public radio station held a round-table discussion between the National Park Service, U.S. Fish and Wildlife Service, and Society for the Prevention of Cruelty to Animals, and solicited audience members to call in and identify their viewpoint. The overwhelming majority of callers supported leash law restrictions that would minimize harassment of western snowy plovers.

Our Arcata Fish and Wildlife Office has formally consulted with the U.S. Army Corps of Engineers regarding gravel extraction on the Eel River, California. Gravel mining operations are subject to permits from the U.S. Army Corps of Engineers under Section 404 of the Clean Water Act. The western snowy plover breeds on the Eel River gravel bars. Impacts to the western snowy plover and its designated critical habitat associated with gravel mining operations have been assessed based on nesting

surveys and changes to habitat resulting from gravel extraction. The Arcata Fish and Wildlife Office has also worked with Humboldt County, the California Department of Fish and Game, and the California Department of Parks and Recreation to implement additional protections for nesting western snowy plovers at MacKerricher, Manchester, Little River, Humboldt Lagoons, and Prairie Creek State Parks; Clam Beach County Park, and the Eel River Wildlife Area. These measures include installation of nest exclosures, signing, and development of educational material for kiosks. Technical assistance has also been provided to Prairie Creek State Park and MacKerricher State Park on exotic vegetation management programs (J. Watkins *in litt.* 1999, pers. comm. 2001). A section 7 consultation with the Bureau of Land Management on finalization of a management plan for Humboldt Bay South Spit is expected to be initiated soon (J. Watkins, pers. comm. 2006).

Our Ventura Fish and Wildlife Office is attempting to initiate a regional approach to habitat conservation planning for western snowy plovers and other listed species along Monterey Bay in Monterey County, California. Currently, there are several proposed development projects within the city of Sand City and a "city wide" habitat conservation plan has been prepared for these projects. The City of Sand City has yet to present a complete draft of their habitat conservation plan to the Ventura Fish and Wildlife Office for review. Formerly, the City of Marina was also proposing several coastal developments that were expected to have adverse effects on western snowy plovers, but these projects are no longer planned due to changes in land ownership and other factors. The City of Marina has halted the drafting of a habitat conservation plan for lands within their jurisdiction. We have expressed concerns about projects being presented in a piecemeal fashion, which does not allow an adequate assessment of their cumulative effects, and have recommended a regional approach through preparation of a regional habitat conservation plan. This plan would provide greater conservation benefits to the western snowy plover. In addition to the adverse effects of development on western snowy plovers and their habitat, recreation on the extensive public lands along Monterey Bay is also adversely affecting western snowy plovers. Therefore, public land managers, including our Refuges Division, the California Department of Parks and Recreation, the California Department of Fish and Game, and the Monterey Peninsula Regional Park District, need to be involved in planning efforts along Monterey Bay.

Through the consultation process, our Ventura Fish and Wildlife Office determined that a draft biological opinion on Vandenberg Air Force Base's initial proposed beach management plan for the western snowy plover, concluding that the plan would "likely jeopardize the continued existence of the western snowy plover and adversely modify its critical habitat." Our draft biological opinion of January 2001 pointed out that the Air Force's beach plan would have allowed twice as much nesting habitat to be open to public recreation as was allowed during the 2000 breeding season, and it would have reduced the time the Air Force spends patrolling the beaches by about 80 percent. Based on this feedback, the Air Force subsequently reinitiated consultation on a modified version of the beach management plan, including commitments to signage, information kiosk, and enforcement patrols. The Ventura Fish and Wildlife Office issued a non-jeopardy biological opinion on the modified action in March 2001. Beach opening and full implementation of conservation measures was implemented on May 25, 2001, with hours and days of open beach limited due to limited availability of enforcement personnel. For the next three breeding seasons (2002, 2003, 2004), the Service issued biological opinions on annual beach management plans proposed by the Air Force. In 2004, we had a series of meetings with the Air Force to discuss their beach management strategy and its effects on the western snowy plover. Through a cooperative effort, the Service and the Air Force came to agreement on a 5-year beach management plan that includes many of the same protective measures that had been in place the last several years, yet allows the Air Force to provide recreational access seven days a week. On March 1, 2005, the Ventura Fish and Wildlife Office issued a new non-jeopardy biological opinion on the Air Force's proposed 5-year beach management plan (2005-2009).

Our Ventura Fish and Wildlife Office is also involved with the development of a habitat conservation plan being funded by the Off-Road Vehicle Division of the California Department of Parks and Recreation for the Pismo/Oceano Dunes State Vehicular Recreation Area and other State parks within the San Luis Obispo District of the California Department of Parks and Recreation. The Ventura Fish and Wildlife Office is also involved in the development of a HCP for the Rancho Guadalupe County Park, Santa Barbara, California. These habitat conservation plans will evaluate and mitigate for effects that recreation and park management activities are having on the covered species, including the western snowy plover.

Recent consultations handled by our Newport Field Office include those in response to the New Carissa Oil Spill, a consultation on BLM management actions at the New River Area of Critical Environmental Concern (ACEC), and a consultation on the Integrated Predator Damage Management Program 2002 to 2007. The Oregon Parks and Recreation Department is currently developing a Habitat Conservation Plan that proposes restrictions on some Oregon beaches to help the plover population recover.

The New Carissa oil spill was a long and complicated incident involving a variety of Federal, State, local and private participants. On February 4, 1999, the *New Carissa,* carrying 359,000 gallons of bunker oil and 37,400 gallons of diesel, grounded on the north spit of Coos Bay and began leaking oil shortly thereafter. Subsequently, oil and oiled wildlife were observed on the beach. Attempts were made to burn off the oil. The vessel broke into two pieces during the second attempt. There were three formal consultations associated with the *New Carissa* between 1999 and 2000. The first consultation addressed the effects of issuing permits for salvage of the *New Carissa* stern section, the second the effects of restoring recreational access to the Coos Bay north spit, and the third the response efforts led by the Coast Guard. In all three consultations, it was concluded that the proposed actions would not jeopardize the western snowy plover if protective measures required to limit take were implemented.

A consultation on the New River ACEC was completed in 2005. The purpose of the biological opinion was to address a variety of issues: recreation management at Floras Lake where measures were not adequately protecting nesting plovers; the periodic construction of a breach on the New River spit to improve fish and wildlife habitat and alleviate flooding; increased habitat restoration; and the development of a primitive beach camping area.

A consultation on Oregon's Integrated Predator Damage Management Program was completed in 2001. The objective of this program is to assist in recovery of the western snowy plover in Oregon by improving western snowy plover nesting and fledging success, through 1) expanding assessment efforts to all western snowy plover breeding and nesting locations to determine predator species responsible for nest, chick and adult predation; and 2) reducing the local predator populations where feasible and where the predator species or individual is known. The consultation

calls for a variety of lethal and non-lethal methods to be used by APHIS-WS personnel to control the predator population.

The Oregon Parks and Recreation Department has been working with various cooperating agencies to develop a Habitat Conservation Plan for Oregon beaches. The Oregon Parks and Recreation Department is responsible for various management activities for most of Oregon's coast, including recreation management, general beach management, and the management of natural resources. In addition, the Oregon Parks and Recreation Department is responsible for issuing various permits along the Oregon coast. Some of these activities may result in "take" of or harm to the snowy plover. A draft version of the Habitat Conservation Plan was distributed to the public in January 2004. The Oregon Parks and Recreation Department conducted public meetings in seven coastal communities to solicit public comment. The area covered under the HCP includes the portions of the ocean shore along the Oregon coast that extend between the mouth of the Columbia River South Jetty on the north and the California/Oregon border on the south (approximately 230 miles of beach). In addition, specific portions of six key state parks, state natural areas, and state recreation areas are included in the covered lands to be managed for snowy plover recovery. Implementation of the plan will begin after approval and completion of the Habitat Conservation Plan and its associated documents.

In southern California, we, through our Carlsbad Fish and Wildlife Office, have worked with local jurisdictions to develop regional habitat conservation plans under section 10 of the Endangered Species Act. The Multiple Species Conservation Program addresses southwestern San Diego County, including, for example, western snowy plover breeding habitat in south San Diego Bay through the City of San Diego. The Multiple Habitat Conservation Program addresses northwestern San Diego County. This plan provides for the conservation of western snowy plover breeding habitat and will potentially result in more management in association with a proposed preserve.

Also in San Diego County, we have been working with the Navy and the Marine Corps to avoid and minimize impacts to western snowy plovers. For example, with the assistance of our programmatic biological opinion in 1995, the Marine Corps has addressed training-related impacts on western snowy plovers and other species on

approximately 17 miles of coastline on Camp Pendleton. We have likewise worked with the Navy at Naval Base Coronado to develop a program to conserve western snowy plover nesting and breeding habitat and allow necessary military training. As a result of successful management on these San Diego County military installations, they support a majority of the western snowy plover population in Recovery Unit 6 (*e.g.*, roughly 65 percent in 2006 from window survey data) while the military installations accomplish their respective training missions.

In the past, several instances were documented of western snowy plover nests being trampled by cattle belonging to the Vail and Vickers Company on Santa Rosa Island within the Channel Islands National Park, owned and managed by the National Park Service. In 1996, a lawsuit to remove cattle from Santa Rosa Island was initiated by the Environmental Defense Center, Santa Barbara, on behalf of the National Park Conservation Association. It was initiated under the authority of the Clean Water Act and the Endangered Species Act, based on concerns about management of livestock by the National Park Service and associated impacts to water quality and sensitive plant and animal species. As a result of a lawsuit settlement, all cattle were removed from Santa Rosa Island in early 1998.

7. Regulatory Protection and Policies of Local Governments

Local governments regulate municipal land uses through development of local land use plans, general plans, comprehensive plans, and zoning policies. On April 21, 1998, we requested that county and coastal city planners within the states of Washington, Oregon, and California complete land-use management surveys regarding the western snowy plover. We sent surveys to 91 State, county, or coastal city planners and received responses from 37 percent of the recipients. Approximately 50 percent of the respondents were aware that western snowy plover habitats occur within their jurisdictions. However, only about one-third knew whether sandy beach and other habitats within their jurisdictions provided breeding and/or wintering habitat for western snowy plovers. Many general plans, coastal zone programs, and comprehensive plans prepared by local governments contain land use designations that are protective of western snowy plover habitats (*e.g.*, parkland, open space, and conservation designations for sandy beach). However, allowable uses in or adjacent to these zones, such as development (*e.g.*, seawalls, recreational

facilities, single-family homes), recreation and public access, could cause direct or indirect threats to breeding or wintering western snowy plovers.

Whereas 43 percent of the respondents include regulatory policies that protect western snowy plover habitat (*e.g.*, sandy beach) in their general plans, local coastal programs or comprehensive plans, only 8 percent have developed regulatory policies specifically to protect the western snowy plover. These respondents included the City of Half Moon Bay, California, and Coos and Curry Counties, Oregon. Only 23 percent of the respondents specifically explain the threatened status of the western snowy plover, identify western snowy plover breeding/wintering locations, or specify shorebird nesting/roosting habitats as environmentally sensitive habitat areas in their jurisdictions. About 50 percent of the respondents indicated they either (1) have approved development within or adjacent to sandy beach or other habitats used by the western snowy plover, or (2) did not know whether such development had been approved by their agency. About half of these same respondents could provide some information on the number of permits authorized, area or linear distance affected, percentage of development types (*e.g.*, housing, recreational) permitted, and permit conditions.

Based on these responses, it seems that specific locations of, and protective measures for, western snowy plover breeding and/or wintering locations are not included in most of the existing general plans, comprehensive plans, local coastal programs, or their implementing ordinances. Also, to better assess cumulative impacts, these responses indicate a need for a better tracking method regarding development projects approved within and adjacent to western snowy plover habitat.

8. Interagency Coordination

Each of the six recovery units for the western snowy plover is represented by a working group which meets at least once a year to coordinate western snowy plover recovery efforts. The working groups have provided a forum for the participation of affected Federal and State agencies and others in discussion, implementation, and adjustment of recovery efforts. Items addressed include research and monitoring needs, predator control, recreation management, habitat restoration, public outreach and law enforcement. In addition, a joint meeting of all six working groups is held

annually. This group, consisting of beach managers, researchers, and outreach staff, meet to discuss range-wide issues (within the United States), to coordinate recovery actions, to learn from the experience of others, and to share information and research. Attendees have included local, State, and Federal agency staff, non-governmental organizations, consulting firms, private citizens, and volunteers.

The recovery unit working groups vary somewhat in organizational structure depending on major local issues, patterns of land ownership within the area, and specific agencies responsible for management. For example, the Oregon/Washington working group is composed of several subcommittees, including Outreach, Media, Predator Control, Research, Law Enforcement, and Recovery Plan Implementation. They facilitate funding partnerships for monitoring and management programs, thus promoting the best use and leveraging of limited funds. They also act as the main forum for discussing and tracking the status and trends of the snowy plover population. The subcommittees have worked on or supported a variety of cooperative projects, such as monitoring of yearly reproductive success, predator control, and outreach materials. Products developed by the Outreach subcommittee include an outreach plan for Oregon/Washington and "Share the Beach" bookmarks, table tents, dog leashes, brochures, interpretive signs, and coloring books. The Media subcommittee is producing a media outreach CD for distribution to various media outlets and inter-agency press releases. The Predator Control subcommittee approved a predator management plan for Oregon, which first went into effect in 2002. The purpose of the Research subcommittee is to identify research and monitoring priorities, establish criteria for setting priorities, review proposed projects, and address funding mechanisms. The Law Enforcement subcommittee focuses on improving compliance with rules and regulations in plover nesting areas and the Recovery Plan Implementation subcommittee is working on guidance that would assist in "stepping down" the recovery plan for Oregon and eventually Washington.

In 1998, an interagency effort in Oregon produced a slide show and portable display to educate beach visitors about western snowy plover conservation. Outdoor education specialists and/or western snowy plover biologists from the U.S. Bureau of Land Management, U.S. Forest Service, Oregon Department of Fish and Wildlife, Oregon Parks and Recreation Department, and U.S. Fish and Wildlife Service participated in this effort. The show provides basic information about the western

snowy plover, the reasons for its decline, and actions needed for its recovery, emphasizing the contribution that beach visitors can make.

II. RECOVERY

A. RECOVERY STRATEGY

The recovery strategy for the Pacific coast population of the western snowy plover (western snowy plover) includes three major components: 1) increase population numbers distributed across the range of the Pacific coast population of the western snowy plover; 2) ameliorate or eliminate threats by conducting intensive ongoing management for the species and its habitat, and developing mechanisms to ensure management in perpetuity; and 3) monitor western snowy plover populations and threats to determine success of recovery actions and to refine management actions. Developing and implementing intensive adaptive management actions, ensuring that management will continue in perpetuity, and monitoring to refine management actions, are all necessary to achieve the targeted population increases across the range. These three major components of the recovery strategy each include many actions and multiple partners that are described in further detail below.

1. Recovery Strategy Components

The following recovery strategy components will guide future recovery efforts for the U.S. Pacific coast population of the western snowy plover.

a. Population increases should be distributed across the western snowy plover's Pacific coast range.

A key component of recovering western snowy plovers is to ensure that population increases are distributed throughout the species' Pacific coast range. In order to achieve this, management goals (Appendix B) and needed management actions (Appendix C) have been determined for 155 sites distributed along the coasts of southern Washington, Oregon, and California. Additionally, the population's range has been divided into six recovery units (see discussion below) with population goals established for each recovery unit. The six recovery units correspond to regions of the U.S. Pacific coast and to the six subpopulations used in the Population Viability Analysis for the Pacific coast Snowy Plovers (Appendix D). In the population viability analysis, the Pacific coast population of the western snowy plover is treated

as a metapopulation, defined as a set of subpopulations among which there is limited dispersal.

The population viability analysis assumes dispersal among subpopulations is limited; however, even limited dispersal among subpopulations is important to species survival and recovery. Dispersal of the population across its breeding range helps to counterbalance catastrophes, such as extreme climatic events, oil spills, or disease that might depress regional survival and/or productivity. Maintaining robust, well-distributed subpopulations should reduce variance in survival and productivity of the Pacific coast population of the western snowy plover as a whole, facilitate interchange of genetic material between subpopulations, and promote recolonization of any sites that experience declines or local extirpations due to low productivity and/or temporary habitat loss.

This recovery plan and the population viability analysis (Appendix D) consider the U.S. Pacific coast population of the western snowy plover to be a single management entity, and population goals and objectives are based on that premise. No portion of the Pacific coast population of the western snowy plover appears to function as a distinct population segment. The Recovery Team therefore recommends that no State, geographic region, or subpopulation of the Pacific coast population of the western snowy plover be considered for delisting separately from the others.

b. Remove or reduce threats by conducting intensive ongoing management for the species and its habitat, and develop mechanisms to ensure management in perpetuity to prevent a reversal of population increases following delisting under the Endangered Species Act.

Management consists of multiple components, including identifying actions to ameliorate or eliminate threats, developing mechanisms to ensure management in perpetuity, continuing outreach and education to provide information to the public, partners, and stakeholders on recovery needs and opportunities, and developing of partnerships among Federal, State, and local agencies and groups to develop and implement effective management. Management actions for the western snowy plover are described in the recovery action outline and in Appendix C. These management actions are necessary to eliminate or ameliorate threats to the western snowy plover,

including loss, degradation, and alteration of habitat; disease, predation; and other manmade factors including disturbance of breeding and wintering birds, contaminants, and oil spills.

In addition to specific management recommendations to ameliorate or eliminate threats, the recovery action outline and recovery strategy for the western snowy plover include several recovery actions to develop mechanisms to ensure that management actions continue in perpetuity to ensure that threats remain neutralized. These include establishing working groups and developing participation plans for each recovery unit; ensuring sufficient U.S. Fish and Wildlife Service staff to coordinate recovery of the Pacific coast population of the western snowy plover; developing and implementing management plans for publicly owned lands; assisting local governments and private land owners in developing habitat conservation plans, developing land use protection measures, and developing landowner agreements; and acquiring habitat where necessary. A key component of these efforts includes education and outreach to inform partners and the public about recovery needs and opportunities for the western snowy plover. Actions for outreach are included in the recovery action outline, and the Information and Education Plan (Appendix K) provides greater detail on implementing these outreach and education actions.

Participation of many different groups will be essential to achieve both short-term and long-term management for the western snowy plover and its habitat. The roles of various groups, potential conservation tools and funding available, and the Recovery Team's vision for participation and coordination of partners are further described below.

c. Annual monitoring of western snowy plover subpopulations and reproductive success, and monitoring of threats and effects of management actions in reducing threats, is essential for adaptive management and to determine the success of recovery efforts.

The recovery action outline describes monitoring for breeding, wintering, and migration areas both to determine whether population numbers and survival of western snowy plovers is increasing and whether threats continue to limit population increases. Additional research actions are also recommended to study certain threats

and develop management techniques and monitoring methods. Results from research and monitoring efforts will be used to develop, refine, and improve management of western snowy plovers and their habitat. Monitoring of demographic characteristics will be necessary to demonstrate that population goals in the recovery criteria are being achieved. Monitoring of threats and effects of management actions in reducing those threats also is essential in demonstrating progress toward recovery and ultimately will assist in threats analyses necessary to make a delisting determination.

2. Roles of Federal, State, Local, and Private Sectors

a. Role of Federal Lands

Federal lands administered by the U.S. Fish and Wildlife Service, National Park Service, U.S. Forest Service, U.S. Bureau of Land Management, the National Marine Sanctuary Program, U.S. Marine Corps, and the U.S. Departments of the Army (including Corps of Engineers), Navy, and Air Force are extremely important to the conservation of the western snowy plover. In California, breeding occurs on National Wildlife Refuge lands, Department of Defense lands, Bureau of Land Management lands, and National Park Service lands. In Oregon, the major Federal landowners are the U.S. Forest Service and Bureau of Land Management, although the State also has jurisdiction over much of the Federally owned area (from mean high tide to the vegetation line) through a recreational easement (E.Y. Zielinski and R.W. Williams *in litt.* 1999). In Washington, the breeding area at Leadbetter Point is within a National Wildlife Refuge.

Under section 7(a)(1) of the Endangered Species Act, Federal agencies are required to actively promote the conservation of listed species. The western snowy plover cannot be recovered simply through general habitat protection or complying with required section 7(a)(2) consultations. The western snowy plover must be actively monitored and managed for the purpose of recovery or its population size will decline. Federal agencies alone cannot assure recovery of the western snowy plover, but should have a leading role in monitoring and management efforts to assure survival and recovery of this species. Some Federal lands contain large areas of contiguous habitat, including adjacent inland areas that are easier to manage for conservation of natural resources than fragmented, linear strips of land that may be

owned by states, counties, cities, and private landowners. Protection of western snowy plovers and their habitat on Federal lands is important not only because of the direct benefits to plovers that use these areas, but also because plover protection programs on Federal lands frequently utilize state-of-the art management measures and therefore serve as examples to non-Federal landowners. The Federal Government also should take the lead in addressing the sensitive issue of predator control.

b. Role of State Lands

State lands administered by the California Department of Parks and Recreation, California Department of Fish and Game, Oregon Department of Fish and Wildlife, Oregon Parks and Recreation Department, Washington Department of Fish and Wildlife, Washington State Parks and Recreation Commission, and Washington Department of Natural Resources play an important role in conservation of western snowy plovers and their habitats. Intensive management for western snowy plovers occurs at a number of State-owned plover habitat areas. The western snowy plover cannot be preserved simply through general habitat protection. Western snowy plovers must be actively monitored and managed to achieve recovery goals on State lands or their population size will decline.

c. Roles of State and Local Governments

State and local government agencies, including state planning agencies and city and county planning and community resources departments, have the primary responsibility for overseeing land uses within their jurisdictions. Therefore, their involvement in future recovery planning and implementing processes is critical. All Appendix B locations should be identified as environmentally sensitive habitat areas requiring protective measures for the western snowy plover in state and local planning documents and zoning designations. Local coastal programs should be amended to include these areas. To facilitate this effort, Federal and State agencies managing western snowy plover habitat should provide technical assistance and information to local governments (see Actions 3.1.6, 3.1.7 and 5.2). We can provide detailed maps of current western snowy plover breeding and/or wintering locations; these maps will be updated periodically as needed.

d. Role of Municipal Lands

Regional, county, and city lands, including regional and municipal park districts, also serve a role in conserving breeding and wintering habitats for western snowy plovers. Because these areas frequently receive heavy pedestrian and recreational use, local jurisdictions with active public outreach programs can reach a large segment of the coastal community regarding the plover's status and habitat needs.

e. Role of Private Lands

Conservation efforts on private lands are needed for the survival and recovery of many listed and other sensitive species. Private landowners can also make important contributions to western snowy plover conservation through facilitating or allowing the monitoring of western snowy plover populations on their land and implementing protective measures.

3. Conservation Tools and Strategies

There are numerous conservation tools and strategies available to Federal, State, municipal, and private landowners interested in western snowy plover protection and recovery. Appendix H includes a summary of conservation tools and strategies that may be adopted by landowners, nonprofit organizations, and regulatory agencies to protect western snowy plover habitat.

4. Funding Sources

Appendix I includes a summary of some potential sources of funds for implementation of recovery actions for the western snowy plover. This list is not intended to be exhaustive, however, and other funding opportunities may also be available.

An essential mechanism for recovery of the western snowy plover is the development and implementation of participation plans for each of the six recovery units (see Action 3.1.2). A key element of these participation plans is the long-term

commitment by participating agencies to seek annual, ongoing funding for western snowy plover management and monitoring activities so that funding within agency budgets can be secured.

In many areas a significant portion of westem snowy plover conservation resources are expended in efforts to minimize the adverse impacts of recreation. Often, the primary objective of signs, ropes, on-site interpretation, and enforcement is to manage the behavior of beach-goers such that impacts to western snowy plovers are reduced as much as possible. In areas that have suffered extensive habitat loss or degradation, such recreation management activities are an extremely high priority in order to protect the western snowy plovers using the limited habitat that remains. For some beach managers, much of the funding and staff time expended on recreation management in and near western snowy plover habitat comes from resources targeted for threatened and endangered species recovery. In absence of the need to coordinate and pay for recreation management activities, more of these limited conservation dollars and staff resources could be directed toward western snowy plover management actions such as biological monitoring, habitat restoration, and predation control.

This situation is unique in the experience of many resource biologists. More typically, avoidance, minimization, and mitigation measures are integral components of projects or programs that entail adverse impacts to sensitive resources, and the costs of these activities are regarded as part of the overall cost of the project or program. Applying this traditional construct to recreation projects and programs could significantly promote western snowy plover recovery in several ways. First, it would require impacts to western snowy plovers to be considered up front when planning beach access or other recreation projects. Second, it would encourage impact avoidance and minimization since such measures are often less expensive than mitigation. Third, it would promote involvement of recreation professionals in designing and implementing recreation management measures. And fourth, it would eliminate or reduce the diversion of biological resource management funds toward recreation management activities, thus enabling more of those dollars to be spent on western snowy plover recovery actions.

5. Coordination, Participation, and Working Groups

We strongly believe that a collaborative stewardship approach to the proactive management of listed species involving government agencies (Federal, State, and local) and the private sector is critical to achieving the ultimate goal of recovery of listed species under the Endangered Species Act. An essential mechanism to achieve recovery of the western snowy plover is the formation and maintenance of working groups for each of the six recovery units (Appendix A), (see Action 3.1.1). Representation from the full range of Federal, State, local, and private landowners and other parties who have a stake in western snowy plover conservation within each of these six recovery units is needed to advance the recovery actions recommended in this recovery plan. Working group membership should include land managers, environmental groups, user groups, and groups involved in conservation projects (including local chapters such as the National Audubon Society, Sierra Club, Native Plant Society, Americorps, California Conservation Corps, Boy Scouts, Surfrider Foundation, and other recreational use groups). These groups can provide large networks of volunteers who can be mobilized to assist public resource agencies in the implementation of management measures for protection and recovery of the western snowy plover.

Working groups for each of the six recovery units currently exist and convene annually for regional and rangewide meetings. Through evaluation, communication, and coordination, members of each of the six working groups should manage the western snowy plover population and monitor progress towards recovery. They should produce annual reports on population monitoring and the effectiveness of management activities for the working group and our Arcata Fish and Wildlife Office. Each of the six working groups should prepare a participation plan, thereby formalizing recovery implementation efforts and the intentions of responsible agencies to seek ongoing, annual funding for recovery implementation. The Recovery Coordinator should coordinate and communicate with each recovery unit to support recovery efforts and assure implementation of the recovery plan (see Actions 3.1 through 3.4, 6, and 7). The Recovery Coordinator also should coordinate with other western snowy plover survey efforts and assessments throughout the west and throughout North America. Coordination with these other efforts may provide valuable information on the status and distribution of the western snowy plover, as

well as valuable information on management actions that may benefit the Pacific coast population of the western snowy plover. A coordinated international conservation program with Mexico also should be established to protect western snowy plover populations and their habitat in that country (see Action 8).

B. RECOVERY UNITS

The Pacific coast population of the western snowy plover has been divided into six recovery units (Appendix A, Figures A-1 through A-7). Establishing recovery units with specific recovery goals for each recovery unit will assist in meeting the objective of ensuring that population increases are distributed throughout the western snowy plover's Pacific coast range. A recovery unit is a special unit of a listed species that is geographically or otherwise identifiable and is necessary to the survival and recovery of the entire listed entity. Recovery units are individually necessary to conserve genetic robustness, demographic robustness, important life history stages, or other features for long-term sustainability of the entire listed species. However, recovery units are not listed as separate entities and cannot be delisted individually. Each recovery unit must be recovered before the species can be delisted.

The resilience to extinction of a widespread species can be negated if the species is subjected to a new stress over a large area (Raup 1991:122, 182). For the western snowy plover the primary stresses that led to the listing of the species were the loss of habitat due to encroachment of European beachgrass and urban development. As a consequence of such widespread habitat loss and the subsequent reduction in the range and vigor of the species, the western snowy plover is now more vulnerable to environmental fluctuations and catastrophes that the species would otherwise be able to tolerate. Chance events such as oil and contaminant spills, windstorms, and continued habitat loss from European beachgrass expansion, described earlier in this plan, could now cause or facilitate the extirpation of the entire listed species or one or more of the breeding populations.

The recovery unit approach in this recovery plan addresses this risk to the long-term survival and recovery of the western snowy plover by employing two widely recognized and scientifically accepted goals for promoting viable populations of listed species: (1) creation or maintenance of multiple populations so that a single or

series of catastrophic events cannot destroy the whole listed species; and (2) increasing the size of each population in the respective recovery unit to a level where the threats of genetic, demographic, and normal environmental uncertainties are diminished (Mangel and Tier 1994; National Research Council 1995:91; Tear *et al.* 1993; Meffe and Carroll 1994:192).

In general, the larger the number of populations and the larger the size of each population, the lower the probability of extinction (Raup 1991:182; Meffe and Carroll 1994:190). This basic principle of redundancy applies to the western snowy plover. By maintaining viable populations at the breeding locations within multiple recovery units, the threats represented by a fluctuating environment are alleviated and the species has a greater likelihood of achieving long-term survival and recovery. Conversely, loss of one or more important breeding locations within a recovery unit could result in an appreciable increase in the risk that the entire listed species may not survive and recover. Because western snowy plovers tend to exhibit site fidelity, migration to new nesting sites could increase stress to breeding birds and reduce nesting success.

Therefore, when evaluating the potential impact of land management actions that may affect the western snowy plover, we will consider whether a significant loss of western snowy plover breeding or wintering habitat in one recovery unit --without adequate compensation alleviating the impacts of that loss-- would adversely affect the viability of the population in that recovery unit as well as the long-term viability of populations in other recovery units.

Several aspects of the biology and life history of the western snowy plover indicate that designation of recovery units is necessary to ensure the long term health and sustainability of the western snowy plover. A portion of the Pacific coast population of western snowy plovers do not migrate up or down the coast and are year round residents. Additionally, the majority of western snowy plovers that do migrate are site-faithful, returning to the same breeding areas in subsequent breeding seasons (Warriner *et al.* 1986, Stenzel *et al.* 1994). Western snowy plovers occasionally nest in exactly the same location as the previous year (Warriner *et al.* 1986). These two features indicate that the Pacific coast population of western snowy plover likely exhibits subpopulation and metapopulation structure (see also Appendix D).

Designation of separate recovery units across the range will ensure that metapopulation dynamics can be maintained for the species.

The area covered by the six recovery units encompasses all the known breeding and wintering sites for the Pacific coast population of the western snowy plover. In addition to exhibiting site fidelity to breeding locations, western snowy plovers also exhibit fidelity to wintering locations. In contrast to many migratory birds, winter migration of the Pacific coast population of western snowy plovers is not uni-directional. Western snowy plovers may move both north and south along the coast from breeding locations. Nesting birds from Oregon have wintered as far south as Monterey Bay, California, while birds from Monterey Bay in central California have wintered north to Bandon, Oregon and south to Laguna Ojo de Liebre in Baja California, Mexico (Page *et al*. 1995*a*). Nesting birds from San Diego County in southern California have wintered north to Vandenberg Air Force Base in Santa Barbara County and south to Baja California (Powell *et al*. 1995, 1996, 1997). Designation of separate recovery units, each essential to the recovery of the western snowy plover, will ensure that wintering and migratory habitat is distributed across the western snowy plover's Pacific coast range and is protected and managed to maximize western snowy plover population survival.

The six recovery units for the Pacific coast population of the western snowy plover are: (1)Washington and Oregon; (2) Del Norte to Mendocino Counties, California; (3) San Francisco Bay, California; (4) Sonoma to Monterey Counties, California; (5) San Luis Obispo to Ventura Counties, California; and (6) Los Angeles to San Diego Counties, California. These recovery units were designated partly based on gaps in distribution of westem snowy plover breeding and wintering locations, and on gaps in available habitat along the coast. For example, a significant portion of the coast of Sonoma County and southern Mendocino County is rocky and composed of steep bluffs lacking beach, dune, or estuary habitat suitable for the western snowy plover. This area constitutes a gap in the distribution of breeding and wintering locations between recovery units 2 and 4. This situation is repeated along the coast of Monterey County, where a gap in western snowy plover locations and suitable habitat occurs between recovery units 4 and 5. Smaller gaps also occur between recovery units 1 and 2, and between recovery units 5 and 6. Recovery unit 3 is unique and has

been designated as a separate recovery unit because much of the habitat in the San Francisco Bay area consists of salt ponds and salt pond levees.

The six recovery units designated for the western snowy plover also vary significantly in numbers of breeding western snowy plovers. Recovery unit 5 supports the greatest number of western snowy plovers, approximately half of the U.S. population, and has the greatest amount of available suitable habitat. Recovery units 4 and 6 support, or have the potential to support, a lesser number of western snowy plovers, collectively about a third of the population. The population in Recovery Unit 3 is relatively lower but has potential to increase with intensive management of salt pond habitat. Recovery units 1 and 2 also support relatively low numbers of western snowy plovers, probably due to suitable habitat being lesser in extent and more widely separated, but represent about half of the geographic range of the Pacific coast population of western snowy plovers within the United States and provide essential wintering, migratory, and breeding habitats.

Collectively, recovery of western snowy plovers within each of the six recovery units is necessary to maintain metapopulation dynamics, ensure protection and appropriate management of wintering and migratory habitat, and ensure the long term health and sustainability of the Pacific Coast population of western snowy plovers across its current range.

C. RECOVERY GOALS AND OBJECTIVES

The goal of this recovery plan is to ensure the long-term viability of the Pacific coast western snowy plover population so that this population can be removed from the Federal list of endangered and threatened species. The specific objectives to achieve this goal are the major components of the recovery strategy described above:

1) Increase population numbers distributed across the range of the Pacific coast population of the western snowy plover;

2) Conduct intensive ongoing management for the species and its habitat and develop mechanisms to ensure management in perpetuity; and

3) Monitor western snowy plover populations and threats to determine success of recovery actions and refine management actions.

D. RECOVERY CRITERIA

Recovery criteria for the Pacific coast population of the western snowy plover include numeric subpopulation targets, reproductive productivity targets, and establishment of management actions. Under each of these three major recovery criteria are additional subcriteria that must be achieved in order to progress toward the major criteria or that must be achieved in order to determine whether the major criteria are being met. Subcriteria include completing development and implementation of population, demographic and threat monitoring programs, incorporating specific management actions into participation and management plans, and completing research actions necessary to refine management actions.

Recovery criteria in this recovery plan are necessarily preliminary and will need periodic reassessment because additional data upon which to base decisions about western snowy plover recovery are needed (*i.e.*, effective predator management techniques, effective restoration techniques, improved monitoring techniques, additional demographic information for some subpopulations). Research actions, monitoring programs, and periodic recovery implementation review are included as recovery actions in order to obtain this information. The completion of many of these actions have been incorporated into recovery criteria in order to ensure that new information is incorporated into recovery implementation decisions.

The recovery criteria recommend that the Pacific Coast population of the western snowy plover be maintained at 3,000 breeding birds. This population increase to 3,000 breeding individuals could occur within 25 years with intensive management of breeding and wintering sites (see Appendix D. Population Viability Analysis for Pacific Coast Snowy Plovers). This population level must be maintained for at least ten years. In addition, average annual productivity of at least one (1.0) fledged chick per male in each recovery unit must be maintained in the last 5 years prior to delisting. Forty years may be required to achieve these demographic components of the recovery criteria, assuming that mechanisms to assure long-term protection and

management of breeding, wintering, and migration areas necessary to maintain the subpopulation sizes and average productivity have been developed and are in place.

The Pacific coast population of the western snowy plover will be considered for delisting when the following criteria have been met:

Criterion 1. Monitoring shows that an average of 3,000 breeding adults distributed among 6 recovery units as specified below have been maintained for a minimum of 10 years:

Recovery Unit	*Subpopulation Size*
1. Washington and Oregon	250 breeding adults
2. Del Norte to Mendocino Counties, California	150 breeding adults
3. San Francisco Bay, California	500 breeding adults
4. Sonoma to Monterey Counties, California	400 breeding adults
5. San Luis Obispo to Ventura Counties, California	1,200 breeding adults
6. Los Angeles to San Diego Counties, California	500 breeding adults

Subpopulation sizes represent the best professional judgment of the Western Snowy Plover Recovery Team's technical subteam. Numbers are based on a site-by-site evaluation of historical records, recent surveys, and future potential (assuming dedicated, proactive management at breeding and wintering locations). Collectively, these numbers represent an approximately 70 percent increase in the Pacific coast population size from the time of listing. On a cumulative range-wide basis the recovery criteria are approximately 83 percent of the total of the "Management Goal Breeding Numbers" identified in Appendices B and C, which represent site-specific target populations under an intensive management scheme. The recovery criteria for population size and distribution for the Pacific coast population of the western snowy plover represent only a portion of its historical abundance and distribution.

To reach these subpopulation sizes will require proactive management to attain a level of productivity that will allow the population to grow. The population viability analysis (Appendix D) suggests that reproductive success between 1.2 to 1.3 fledglings per male per year, with adult survival of 76 percent and juvenile survival of 50 percent, provides a 57 to 82 percent probability of reaching a population of 3,000 western snowy plovers within 25 years. Enhancing productivity is critical to population growth. Once the population size criterion is met, a lower rate of productivity can sustain the population.

1a. A program is developed and implemented to monitor the western snowy plover breeding population and wintering locations (see Actions 1.1 and 1.2) to determine whether recovery unit subpopulation criteria are being achieved.

The monitoring program must include monitoring of population size and distribution, survival, and productivity. Monitoring population size and distribution are necessary as a means of measuring whether the recovery criterion is being met. Monitoring demographic characteristics such as survival and productivity also will be necessary to determine population trends and progress toward achieving the recovery criterion. The monitoring program should also assess whether management goals for breeding and wintering sites listed in Appendix B are being achieved. Collectively, the breeding management goal numbers are about 20 percent higher than the recovery criteria subpopulation sizes. Monitoring of individual sites will assist in determining the effectiveness of management actions and whether any refinements are necessary. Monitoring of wintering sites will assist in indicating whether survival of western snowy plovers is sufficient to make progress toward meeting breeding population size criteria.

When the species has recovered sufficiently to be delisted, the ongoing program of monitoring actions should be integrated into a post-delisting monitoring plan to cover a minimum of 5 years after delisting and ensure ongoing recovery and effectiveness of management actions. This monitoring plan should be developed and ready for implementation before delisting.

1b. A program is developed and implemented to monitor the site-specific threats identified in Appendix C (Action 1.3) and monitoring results are used to refine site-specific management actions identified in Appendix C.

In conjunction with monitoring of breeding subpopulation sizes and distribution and demographic characteristics, threats at each breeding and wintering site must be monitored in order to determine whether management actions are effective in increasing western snowy plover survival and reproduction. If threats continue limiting population increases, or additional threats are identified, management actions recommended in Appendix C may require modification.

1c. Management activities identified in Appendix C that are necessary to ameliorate threats and achieve increases in reproductive success, survival, and overall population size are incorporated into participation and management plans developed and implemented under Criterion 3.

Appendix C provides location-specific summaries of current management activities at western snowy plover breeding and wintering sites based on: 1) responses by public land managers and private conservation organizations to a survey prepared by the Recovery Team on western snowy plover management and beach use; and 2) supplemental information from the Recovery Team and from our field office staff. Appendix C also identifies additional management activities needed at each site to ameliorate threats and achieve management goals. These management recommendations are intended to provide preliminary guidance but additional management needs likely will be identified through monitoring, research, and site-specific experience.

1d. Research actions (Action 4) are completed and incorporated into management and participation plans and into monitoring plans.

Several research needs identified under Action 4 are necessary to refine and improve management activities for the western snowy plover and also to improve monitoring of western snowy plover population sizes, demographics, and threats. Improving and refining management actions will increase the effectiveness of management actions in increasing population numbers, survivorship, and productivity. Improved monitoring

techniques are needed to ensure that monitoring efforts are adequate to determine whether recovery actions are successful and recovery criteria are being met.

Criterion 2. A yearly average productivity of at least one fledged chick per male has been maintained in each recovery unit in the last 5 years prior to delisting.

From currently available data, it is estimated that males must average one fledged young annually for population equilibrium (see Appendix D). Higher rates of productivity will be necessary to reach the target population size of 3,000 breeding adults. After this population size is achieved and maintained for a minimum of 10 years, a lower rate of productivity of one fledged chick per male will be necessary to maintain the population size at an average of 3,000 breeding adults. Monitoring programs developed and implemented under criteria 1a and 1b should continue throughout this period. We also assume that management designed to ameliorate threats (criteria 1c and 3) will continue through this period and after delisting.

Criterion 3. Mechanisms have been developed and are in place to assure long-term protection and management of breeding, wintering, and migration areas listed in Appendix B to maintain the subpopulation sizes and average productivity specified in Criteria 1 and 2.

Development of mechanisms to ensure long-term management and protection of western snowy plovers and their habitat are listed under Action 3, which outlines the recovery actions recommended to meet these recovery criteria. The recovery action outline section describes each action in detail. The recovery action outline lists all subactions necessary to fulfill the main recovery action. It also represents a prioritization of measures to be implemented. Completion of these actions will ensure that threats to western snowy plovers and their habitat are ameliorated and that management will continue after delisting to prevent a reversal of population increases.

3a. Working groups for each of the six recovery units are established.

Action 3.1 recommends the establishment of working groups for each recovery unit. Working groups should be diverse and include representatives from Federal, State,

local, and private sectors. At present working groups are in existence for all recovery units, and should continue to be maintained and meet regularly. The roles of the working groups are to coordinate and facilitate recovery efforts within each recovery unit, assess population trends, and carry out outreach activities.

3b. A participation plan for each recovery unit working group has been developed and implemented.

Each working group is tasked with developing a participation plan that delineates and prioritizes recovery activities within each recovery unit and for each location identified in Appendix B. These plans should identify the roles and responsibilities of each member of the working group and their commitments to carry out identified recovery actions.

3c. Management plans for all Federal and State lands identified in Appendix C have been developed and implemented.

Appendix C identifies the landowners of western snowy plover wintering and breeding sites. Many of the sites are owned or managed by Federal or State agencies. Development and implementation of management plans that incorporate the management goals and recommendations in Appendix C for all these sites are necessary to ensure that population goals are reached, threats ameliorated, and long-term protection and management of westem snowy plovers and their habitat are in place.

3d. Mechanisms to protect and manage western snowy plover breeding and wintering sites identified in Appendices B and C are in place for all areas owned or managed by local governments or private landowners.

Appendix C also identifies many western snowy plover breeding and wintering locations that are owned or managed by local governments, private conservation organizations, or private landowners. These lands also require protection and management to ensure that population goals are reached, threats ameliorated, and long-term protection and management of western snowy plovers and their habitat are in place. Because of the diverse ownership and management of these lands, many

different mechanisms may be used to ensure protection and management of these locations. These mechanisms are further described in the recovery action outline and Appendices H and I.

3e. Public information and education programs are developed and implemented.

Outreach is a major component of developing and putting in place mechanisms to assure long-term protection and management of breeding, wintering, and migration areas listed in Appendix B. Outreach efforts will be needed to solicit participation of the many Federal, State, local, and private groups in recovery efforts and notify groups and individuals of recovery opportunities and incentives for the western snowy plover. Outreach efforts also must be used as a component of management of western snowy plovers and their habitats. These efforts will include informing the public and gaining their support for measures intended to protect western snowy plovers.

E. RELATIONSHIP OF RECOVERY ACTIONS AND CRITERIA TO THREATS

The goal of this recovery plan is to ensure the long-term viability of the Pacific coast population of western snowy plovers so that they can be removed from the Federal list of endangered and threatened species. The delisting process requires demonstrating that threats to the western snowy plover have been reduced or eliminated such that the species survival in the wild is assured. Table 8 lists the threats to the western snowy plover that have been identified during and since the listing process and indicates the actions and recovery criteria in the recovery plan that address each threat.

The western snowy plover faces multiple threats throughout its Pacific coast range. Major threats to the western snowy plover include habitat destruction and modification and lack of habitat protection mechanisms (listing factors A and D), disease or predation (listing factor C), and manmade factors that primarily result in disturbance or mortality of breeding birds (listing factor E). Effects of research on western snowy plovers (listing factor B) is also a threat but is comparatively minor

and easily addressed through permitting processes. Many of the threats to western snowy plovers are interrelated or have complex interactions with each other. For example, coastal development that destroys or modifies habitat (listing factor A) also results in increased disturbance from recreational activities (listing factor E) and in increased predator populations (listing factor C). Recovery actions and criteria therefore may address multiple threats.

The majority of threats to the western snowy plover, other than habitat destruction or modification, affect the western snowy plover's productivity (breeding success) and survival within otherwise suitable habitat. Criteria 1 and 2 are directed at determining whether the effects of threats on productivity and survival have been removed and expected population and productivity increases are being achieved. Threats addressed by these recovery criteria primarily fall under listing factors B, C, and E. Reduction and elimination of these threats, and the expected increases in productivity and survival, rely primarily on developing intensive management and monitoring programs for the western snowy plover. Criterion 3 is directed at achieving the management and habitat protections necessary to reduce and eliminate threats that fall primarily under listing factors A and D, but also address threats under listing factors B, C, and E that can be eliminated or ameliorated by ensuring long-term management.

Table 8. Threats to the Pacific coast population of the western snowy plover and steps within the recovery plan to reduce or eliminate threats.

Factor*	Threat	Action	Criterion
A	**The present of threatened destruction, modification, or curtailment of its habitat or range.**		
A*	Encroachment of introduced beachgrass and nonnative vegetation.	1.1-1.3, 2.2.1, 3.1-3.10, 4.1.1, 5.1-5.7	1b-d, 2, 3a-e
A*	Shoreline stabilization	1.1-1.3, 2.1, 3.1-3.10, 5.1-5.7	1b, 1c, 3a-e
A*	Urban development and construction	1.1-1.3, 2.1, 3.1-3.10, 5.1-5.7	1b, 1c, 3a-e
A	Dredging disturbance and tailings deposit	1.1-1.3, 2.1, 3.1-3.10, 5.1-5.7	1b, 1c, 3a-e
A*	Sand mining	1.1-1.3, 2.1, 2.2.2, 3.1-3.10, 5.1-5.7	3a-e
A	Beach nourishment with inappropriate design and/or sand type	1.1-1.3, 2.2.3, 3.1-3.10, 5.1-5.7	3a-e
A	Driftwood removal	1.1-1.3, 2.3.4, 3.1-3.10, 5.1-5.7	1b, 1c, 2 3a-e
A	Beach fires and camping	1.1-1.3, 2.3.3, 3.1-3.10, 5.1-5.7	1b, 1c, 2 3a-e
A	Water course diversion, impoundment, or stabilization	1.1-1.3, 3.1-3.10, 5.1-5.7	1b, 1c, 3a-e

Factor*	Threat	Action	Criterion
A	Habitat conversion for other species	1.1-1.3, 3.1-3.10, 5.1-5.7	1d, 3a-e
A	Operation of salt ponds	1.1-1.3, 3.1-3.10, 5.1-5.7	1b, 1c, 3a-e
B	**Overutilization for commercial, recreational, scientific or educational purposes.**		
B*	Egg collecting	1.1-1.3, 2.3.8	none, 1c
B	Studying and monitoring plovers	1.4, 1.5, 3.1-3.2, 4.3	1a-d 2
B	Banding	4.6	1a-d
C	**Disease or predation.**		
C*	Introduced nonnative predators	1.1-1.3, 2.4, 4.2, 3.1-3.10, 5.1-5.7	1b, 1c, 2 3a-e
C	Increased populations of native predators due to human influences	1.1-1.3, 2.4, 4.2,3.1-3.10, 5.1-5.7	1b, 1c, 1d, 2, 3a-e
C*	Predator attractants	1.1-1.3, 2.4, 4.2, 3.1-3.10, 5.1-5.7	1b, 1c, 1d, 2, 3a-e
C	Predation by domestic and feral cats	1.1-1.3, 2.4, 4.2, 3.1-3.10, 5.1-5.7	1a-d, 2, 3a-e
D	**The inadequacy of existing regulatory mechanisms.**		
D*	Limited habitat protection under the Migratory Bird Treaty Act and State laws	2.3.8, 3.1-3.10, 5.1-5.7	3a-e

Factor*	Threat	Action	Criterion
D	Conflicting beach management methods and mandates	1.1-1.3, 2.3.8, 3.1-3.10, 5.1-5.7	1b, 1c, 3a-e
D*	Sections 404 of Clean Water Act and 10 of Rivers and Harbors Act apply to limited amount of habitat	2.3.8, 3.1-3.10, 5.1-5.7	1b-d 3a-e
D*	Lack of protection in Baja California, Mexico	8	
E	**Other natural or manmade factors affecting its continued existence.**		
E*	Loss of nests and habitat due to natural events	1.1-1.3, 1.6, 2.1, 2.2, 2.3.8, 3.1-3.10, 4.4, 4.5, 4.10	1b, 1c, 3a-e
E*	Disturbance by pedestrians	1.1-1.3, 2.3.1, 2.3.8, 3.1-3.10, 4.9, 5.1-5.7	1b, 1c, 2, 3a-e
E*	Disturbance by dogs	1.1-1.3, 2.3.1, 2.3.2, 2.3.8, 3.1-3.10, 4.9, 5.1-5.7	1b, 1c, 2, 3a-e
E*	Disturbance by motorized vehicles	1.1-1.3, 2.3.5, 2.3.8, 3.1-3.10, 4.9, 5.1-5.7	1b, 1c, 2, 3a-e
E*	Disturbance by beach cleaning	1.1-1.3, 2.3.5, 2.4.1, 3.1-3.10, 4.9, 5.1-5.7	1b, 1c, 2, 3a-e
E*	Disturbance from equestrian traffic	1.1-1.3, 2.3.6, 2.3.8, 3.1-3.10, 4.9, 5.1-5.7	1b, 1c, 2, 3a-e

Factor*	Threat	Action	Criterion
E	Disturbance from fishing activities	1.1-1.3, 2.3.3, 2.3.8, 3.1-3.10, 4.9, 5.1-5.7	1b, 1c, 2 3a-e
E	Disturbance by fireworks	1.1-1.3, 2.3.3, 2.3.8, 3.1-3.10, 4.9, 5.1-5.7	1b, 1c, 2, 3a-e
E	Disturbance by kites and model airplanes	1.1-1.3, 2.3.3, 2.3.8, 3.1-3.10, 4.9, 5.1-5.7	1b, 1c, 2, 3a-e
E*	Military exercises and aircraft overflights	1.1-1.3, 2.3.8, 2.3.9, 3.1-3.10, 5.1-5.7	1b, 1c, 2 3a-e
E	Large crowds associated with special events	1.1-1.3, 2.3.3, 2.3.8, 3.1-3.10, 4.9, 5.1-5.7	1b, 1c, 2 3a-e
E	Increased coastal access to beaches	1.1-1.3, 2.3.1.2, 2.3.8, 3.1-3.10, 4.9, 5.1-5.7	1b, 1c, 2 3a-e
E	Livestock grazing	1.1-1.3, 2.3.7, 2.3.8, 3.1-3.10, 5.1-5.7	1b, 1c, 3a-e
E	Oil spills and disturbance from oil spill clean-ups	1.1-1.3, 2.5, 4.7, 5.6	1b-d 3a-e 1b, 1c, 2 3a-e
E	Environmental contaminants	1.1-1.3, 4.8, 5.6	1b-d, 3a-e
E	Litter, garbage, & debris	1.1-1.3, 2.3.8, 2.4.1, 3.1-3.10, 4.9, 5.1-5.7	1b, 1c, 2 3a-e

Factor*	Threat	Action	Criterion
E	Urban runoff and impaired water quality	1.1-1.3, 2.1, 2.3.8, 3.1-3.10, 5.1-5.7	3a-e
E	Management for other special status species	1.1-1.3, 1.7, 2.6, 2.7, 2.3.3, 3.1-3.10, 4.2.2, 5.1-5.7	3a-e

* Indicates threats originally identified during the listing process.

III. NARRATIVE OUTLINE OF RECOVERY ACTIONS

1 Monitor breeding and wintering population and habitats of the Pacific coast population of the western snowy plover to determine effects of recovery actions to maximize survival and productivity. To assure the long-term viability of western snowy plover populations, their populations and breeding and wintering habitat should be monitored and managed in a systematic, ongoing fashion. Systematic, ongoing monitoring of breeding birds and wintering birds should be undertaken at the recovery-unit level to measure progress towards recovery and identify management and protection efforts that are needed. In addition to the known breeding sites, all known wintering locations (Appendix B) are considered currently important to western snowy plover conservation. These sites include both wintering locations that currently support breeding birds and locations that may potentially support nesting birds in the future. These locations also may support migrating western snowy plovers. There is a need for better information about wintering and migration sites, including spatial and temporal use patterns, feeding areas, habitat trends, and threats. Appendix C, Table C-1 identifies 147 locations where monitoring western snowy plover populations is occurring or recommended to achieve management goals.

1.1. Annually monitor western snowy plover abundance, population size, and distribution at breeding and wintering locations in each recovery unit using window surveys. Comprehensive range-wide window surveys of breeding locations and wintering locations (Appendix B) should be conducted annually to determine population trends and fluctuations, and to determine whether management goal breeding numbers (Appendix B) are being achieved. The window survey described in Appendix J (Monitoring Guidelines) should be employed as the primary index of population size to minimize the probability of double-counting birds nesting at multiple locations during the same season. Window surveys are conducted over a relatively short time period to minimize double-counting of birds that change location during the season, but may not fully account for all breeding or wintering birds. Window survey methodology should be improved and correction factors estimated (Action 4.3.1) to improve the

accuracy and utility of population indices. This correction may require some banding at sites where there are currently no marked birds on which to base correction factors.

1.2 Develop and implement a program to monitor western snowy plover productivity and annual survival in each recovery unit. Development and implementation of a program to monitor western snowy plover productivity and survival, in addition to comprehensive population size and distribution monitoring, is necessary to measure progress toward achieving recovery criteria and to assess the effectiveness of management in removing threats that affect nesting success and survival. Results from this monitoring program also may be used to update the population viability analysis and assess progress toward recovery goals (Actions 4.11 and 6). Monitoring productivity and survival likely will be much more intensive than monitoring population sizes and distribution (Action 1.1), and cannot be implemented at all breeding sites because of insufficient color band combinations to monitor the entire Pacific coast population. Plans for monitoring these demographic characteristics instead should utilize methods to sample demographic characteristics across the breeding range and in each recovery unit. Actions 4.3.2 and 4.3.3 recommend developing methodologies to estimate productivity and survival. The monitoring program should incorporate these methods and should specify the number of sites sampled in each recovery unit, how sites will be selected, and indicate control sites from intensively monitored breeding locations (*i.e.*, the coast of Oregon, extreme northern California, and the shoreline of Monterey Bay).

1.3 Develop and implement a program to monitor at all breeding and wintering sites the habitat conditions, disturbances, predation, and other threats limiting abundance of breeding and wintering birds, clutch hatching success, chick fledging success, and survival. Monitoring of threats to the western snowy plover is necessary to determine effectiveness of recovery actions in ameliorating or eliminating threats, assess progress toward recovery, and refine site-specific managements as necessary. A standardized threats monitoring program

should be developed and applied to all breeding and wintering sites in conjunction with monitoring developed and implemented under actions 1.1 and 1.2. At a minimum, monitoring should include determining substrate characteristics and vegetation composition (level of nonnative species), frequency and levels of disturbance (*e.g.*, recreational activities, pets, vehicles, horses), and presence and abundance of predators. Appendix J (Monitoring Guidelines) provides general guidance on monitoring but may require revision as research actions under action 4 are completed. Opportunities to incorporate monitoring into Federal activities subject to section 7 of the Endangered Species Act, such as dredging and discharges regulated by the U.S. Army Corps of Engineers, should be utilized when possible.

1.4 Develop and implement training and certification programs for western snowy plover survey coordinators and observers, consistent with recommendations in Appendix J (Monitoring Guidelines). Classroom and field training are required for observers who survey for western snowy plovers, and before we can issue a section 10(a)(1)(A) permit. Instruction programs and materials should be developed for comparable training to occur throughout the western snowy plover range to improve consistency of data collection. Classroom topics should include, but not be limited to: (1) biology, ecology, and behavior of breeding western snowy plovers; (2) identification of adult plovers, their young, and their eggs; (3) threats to plovers and their habitats; (4) survey objectives, protocols, and techniques; (5) regulations governing the salvage of carcasses or eggs; (6) special conditions of existing recovery permits; (7) field identification of potential western snowy plover predators; (8) biology and behavior of predator and scavenger species; and (9) other activities (*e.g.*, banding). Field training should include, as appropriate: (1) locating, identifying, and monitoring nests; (2) handling eggs and capturing and handling adults or chicks; (3) specifics on the target activity for which a recovery permit is to be issued, or under which an observer will work; (4) practical field exercises; and (5) field review of appropriate classroom topics.

1.5 Develop a submittal system for monitoring data to ensure consistent reporting among recovery units and sites, and annually review and revise the system as necessary. Initially, range-wide survey data will be limited to results from 2 annual window surveys. As population and demographic monitoring methods are developed and implemented (Actions 1.1, 1.2, 4.3.1, 4.3.2, and 4.3.3), a more sophisticated reporting and compiling system will be necessary. Our lead office should coordinate with researchers involved with monitoring to ensure that data collection, submittal, and entry systems remain current, include correction factors that account for lack of detections during surveys, and are consistent among recovery units and sites. An annual range-wide report should be developed and distributed to all interested parties. Additionally, consistent reporting of sightings of banded western snowy plovers is needed. Sightings of banded birds provide information on the wintering sites of breeding birds, use of multiple sites by breeding and wintering plovers, and survival and dispersal of adults and juveniles. In accordance with procedures of the U.S. Geological Survey, Bird Banding Laboratory, the Point Reyes Bird Observatory should continue to act as the color band coordinator for the Pacific coast population to avoid use of duplicate color banding schemes among researchers.

1.6 Assess and evaluate new breeding, wintering, and migration areas as they are discovered to determine threats and management needs and update lists of areas identified in Appendices B and C as data become available. As new western snowy plover breeding and wintering areas are discovered, data should be collected to assess site boundaries, habitat characteristics, population levels, and any significant threats. The current list of important breeding and wintering locations (Appendix B) should be expanded or refined as appropriate, and any new areas incorporated into management and monitoring plans. Areas determined to be important for migration through action 4.4.4 also should be evaluated and added to the list of areas requiring protection, management, and monitoring. Management goals and needed management to ameliorate or eliminate threats should be developed for all new breeding, wintering, and migration

areas and should be included in periodic revisions of Appendices B and C of this recovery plan.

1.7 Annually coordinate monitoring of western snowy plovers and California least terns to minimize effects of disturbance to both species. Coordination with least tern monitors and managers is needed in all areas where western snowy plovers share breeding sites with California least terns. Coordination should take place at biannual pre-and post-season California least tern monitoring meetings. Protocols for monitoring California least terns should be revised as necessary so that western snowy plovers are not detrimentally affected. Human activities within some least tern colonies in southern California include monitoring by one to four people several days per week; maintenance of tern fences; predator management; site preparation; and banding/observation efforts. Human activities associated with tern monitoring must be recognized as additional disturbance to western snowy plovers. Section 10(a)(1)(A) permits, issued under the authority of the Endangered Species Act for western snowy plovers and least terns, should include both species where applicable. Monitoring efforts for both species should be kept separate because of differences in monitoring techniques and species' behaviors. Monitors of least terns and western snowy plovers should be aware of species' differences in nest spacing, brood-rearing, foraging behavior, time of breeding, vulnerability to disturbance, and monitoring and banding techniques.

Western snowy plovers generally begin nesting at least 1 month before the arrival of breeding least terns; thus, tern management often begins well after western snowy plovers have initiated nests. Site preparation (vegetation removal and fence construction) should be coordinated to minimize disturbance to nesting western snowy plovers, and if possible to enhance breeding success for both species (as well as considering other sensitive species, including plants, that may be present). Predator management also should be coordinated to benefit both species.

1.8 **Develop post-delisting monitoring plan.** Prior to delisting a five-year monitoring plan should be developed. Methodology and scope of post-delisting monitoring should be appropriately integrated with existing monitoring efforts for continuity and comparability. Monitoring and research results should be used to guide the long-term conservation of the species.

2 **Manage breeding and wintering habitat of the Pacific coast population of the western snowy plover to ameliorate or eliminate threats and maximize survival and productivity.** The Pacific coast population of the western snowy plover is sensitive to changes in productivity and in adult and juvenile survival rates (see Appendix D). Furthermore, recovery of this species is contingent on intensive management of breeding habitat and availability of wintering habitat for more than the current number of westem snowy plovers (see recovery criteria). Appendix C provides a summary of site-specific management needs at 155 breeding and wintering locations (actions 2 and 3). Management efforts may be time-consuming, costly, and sometimes require intensive management. Western snowy plover breeding habitat is extremely dynamic and factors affecting breeding success, such as types and numbers of predators, can change quickly; therefore, managers should be prepared to modify protection as needed. Action 6 recommends annual review of progress toward recovery and revision of site-specific management actions based on monitoring and research results and site-specific experience. Management and protection of western snowy plovers on Federal and State lands are especially important. In addition, protection on Federal and State lands furnishes leadership by example to local land managers. Land managers should recognize that components of breeding habitat include: areas where plovers prospect for nesting sites, make scrapes, lay eggs, feed, rest, and rear broods. Breeding habitat also includes travel corridors between nesting, resting, brood-rearing, and foraging areas. Wintering and migration habitats should also be monitored and managed to maximize survival and recruitment of western snowy plovers into the breeding population.

2.1 Maintain natural coastal processes that perpetuate high quality breeding and wintering habitat by incorporating the following recommendations into development of participation plans, management planning, and habitat protection (action 3) for the sites identified in Appendix C and any additional sites identified through surveys and monitoring. The dynamic nature of beach strand habitats as storm-maintained ecosystems should be recognized and allowed to function. Natural process that contribute to maintaining wide, flat, sparsely-vegetated beach strands preferred by western snowy plovers include: inlet formation, migration, and closure; erosion and deposition of sand dunes; and overwash and blowouts of beach and dune habitat. Coastal development, beach stabilization, construction of rock jetties and seawalls, sand removal and dredging, water diversion and impoundment, and planting of nonnative vegetation interfere with these processes and result in loss and degradation of habitat.

Maintenance of natural coastal processes can be accomplished through establishment of management plans, conservation easements, fee title acquisition, zoning, and other means. Coastal development, beach stabilization, resource extraction, and water diversion and/or impoundment projects should be carefully assessed for impacts to wintering western snowy plovers. Recommendations from U.S. Fish and Wildlife Service offices (under the Endangered Species Act and Clean Water Act) and/or State agencies should focus on avoiding or minimizing adverse impacts to wintering habitat. Where adverse effects cannot be avoided, agencies should document impacts so that cumulative effects on this species' habitat can be assessed and compensated. When beach development cannot be avoided, the following protections should be implemented: (1) construction should take place outside the nesting season, (2) developers and others should be advised during planning stages that stabilization of shorelines will result in additional habitat degradation and that these impacts may affect evaluation and issuance of permits under the jurisdiction of the U.S. Army Corps of Engineers or State coastal management agencies, and of measures to minimize the impacts, (3) property owners (*e.g.*, hotel or resort owners) should tailor

recreational activity on the beach and dunes to prevent disturbance or destruction of nesting western snowy plovers, their eggs, and chicks, (4) lights for parking areas and other facilities should not shine on western snowy plover habitat, (5) sources of noise that would disturb western snowy plovers should be avoided, and (6) the establishment of predator perches and nesting sites should be avoided when designing facilities. Appendix C, Table C-1 identifies 86 locations which currently have development restrictions in place and 16 locations where development should be restricted or avoided to achieve management goals.

2.1.1 Develop a prioritized list of western snowy plover wintering and breeding sites where natural coastal processes need protection, or where impaired natural coastal processes should be enhanced or restored. Recovery Unit working groups should evaluate the sites within their recovery unit and determine where natural processes are likely to be disrupted or are in need of being enhanced or restored, or are of particular importance to maintaining high quality western snowy plover habitat. Sites should be prioritized based on their importance to western snowy plover breeding and the degree of threat to the western snowy plover and its habitat should natural processes be disrupted.

2.1.2 Identify mechanisms necessary to protect, enhance, or restore natural coastal processes for the sites identified in action 2.1.1 and implement through incorporating into actions 3.1 -3.10. Mechanisms to protect, enhance, or restore natural processes may include development of management plans that prohibit or restrict activities that disrupt natural process (*i.e.* dredging or sand removal, recreational activities that contribute to excessive erosion or compaction), acquisition of habitat, landowner agreements, local land use protection measures, or enhancement activities. Identification of these sites and mechanisms should be used to guide implementation of long-term management and protection under action 3.

2.2 Create and enhance existing and potential breeding and wintering habitat. Past and ongoing impacts to western snowy plover breeding habitat from development, artificial beach stabilization, and other projects have resulted in loss and degradation of western snowy plover habitat. Habitat enhancement and creation are needed at multiple sites to offset these losses. Where impacts cannot be avoided, projects should remediate and compensate habitat loss and degradation by maintaining natural long-shore sand budgets and minimizing interference with natural patterns of sand accretion and depletion. When these types of projects are planned, complex natural sand movement patterns should be taken into account. Beach management policies should recognize that many current erosion and sedimentation problems are the result of past property and/or inlet "protection" efforts. Habitat restoration projects in historic or potential breeding sites, where feasible, is encouraged. Creation of habitat should be emphasized in areas not subject to recreational impacts.

2.2.1 Remove nonnative and other invasive vegetation from existing and potential habitat and replace with native dune vegetation. Land managers should implement remedial efforts to remove or reduce vegetation that is encroaching on western snowy plover breeding habitat or obstructing movement of chicks from nesting to feeding areas. Particular attention should be given to the eradication of introduced beachgrass (*Ammophila* spp.) within coastal dunes.

2.2.1.1 Develop and implement prioritized removal and control strategies for introduced beachgrass and other nonnative vegetation for each recovery unit. These strategies should include early intervention to prevent expansion into breeding areas where introduced beachgrass and other nonnative vegetation have not yet spread or are in early stages of spreading. Attention also should be given to the removal of giant reed, Scotch broom, gorse, iceplant, and shore pine. Remove/manage vegetation on salt ponds, including levees.

Schedule/coordinate removal efforts to avoid disturbing nesting western snowy plovers. Appendix C, Table C-1 identifies 86 locations where removal of nonnative and other vegetation is either currently occurring or needs to be initiated to achieve management goals.

2.2.1.2 Replace exotic dune plants with native dune vegetation where it is likely to improve habitat for western snowy plovers. Land managers should make special efforts to reestablish native dune plants in western snowy plover nesting habitat, while concentrating on removal of nonnative vegetation. Native dune vegetation includes American dunegrass (*Leymus mollis*), beach morning glory (*Calystegia soldanella*), pink sand-verbena (*Abronia umbellata*), yellow sand verbena (*Abronia latifolia*), beach bursage (*Ambrosia chamissonis*), grey beach pea (*Lathyrus littoralis*), whiteleaf saltbush (*Atriplex leucophylla*), and California saltbush (*Atriplex californica*). These efforts should be targeted for coastal dune sites that currently support nonnative vegetation species such as introduced beachgrass (*Ammophila* spp), and should be combined with removal of this invasive plant. Seeds of local native dune plants collected within approximately 32 kilometers (20 miles) of the site to be planted should be used as replacement plant stock. Revegetation efforts should be monitored to ensure that the amount of vegetative cover is compatible with suitable breeding habitat for plovers.

2.2.2 Deposit dredged material to enhance or create nesting habitat. Near-shore (littoral drift) and on-shore disposal of dredged material seems to be beneficial for perpetuating high quality western snowy plover nesting habitat in some instances and should be encouraged where appropriate. However, monitoring of habitat characteristics before, during, and after projects is needed,

particularly in cases of large operations occurring on sites where western snowy plovers nest or are deemed likely to nest following the disposal operation. On-shore disposal of dredged material should be scheduled outside the nesting season and, where possible, during seasons when birds are not present. In addition, dredged material must be clean sand or gravel of appropriate grain size and must be graded to a natural slope.

2.2.2.1 Evaluate western snowy plover breeding and wintering sites listed in Appendix C and potential breeding sites to determine whether dredged materials may be used to enhance or create nesting habitat. Recovery Unit working groups should identify sites where dredged material may be used to enhance or create nesting habitat. Evaluation of sites should include impacts (short- and long-term) to existing western snowy plover habitat, likelihood of use by western snowy plovers, whether appropriate sources of clean dredged material exist, and opportunities to utilize material from dredging projects.

2.2.2.2 Develop and implement plans, including pre- and post-project monitoring, to use dredged material to enhance or create nesting habitat at the sites identified in action 2.2.2.1. Plans to implement use of dredged material to enhance or create nesting habitat should be developed for sites identified in action 2.2.2.1. Plans should include measures to minimize impacts to western snowy plovers and existing habitat and should include pre- and post-project monitoring to determine effectiveness of the project in enhancing or creating nesting habitat.

2.2.3. Implement beach nourishment activities if action 4.1.2 indicates beach nourishment activities are effective in enhancing western snowy plover habitat. Beach nourishment activities have the potential to enhance western snowy plover habitat, but should be carefully evaluated to weigh the probable adverse and beneficial effects on plovers and on other sensitive coastal dune species.

2.2.3.1 Evaluate and identify sites where beach nourishment activities may be effective in creating and enhancing western snowy plover habitat. Potential sites include those sites where natural coastal processes have been disrupted (*i.e.* by coastal development, beach stabilization, construction of rock jetties and seawalls, etc.). Evaluation of sites should consider potential for adverse effects to existing western snowy plover habitat, whether appropriate sand sources are available, and whether long-term benefits are likely to occur.

2.2.3.2 Develop and implement beach nourishment plans, including pre- and post-project monitoring for the sites identified in action 2.2.3.1. Plans to implement beach nourishment activities to enhance or create nesting habitat should be developed for sites identified in action 2.2.3.1. Plans should include measures to minimize impacts to western snowy plovers and existing habitat and should include pre- and post-project monitoring to determine effectiveness of the project in enhancing or creating nesting habitat.

2.2.4 Create, manage, and enhance coastal ponds and playas for breeding habitat. Coastal ponds and playas, including salt ponds, should be enhanced and created to improve breeding habitat. Significant opportunities for management of nesting plovers currently exist within San Francisco Bay salt ponds, Moss Landing

Wildlife Area, Bolsa Chica wetlands, and south San Diego Bay salt ponds. However, salt ponds should only be created or enhanced at existing salt pond habitat; they should not be used for mitigation or compensation of coastal beach-dune or other western snowy plover habitats. Creation of habitat should be emphasized in areas that would preclude or reduce recreational impacts. Appendix C, Table C-1 identifies 15 locations where habitat enhancement is either currently in place or needs to be initiated to achieve management goals. Additional sites also may provide opportunities to enhance western snowy plover breeding habitat.

2.3 Prevent disturbance of breeding and wintering western snowy plovers by people and domestic animals. Disturbance by humans and domestic animals causes significant adverse impacts to breeding and wintering western snowy plovers. Because human disturbance is a primary factor affecting western snowy plover reproductive success, land managers should give the highest priority to implementation of management techniques to prevent disturbance of breeding birds. Western snowy plover breeding and wintering sites are highly variable in their amount of recreational activity. Land managers should conduct site-specific evaluations to determine whether recreational activities, domestic animals, and off-road vehicles pose a threat to plovers and implement appropriate measures. As information is gathered, it should be incorporated into conservation efforts. Management plans (Actions 3.3.1, 3.3.2, and 3.4) should include appropriate human/domestic animal access restrictions to prevent disturbance of western snowy plovers. Management techniques described below can reduce impacts of beach recreation on western snowy plovers, but they must be implemented annually as long as the demand for beach recreation continues.

2.3.1 Prevent pedestrian disturbance. Management measures to protect western snowy plovers should be determined on a site-by-site basis; factors to consider include the configuration of habitat as well as types and amounts of on-going pedestrian activity. On national wildlife refuges and State natural preserves within the

California State Parks system, where protection of wildlife is the paramount purpose of Federal and State ownership, western snowy plover habitat should be closed during the breeding season. Other areas also should be closed when necessary to adequately protect breeding western snowy plovers.

2.3.1.1 Restrict access to areas used by breeding western snowy plovers, as appropriate. Unless a beach is closed to public entry, or use is minimal, posting and/or fencing of nesting areas is recommended to discourage pedestrian use of the area and allow for plover courtship and prenest site selection, to prevent obliteration of scrapes, crushing of eggs or chicks, and repeated flushing of incubating adults. Any access restrictions should be accompanied by outreach programs to inform the public of any restrictions and provide educational material on the western snowy plover (see action 5).

2.3.1.1.1 Seasonally close areas used by breeding western snowy plovers. Dates of seasonal closures/restrictions should be based on the best data available, and be coordinated by geographic region for consistency in communicating with the public. Closures may be determined on a year-to-year basis and other options such as fencing may be considered first. To provide broods with access to foraging areas, closures should cover the area down to and including the water line, where practical. Areas where territorial plovers are observed also should be closed to prevent disruption of territorial displays and courtship. Because nests can be difficult to locate, especially during egg-laying, closure of these areas will also prevent accidental

crushing of undetected nests. Appendix C, Table C-1 identifies 81 locations where public access is either currently restricted or it is recommended it be restricted to achieve management goals.

2.3.1.1.2 Fence areas used by breeding western snowy plovers. Fencing to keep people and beach activities out of nesting/brood rearing areas should not hinder chick movements, unless fencing is specifically meant to keep chicks from being harmed. Areas with a pattern of nesting activity in previous year(s) or where territorial plovers are observed should be fenced before plovers begin nest-site selection. Because nests can be difficult to locate, especially during egg-laying, closure of these areas will also prevent accidental crushing of undetected nests. Symbolic fences (one or two strands of 1/4 inch plastic-coated steel cable strung between posts) with signs identifying restricted areas substantially improve compliance of beach-goers and decrease people's confusion about where entry is prohibited. On portions of beaches that receive heavy human use during the breeding season, fencing of prime brood-rearing areas to exclude or reduce numbers of pedestrians also should be implemented to contribute to the survival and well-being of unfledged chicks. Appendix C, Table C-1 identifies 64 locations where nesting areas are fenced or where fencing is recommended to achieve management goals.

2.3.1.1.3 Post signs in areas used by breeding western snowy plovers. Areas with a pattern of nesting activity in previous year(s) should be posted before plovers begin nest-site selection. On portions of beaches that receive heavy human use during the breeding season, posting of prime brood-rearing areas to exclude or reduce numbers of pedestrians also should be implemented to contribute to the survival and well-being of unfledged chicks. Appendix C, Table C-1 identifies 65 locations where exclusionary signs are in place or recommended to achieve management goals.

2.3.1.2 Locate new access points and trails well away from western snowy plover nesting and wintering habitat, and modify existing access and trials as necessary. Recreational users such as campers, clammers, anglers, equestrians, collectors, *etc.*, should be encouraged to consistently use designated access points and avoid restricted areas. Roads, trails, designated routes, and facilities should be located as far away from western snowy plover habitat as possible. Recreationists using boats should be restricted or prohibited from areas being used by the western snowy plover. Appendix C, Table C-1 identifies 67 locations where boat use is currently and/or is recommended to be prohibited or restricted, and 81 locations where access is currently and/or is recommended to be prohibited or restricted to achieve management goals.

2.3.1.2.1 Evaluate existing and planned access at all breeding and wintering locations and determine whether access may adversely affect western snowy plovers and their habitat. Review of access points should include evaluating level of and timing of use by recreational users and level of effects on the western snowy plover.

2.3.1.2.2 For sites where access is determined in action 2.3.1.2.1 to adversely affect western snowy plovers, develop and implement plans to minimize effects. Actions that could minimize effects of access include seasonal restrictions, signs, fencing, or relocation or modification of access points or trails.

2.3.2 Implement and enforce pet restrictions. It is preferable that land managers prohibit pets on beaches and other habitats where western snowy plovers are present or traditionally nest or winter because any noncompliance with leash laws can cause serious adverse impacts to western snowy plovers. If pets are not prohibited, they should be leashed and under manual control of their owners at all times. Pets should be prohibited on beaches and other western snowy plover habitats if, based on observations and experience, pet owners fail to keep pets leashed and under full control.

Land managers should document the type and frequency of infractions of rules and regulations requiring pets on leash. This information, including the number of verbal warnings, written warnings, and notices to appear (citations), should be documented so that comparisons can be made between locations. This documentation could help ensure that adequate effort is being

made to enforce pet regulations. Appendix C, Table C-1 identifies 120 locations where pets are currently prohibited or restricted and where they are recommended to be prohibited or restricted to achieve management goals.

2.3.3 Annually review existing recreational activities at breeding and wintering sites listed in Appendix C and develop and implement plans to prevent disturbance from disruptive recreational activities where western snowy plovers are present. Some recreational activities may disrupt western snowy plover breeding and foraging, attract predators, destroy nests, or degrade habitat. Management of a variety of recreational activities is needed to minimize these effects. Special events, including sporting events, media events, fireworks displays, and beach clean-ups, attract large crowds and require special attention. Special events planned in western snowy plover nesting areas should not be held during the plover nesting season. Early planning and coordination with local resource agencies should be emphasized. Fireworks should be prohibited on beaches where plovers nest. When fireworks displays are situated to avoid disturbance to western snowy plovers, careful planning also should be conducted to assure that spectators will not walk through and throw objects into plover nesting and brood-rearing areas. Sufficient personnel also must be on-site during these events to enforce plover protection measures and prevent use of illegal fireworks in the vicinity of the birds.

Flying of kites and model airplanes should be managed to avoid adverse impacts in areas where nesting plovers are present. Sports such as ball- and frisbee-throwing should be managed within hitting and throwing distance of western snowy plover nesting areas because of tendencies for stray balls and frisbees to land in closed areas where they can smash nests and where efforts to remove them can disturb territorial or incubating birds. Camping and beach fires should be prohibited in western snowy plover

nesting areas during the nesting season. Appendix C, Table C-1 identifies 11 locations where kites are and/or should be prohibited and/or restricted to achieve management goals, but additional recreational activities also should be reviewed for potential adverse effects to western snowy plovers.

2.3.4 Inform beach users of restrictions on driftwood removal through posting of signs. Driftwood removal should not be allowed unless needed to create sufficient open habitat to induce nesting activities. In such cases, driftwood removal should occur outside of the breeding season. Appendix C, Table C-1 identifies 26 locations where driftwood collection restrictions currently occur and/or are recommended for restriction to achieve management goals. Driftwood removal should also be minimized through enforcement as identified in Action 2.3.8.

2.3.5 Prevent disturbance, mortality, and habitat degradation by prohibiting or restricting off-road vehicles, including beach-raking machines. Recreational off-road vehicles should be prohibited or restricted at western snowy plover breeding areas, as appropriate. Violations associated with unauthorized entry of recreational off-road vehicles into closed or fenced nesting areas should be strictly enforced. During the nonbreeding season, enforcement of violations regarding recreational off-road vehicle use should continue where western snowy plover use of beaches occurs year-round. Because of potential habitat degradation caused by mechanized beach cleaning, alternatives to this type of beach cleaning are recommended, including manual beach cleaning by agency staff and volunteers knowledgeable about the need to maintain coastal dune habitat characteristics and to protect western snowy plovers. Appendix C, Table C-1 identifies 101 locations where off-highway vehicles are currently and/or recommended for prohibition or restriction to achieve management goals.

Essential vehicles within western snowy plover nesting areas should: (1) travel on sections of beaches where unfledged chicks are present only if absolutely necessary; (2) when possible, travel through chick habitats only during daylight hours; (3) travel at less than 8 kilometers (5 miles) per hour; (4) use a guide familiar with western snowy plovers; (5) use open four-wheel motorized off-highway vehicles or nonmotorized all-terrain bicycles to improve visibility; (6) avoid driving on the wrack (marine vegetation) line and during high-tide periods; (7) travel below the high tide mark and as close to the water line as is feasible and safe; and (8) avoid previous tracks on the return trip.

2.3.6 Implement restrictions on horseback riding in nesting areas through annual coordination with commercial and private equestrian operations and groups. Strategies to reduce adverse impacts to nests from commercial and private equestrian use of western snowy plover habitat should include: (1) use of designated trail systems or, when absent, use of the wet sand area in areas not closed to the water line; (2) advance coordination with local resource agencies regarding locations of nests and broods; (3) compliance with closed or restricted areas; and (4) informing riders of the need for restrictions to protect habitats used by western snowy plovers and other sensitive coastal dune species. Avoid high-tide periods. Violations regarding unauthorized entry into closed or restricted breeding areas by equestrians should be strictly enforced. Appendix C, Table C-1 identifies 72 locations where restriction or prohibition of horses currently exists or is recommended to achieve management goals.

2.3.7 Implement and enforce restrictions on livestock in nesting areas through annual coordination with land managers, landowners, and grazing lessees. Strategies to reduce adverse impacts to nests from livestock grazing in western snowy plover habitat should include: (1) advance coordination with local resource agencies regarding locations of nests and broods; (2)

compliance with closed or restricted areas; and (3) informing landowners of the need for restrictions to protect habitats used by western snowy plovers and other sensitive coastal dune species. Violations regarding unauthorized entry into closed or restricted breeding areas by livestock should be strictly enforced. Appendix C, Table C-1 identifies 18 locations where restriction or prohibition of livestock currently exists or is recommended to achieve management goals.

2.3.8 Enforce regulations in areas used by breeding western snowy plovers. Land managers should monitor violations and enforce regulations within all closed and restricted areas, with particular attention to areas where nests or broods are present.

2.3.8.1 Determine enforcement needs for western snowy plover breeding and wintering sites and provide sufficient wardens, agents, or officers to enforce protective measures in breeding and wintering habitat. Wardens are especially needed on heavily-used beaches during the peak recreational season, which coincides with the western snowy plover breeding season in many locations. Federal, State, and local authorities should provide a coordinated law enforcement effort to eliminate activities that may adversely impact western snowy plovers, such as illegally-parked vehicles, trespassing off-road vehicles, pedestrians, pets in restricted areas, illegal or unauthorized activities (*e.g.*, fireworks, beach fires, driftwood removal), pets off leash, and littering. Patrols and enforcement are needed to ensure compliance and to make sure restrictive measures are successful. Specific actions to be implemented include patrols in protected areas (see action 2.3.8.2) and car patrols to prevent illegal driving and parking. Appendix C, Table C-1 identifies 105 locations where

enforcement of regulations currently occurs or is recommended to occur to achieve management goals.

2.3.8.2 Develop and implement annual training programs for enforcement personnel and others who work in western snowy plover breeding habitat to improve enforcement of regulations and minimize effects of enforcement actions on western snowy plovers and their habitat. Federal, State, and local enforcement personnel and others who work in western snowy plover habitat should be trained to be familiar with the Endangered Species Act and other wildlife conservation statutes, and with the measures recommended in this recovery plan. Training, especially specific training for professional law enforcement agents regarding investigation of potential wildlife and Endangered Species Act violations, should be coordinated with local U.S. Fish and Wildlife Service Law Enforcement offices. It is essential that wardens, whether professional or volunteers, (1) be thoroughly trained in procedures for conducting patrols in a manner that minimizes risk to plovers; (2) have at least basic knowledge of western snowy plovers for public education purposes; and (3) be trained to handle potentially confrontational situations. *In cases involving take of listed species, it is essential that investigations be conducted only by trained, certified, and professional law enforcement agents.* Our local Law Enforcement office should be informed *immediately* whenever evidence of suspected take of western snowy plovers is encountered.

Enforcement personnel should be instructed in measures that can minimize effects of enforcement actions on western snowy plovers. Where the extent of habitat to be protected is large, making foot patrols infeasible, horses,

four-wheel all-terrain vehicles/off-road vehicles, or nonmotorized all-terrain bicycles, are preferred over trucks, automobiles, *etc*., because they afford improved visibility for operators. Except during emergencies, vehicle speed should not exceed 8 kilometers (5 miles) per hour and horses should be ridden at a walk only. In addition to providing maximum visibility for operators, horse and foot patrols by uniformed personnel have the added advantage of providing informational/educational interactions with beach visitors to promote compliance with plover protection measures.

Enforcement and emergency response personnel (such as search and rescue, and fire) should be well aware of potential western snowy plover locations. These locations should be named as avoidance areas as a part of their plans and training exercises. Enforcement patrols should use the same access trails as beach visitors; if additional access points are needed, they should be the minimum necessary and as far away from nesting plovers as possible.

2.3.9 Develop and implement a program to annually coordinate with local airports, aircraft operations, and agency aircraft facilities to facilitate compliance with aviation regulations regarding minimum altitude requirements. Each recovery unit working group should develop a list of local airports, aircraft operations, and agency aircraft facilities within each recovery unit. Working groups, land managers, and the U.S. Fish and Wildlife Service should annually inform them of western snowy plover breeding areas that should be avoided by aircraft operations or where minimum altitude requirements should be enforced to minimize disturbance of western snowy plovers. Aircraft operations within western snowy plover habitat should require a minimum altitude of 152 meters (500 feet) for aircraft and a possibly higher altitude for

helicopters. Aircraft operations that have already established guidelines allowing aircraft to fly under the 152-meter (500-foot) threshold should raise the limits to this minimum threshold or higher as needed. Exceptions such as use for low-altitude military training should be addressed in coordination with the appropriate Fish and Wildlife Office through section 7 consultation.

Ultralight aircraft are a new potential source for negative effects to the snowy plover. Ultralight aircraft landed on nesting plover beaches at Point Reyes National Seashore in 2003. These aircraft are sometimes associated with an airport but often are kept on ranches or other private lands (S. Allen *in litt.* 2004).

In addition, land managers should report suspected violations of aviation regulations in western snowy plover nesting areas during the breeding season. Suspected violations and the aircraft's registration number should be reported to law enforcement officers and, if appropriate, the Federal Aviation Administration. If not in violation of aviation regulations (*e.g.*, helicopters), a description of the helicopter should be reported to law enforcement officers so they can notify the operator of the presence of, and potential for take of, western snowy plovers in nesting areas.

2.4 Prevent excessive predation for western snowy plovers. Land managers should employ an integrated approach to predator management that considers a full range of management techniques. Managers may need to reevaluate and clarify their policies on the management of predator populations and/or habitat where predation might be limiting local western snowy plover populations. In particular, policies that prohibit management of native predator populations, even when human-abetted factors have caused substantial increases in their abundance, may be counter-productive to the overall goal of protecting "natural" ecosystems.

In addition to predator management activities by on-site biologists, assistance from the U.S. Department of Agriculture (Wildlife Services Branch) biologists, State wildlife agency furbearer biologists, biologists specializing in avian predators, and professional trappers should be sought and used as needed and appropriate. Federal, State, and local agencies and the general public should be aware of the adverse consequences to listed species if needed predator control measures are prohibited or restricted. Appendix C, Table C-1 identifies 61 locations where predator control currently occurs or is recommended to achieve management goals. Below are specific means of predator control.

2.4.1 Manage litter and garbage and its removal to minimize attracting predators on western snowy plover habitat. Litter and garbage in western snowy plover habitat may increase predation of western snowy plovers by providing food that attracts predators and encourages increased predator populations. Appropriate management of litter and garbage, particularly in areas that receive heavy recreational use, is needed to prevent or minimize excessive predation.

2.4.1.1 Implement and enforce anti-littering regulations. Litter should not be allowed in western snowy plover breeding areas to avoid attracting predators. Littering ordinances should be enforced year-round.

2.4.1.2 Evaluate the effects of current litter and garbage management on predation of western snowy plover at breeding and wintering sites. All sites in Appendix C should be evaluated to determine whether garbage and litter affect predation on western snowy plovers by attracting predators.

2.4.1.3 Develop and implement garbage and litter management plans for all sites identified in action 2.4.1.2 where litter and garbage contribute to

predation on western snowy plovers. Plans for managing litter and garbage should be incorporated into long-term protection and management efforts developed and implemented under action 3. Beachgoers should be discouraged from leaving or burying trash or food scraps on the beach. Trash cans should not be located on the beach unless there is no other recourse to prevent littering. Emptying cans in the evening instead of leaving them overnight is preferable. Fish-cleaning stations should be located well away from plover breeding areas. Land managers should supply covered or scavenger-proof trash receptacles at access points and away from western snowy plover habitat, and receptacles should be routinely emptied. Until predator-proof trash containers can be installed, existing trash cans should be emptied frequently to reduce attractiveness and availability of their contents to scavenging predators. Land managers should also provide toilets at access points and away from western snowy plover habitat to discourage people from using the dunes.

Although removal of trash from the beach reduces predation threats, beach-raking should be avoided year-round to protect breeding and wintering western snowy plovers (see action 2.3.5). Beach-raking of western snowy plover habitat also should be avoided because it removes plover food sources. Trash should be selectively removed from the beach manually, but natural materials, including shells, kelp, and driftwood, should be left intact (see action 2.3.4).

2.4.2 Annually identify predator perches and unnatural habitats attractive to predators and remove where feasible. Planners should not allow unnatural habitats or other predator attractants to be placed near western snowy plover nesting locations. Where

feasible, land managers should remove from western snowy plover breeding locations any exotic vegetation, perches, and other features that attract avian and mammalian predators. Where signs and fences are necessary as part of management to protect plover breeding areas, attempts should be made to design them in a way that will deter their use by predators (*e.g.*, install spikes on fence posts).

2.4.3 Erect predator exclosures to reduce western snowy plover egg predation and improve productivity (number of fledglings per male) where appropriate. Guidelines for the use of predator exclosures to protect nesting western snowy plovers are contained in Appendix F. Exclosures are a valuable tool for countering human-abetted predation threats to western snowy plover eggs, but they are not appropriate for use in all situations, nor do they provide any protection for mobile plover chicks, which generally leave the exclosure within one day of hatching and move extensively along the beach to feed. Exclosures should be used in conjunction with an integrated predator management program. Also, exclosures must be carefully constructed, monitored, and evaluated by qualified persons. In some areas, avian predators have learned over time to associate exclosures with a source of prey (J. Buffa *in litt.* 2004). String (twine) or a more substantial plastic stealth material may be needed on top of exclosures to deter avian predators. Appendix C, Table C-1 identifies 53 locations where exclosures are currently used or recommended for use to achieve management goals.

The use of exclosures (small circular, square, or triangular metal fences that can be quickly assembled) to deter predator and human intrusion is recommended as one of the most effective management tools to protect nests (see Appendix F for exclosure protocols). However, it should be recognized that while exclosures provide nest protection, they do not ensure survival of chicks to fledging age and may contribute to predation on adults,

so their use should be evaluated carefully and may not substitute for other measures that reduce human disturbance (2.3) or control predation (2.4.1, 2.4.2, 2.4.3, 2.4.5).

2.4.4 Evaluate the need for and feasibility of predator removal and implement removal where warranted. Where predators have been identified through monitoring to adversely affect western snowy plover breeding success and/or survival and cannot be adequately controlled through use of exclosures, land managers should evaluate the need for and feasibility of predator removal. Removal of predators should be pursued where it is feasible, warranted, humanely conducted, and useful. Situations that may especially warrant predator removal include those where nonnative predators such as red fox (*Vulpes vulpes regalis*), feral cats, and Norway rats (*Rattus norvegicus*) are present, where predators have been introduced to islands, where predator range extensions have been human-abetted, or where high rates of western snowy plover adult, chick, or egg predation (which cannot be countered with predator exclosures or other aversion methods) are occurring. Nonnative predators should be lethally controlled in plover nesting habitat. Native predators should be removed or controlled by nonlethal means whenever possible. Gulls also should be discouraged from establishing and expanding nesting colonies at western snowy plover nesting areas, and land managers should determine whether existing gull colonies warrant removal. If removal is not warranted, exclosures around plover nests should be used to prevent large flocks of roosting gulls from trampling plover nests.

Federal and State permits must be obtained to legally capture, kill, or hold and release birds protected under the Migratory Bird Treaty Act and State laws. Also, individuals responsible for capturing such birds and the holding facility must have the proper Federal and State permits, and Federal land managers must document that such activities are in compliance with the National

Environmental Policy Act. Biological considerations for determining whether removal of avian predators is appropriate include the time of year (to assess whether the predator is caring for young or is a fledgling itself), whether the predatory bird is a resident or migrating through western snowy plover nesting habitat, and whether the predatory bird is a sensitive species or listed under the Endangered Species Act. Because of the potential for swift and significant losses of plovers by avian predators, land managers should plan in advance to complete the necessary procedures and secure needed permits to effectively deal with cases of high negative impact on western snowy plovers. If feasible, removal of native predators should focus on problem individuals rather than populations. Possible control methods include egg addling, nest removal, translocation of problem individuals, and holding in captivity with later release after plover breeding season. State permits must also be obtained as appropriate for the capture and removal of problem mammals (*e.g.*, raccoons, skunks, and opossums). In 2001, the California Coastal Commission determined that predator management in western snowy plover habitat on Vandenberg Air Force Base was also subject to Coastal Consistency review under the Coastal Zone Management Act.

2.4.5 Remove bird and mammal carcasses in western snowy plover nesting areas. Where practical and not disturbing to western snowy plovers, dead birds and mammals that wash up on the beach in close proximity to plover nests should be removed to reduce the attraction of predators to plover nests. Removal of carcasses of marine mammals and species listed under the Endangered Species Act should be coordinated with the National Marine Fisheries Service and the U.S. Fish and Wildlife Service.

2.5 Protect western snowy plovers and their breeding and wintering habitat from oil or chemical spills. Land managers should develop oil/chemical spill emergency response plans that provide for protection of

known western snowy plover breeding areas. The U.S. Coast Guard should update their emergency response measures to include protective measures for the western snowy plover. In the event of a spill in the vicinity of a western snowy plover nesting or feeding area, efforts should be made to prevent oil/chemicals from reaching these beaches. Clean-up operations should be prompt, but agencies should exercise special care during remediation efforts and coordinate closely with us to prevent accidental destruction of nests and/or excessive disturbance of breeding adults, nests, or chicks. Response plans should include applicable recommendations contained in this recovery plan (*e.g.*, Action 2.3.5 regarding essential vehicles).

Efforts must be made to minimize the likelihood of oil or chemical spills in plover wintering areas. Land managers should develop oil/chemical spill emergency response plans that provide for protection of known plover wintering areas. The U.S. Coast Guard should update their emergency response measures to include protective measures for the western snowy plover. Shorebird or coastal ecosystem protection plans developed by State or local agencies to address oil/chemical spills should also include protection measures for western snowy plovers. In the event of a spill in a known western snowy plover wintering area, efforts should be made to prevent oil/chemicals from impacting plovers and unavoidable impacts should be documented. Restoration efforts should begin expeditiously, but agencies should exercise special care and coordinate closely with us to prevent excessive disturbance to wintering western snowy plovers. Further, habitat restoration efforts must be conducted in compliance with the National Environmental Policy Act and the Coastal Zone Management Act.

If western snowy plovers or their habitat sustain injury due to oil/chemical spills, the responsible parties should restore the areas to their original condition or the Federal Government (U.S. Coast Guard) should lead the clean-up effort; appropriate claims should also be filed under the Natural Resource Damage Assessment regulations to recover damages and undertake relevant restoration work. Assessment of natural resource

damages is facilitated by availability of baseline data on pre-spill conditions. Therefore, whenever possible, agencies that own or manage western snowy plover habitat should collect baseline data on behavior, reproduction, distribution, abundance, and habitat use. The baseline information on plover distribution and habitat use should also be supplied to the Area Committees that develop and update regional spill contingency plans so that this information can be incorporated into pre-spill planning efforts for protection of sensitive environments and species. Oil spill emergency response personnel should be well aware of potential plover locations. These locations should be named as avoidance areas as a part of their training exercises. Appendix C, Table C-1 identifies 4 locations where contaminant removal is occurring or is recommended to achieve management goals.

2.5.1 U.S. Fish and Wildlife Service biologists should participate in Area Committees responsible for maintaining the Area Contingency Plans for the Pacific Coast to facilitate the updating of spill response plans to include protection of western snowy plovers. Active participation in the Area Committees would require funding for staff participation from the six U.S. Fish and Wildlife Service offices responsible for the coastlines of California, Oregon and Washington.

2.5.2 Assign monitors to beaches that are inhabited by western snowy plovers to protect western snowy plovers from injury during spill responses. Monitors would be responsible for identifying areas of beach that are in use by plovers and directing response personnel and vehicles around these sensitive areas. Potential monitors should be identified in advance, and, where necessary, retained under contract so they can begin work immediately in the event of a spill. Spill response may require approximately two weeks of cleanup work that should be monitored, with potentially five incidents of this magnitude per year.

2.6 Reduce adverse impacts of recovery efforts for other sensitive species, including those within the San Francisco Bay Recovery Unit, by compensating for the loss of western snowy plover breeding and wintering habitat. Management and recovery actions for other sensitive species carried out in western snowy plover habitat should be evaluated for adverse effects to western snowy plover habitat. All efforts should be made to conserve western snowy plover habitat and minimize adverse effects. Where this is not possible, any loss of western snowy plover habitat values should be compensated. Within coastal beach-dune habitats in Washington, Oregon, and California, compensation efforts should emphasize the removal of beachgrass (*Ammophila* spp.) for lost western snowy plover breeding habitat resulting from management for other sensitive species.

To compensate for the loss of existing western snowy plover breeding habitat values in San Francisco Bay from planned conversion to tidal marsh, appropriate salt ponds should be designated for protection and enhancement as western snowy plover breeding habitat. Currently, most western snowy plover breeding habitat occurs on levee roads, margins of active salt ponds, and pond bottoms of inactive salt ponds. Roads and levees provide lower quality habitat because of disturbance and ease of predator access. Any losses of western snowy plover breeding habitat should be replaced with habitat that provides similar or higher values (*i.e.*, salt ponds or salt pans) in concert with recovery actions implemented from the Recovery Plan for Tidal Marsh Ecosystems of Northern and Central California (U.S. Fish and Wildlife Service in prep.). Habitat enhancement for western snowy plovers should be phased in with scheduled tidal marsh restoration for other listed species. During this interim period, land managers should make all efforts to achieve the recovery criteria of 500 breeding adults within the San Francisco Bay Recovery Unit by intensively managing existing western snowy plover breeding habitat.

Any replacement of western snowy plover breeding habitat in San Francisco Bay should concentrate on areas where the necessary

components of western snowy plover breeding habitat can be created. These areas include locations where unvegetated salt pans, salt ponds, islets and levees, and tidal mudflats/sandflats can be created or enhanced. Also, attempts should be made to avoid areas that are adjacent to landfills or other high concentrations of potential predators. Unless it is shown to be infeasible, creation and enhancement of western snowy plover breeding habitat should be emphasized in areas that currently support high numbers of breeding plovers and/or are not conducive to salt marsh restoration. The area to be managed for western snowy plovers should be sufficient to support a population of 500 breeding birds, estimated at 809 hectares (2,000 acres) of managed salt ponds. Most of these managed salt ponds should be located in South San Francisco Bay, which supports most of the existing western snowy plover population; however, some should also be located in the North Bay. Created or enhanced salt ponds should be intensively managed, similar to the Moss Landing Wildlife Area salt ponds. Management measures practiced at these salt ponds include maintenance of water control structures to maintain desired water levels, removal of excessive vegetation, and predator control.

2.7 Discourage pinnipeds from usurping western snowy plover nesting areas. Land managers should monitor pinniped colonies adjacent to western snowy plover breeding habitat and seek to keep breeding pinnipeds from occupying western snowy plover nesting areas during the breeding season where possible. Where conflicts occur, breeding pinnipeds should be discouraged from hauling out at western snowy plover breeding areas or be relocated, if feasible. Implementation of this action should be coordinated with the National Marine Fisheries Service to ensure compliance with the Endangered Species Act of 1973 and the Marine Mammal Protection Act of 1972 (16 U.S.C. 1361 *et seq.*).

2.7.1 In coordination with National Marine Fisheries Service, investigate feasibility and methods for discouraging pinniped use of western snowy plover nesting areas. Marine mammal populations have increased in many western snowy plover nesting areas. However, methods, effectiveness, and impacts of

discouraging pinniped use of beaches are unknown and should be investigated. Methods considered should be evaluated for their effects on western snowy plovers and their habitat as well as effectiveness in discouraging pinniped use. Workshops, such as those conducted by NMFS, for developing methods to reduce conflicts between pinnipeds and other species and human users should be held.

2.7.2 Identify areas where pinniped use is negatively affecting western snowy plover nesting and implement any appropriate methods identified in action 2.7.1. If effective methods are determined through action 2.7.1, sites where pinniped use negatively affects western snowy plover nesting should be identified and methods to discourage pinniped use implemented. Implementation of any methods to discourage pinniped use should be closely coordinated with the National Marine Fisheries Service to ensure compliance with the Endangered Species Act of 1973 and the Marine Mammal Protection Act of 1972 (16 U.S.C. 1361 *et seq.*).

3 Develop mechanisms for long-term management and protection of western snowy plovers and their breeding and wintering habitat. Long-term management and protection will be needed on Federal and non-Federal lands to meet recovery criteria for each recovery unit and to meet management goals for individual breeding and wintering locations. Development of long-term protection mechanisms should include opportunities for participation of various stakeholders in development of management options.

3.1 Establish and maintain western snowy plover working groups for each of the six recovery units to facilitate regional cooperative networks and programs. Development of regional cooperative networks and programs, coordinating local public and private land use planning with State and Federal land use planning, recovery planning, and biodiversity conservation is needed (Figure 12). To facilitate and develop regional cooperative programs, working groups have been established for each of

the six recovery units and should be maintained. U.S. Fish and Wildlife Service field offices should facilitate exchange of information among working groups. The working groups should be composed of representatives from the Federal, State, local, and private sectors; and meet regularly to assess western snowy plover population trends and coordinate plover recovery efforts. Each of the six working groups should use this recovery plan as a guide, but members will prioritize in cooperation with our Arcata Fish and Wildlife Office what management measures need to be implemented in their recovery unit because they have on-the-ground, day-to-day, experience about what is currently being done in these areas. Working groups should assist with updating information contained in Appendices B and C, tracking whether management goals are being met, and recommending changes in management goals and site-specific management actions, if necessary. Public outreach also should be a major focus of the working groups. An interchange of ideas between all six working groups should also occur on an on-going basis.

3.2 Develop and implement regional participation plans for each of the six recovery units that outline strategies to implement recovery actions. The 1994 Interagency Cooperative Policy on Recovery Plan Participation and Implementation Under the Endangered Species Act (U.S. Fish and Wildlife Service and National Oceanic and Atmospheric Administration 1994) provides for a participation plan process, which involves all appropriate agencies and affected interests in a mutually-developed strategy to implement recovery actions. Participation plans for implementing recovery actions for the western snowy plover that include all partners should be developed by each of the six recovery unit working groups. In addition to outlining a strategy to implement recovery actions, the participation plan should include strategies for evaluation of progress and needs for plan revision. Participation plans may also achieve the policy's goal of providing for timely recovery of species while minimizing social and economic impacts. Plans should identify and prioritize specific recovery activities for each location identified in Appendices B and C,while considering the needs of the entire Pacific coast population. They

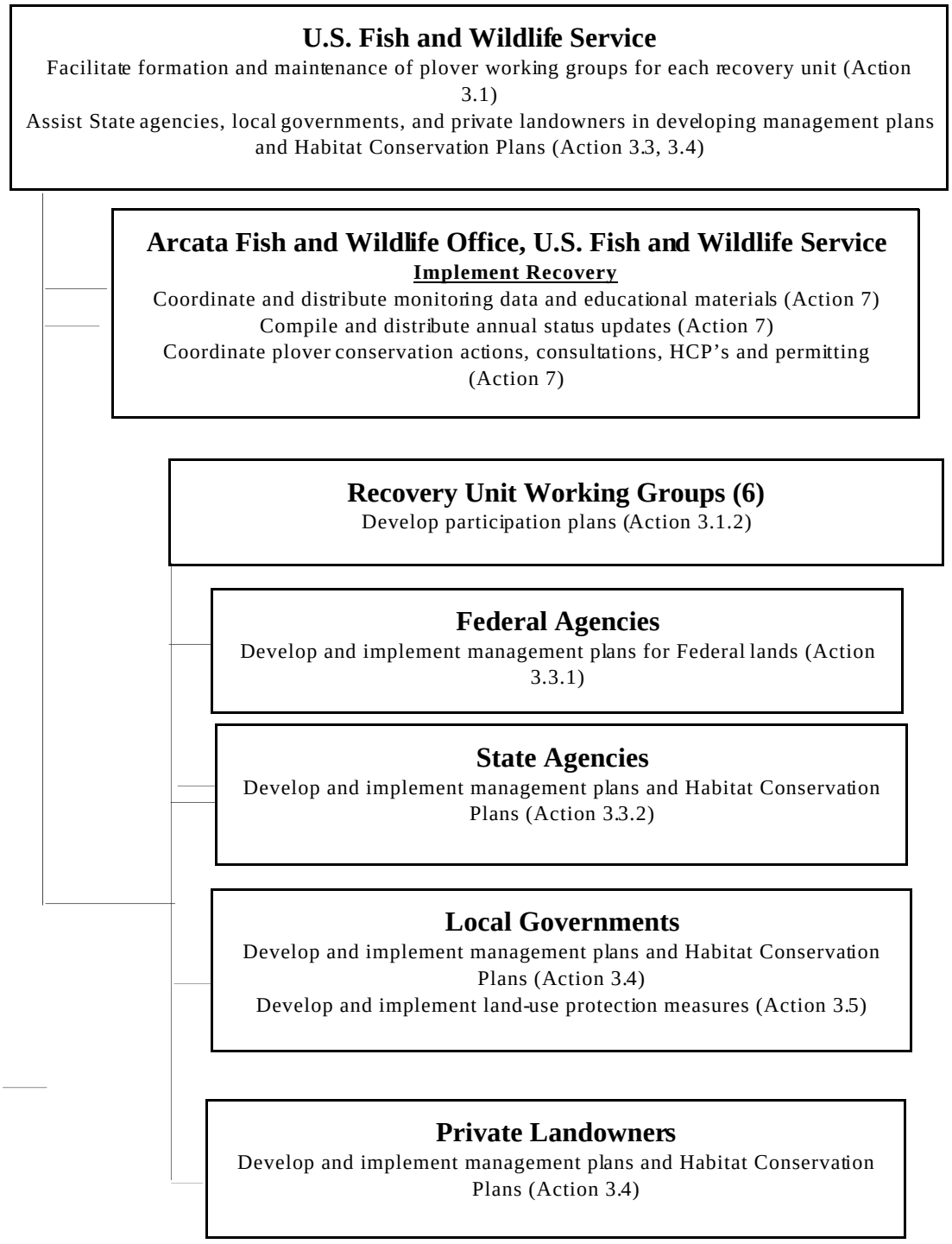


Figure 12. Chart of recovery planning and implementation efforts.

should include, but not be limited to: (1) endorsements by responsible agencies of their intent to seek economic resources for ongoing recovery actions; (2) outreach efforts to enhance the public's understanding of the western snowy plover's habitat needs (including an information and education strategy specific to area demographics and recreational activities); (3) economic incentives for conservation of western snowy plovers on private lands; and (4) all actions necessary to maintain western snowy plover productivity after delisting. Participation plans may also identify ways in which recovery actions for western snowy plovers will be covered as part of coastal ecosystem plans or other conservation measures.

3.3 Develop and implement management plans for all Federal and State lands to provide intensive management and protection of western snowy plovers and their habitat. Federal and State land managers should develop and implement management plans for all breeding and wintering locations (listed in Appendix B) that occur on Federal or State lands. Intensive management programs for western snowy plovers at national wildlife refuges should be implemented and annually evaluated to ensure they provide sufficient plover protection. Intensive management programs also should be implemented and periodically evaluated on lands administered by the National Park Service, U.S. Forest Service, U.S. Bureau of Land Management, U.S. Army Corps of Engineers, and Federal military bases, State wildlife areas, State ecological reserves, and State park lands (including State natural preserves and State seashores).

3.3.1 Develop and implement management plans for Federal lands. Federal agencies should develop or update, as appropriate, site-specific management plans that address threats to western snowy plovers, and adopt management measures for habitat protection and enhancement on Federal lands. Management plans should be implemented on an ongoing basis. Federal agencies also should review their proposed actions under the requirements of sections 7 and 10 of the Endangered Species Act prior to implementing the management plans because they may require authorization under section 7(a)(2) or 10(a)(1)(A).

3.3.2 Develop and implement management plans and habitat conservation plans on State wildlife areas, State ecological reserves, and State beaches. State agencies that manage State beaches, wildlife areas, or ecological reserves should develop and implement site-specific management plans and habitat conservation plans to minimize and mitigate impacts to western snowy plovers, and management measures for habitat protection and enhancement on State lands. State agencies should coordinate the development of habitat conservation plans with us and apply for section 10(a)(1)(B) permits under the Endangered Species Act if their management actions and allowed uses are resulting in incidental take of western snowy plovers.

3.4 Develop and implement habitat conservation plans or other management plans for western snowy plover breeding and wintering sites owned or managed by local governments and private landowners. We should provide assistance in the development of habitat conservation plans or other management plans to: (1) county and city governments that manage western snowy plover habitats; (2) private resource managers; and (3) owners of large amounts of private natural land. Habitat conservation plans are only required if an incidental take permit under section 10(a)(1)(B) of the Endangered Species Act is desired or required.

3.5 Provide technical assistance to local governments in developing and implementing local land use protection measures through periodic workshops. Federal and State agencies should assist local governments with jurisdiction over western snowy plover habitats in developing western snowy plover protection policies as part of new or revised local general plans, zoning policies, implementing measures, land use plans, comprehensive plans, and local coastal programs. For areas where beach closures are necessary, appropriate ordinances, administrative rules, and regulations should be developed by State and local governments to enable law enforcement officers to conduct necessary enforcement actions.

Technical assistance such as maps of western snowy plover habitats, identification of local threats, and recommended site-specific protective measures should be provided to coastal planners. At least two workshops within each recovery unit that provide local governments with basic information on the western snowy plover, its habitats, threats, and recommended protective measures should be conducted during the first 10 years of recovery plan implementation. Additional technical assistance likely will be required but should be provided on an as needed basis as new or revised general plans, policies, ordinances, and other land use protection measures are developed.

3.6 Develop and implement cooperative programs and partnerships with the California State Coastal Commission, the Oregon Department of Land Conservation and Development, the Washington State Parks and Recreation Commission, the Oregon Parks and Recreation Department, the California Department of Parks and Recreation, and the Oregon Department of Fish and Wildlife to ensure that they use their authorities to the fullest extent possible to promote the recovery of the western snowy plover. Federal and State agencies should assist the California State Coastal Commission, Oregon Department of Land Conservation and Development, Washington State Parks and Recreation Commission, Oregon Parks and Recreation Department, California Department of Parks and Recreation, and Oregon Department of Fish and Wildlife in reviewing, updating, and amending local coastal programs and policies for consistency with the western snowy plover recovery plan. This review should include protection of western snowy plover habitats, cumulative impacts to western snowy plovers, and policies or restrictive measures recommended in this recovery plan.

3.7 Obtain long-term agreements with private landowners. Agreements between Federal and State agencies and private landowners interested in western snowy plover conservation should be developed and implemented. Landowners should be informed of the significance of plover populations on their lands and be provided with information about available conservation mechanisms, such as agreements and incentive

programs. For private lands with potential occurrences of western snowy plovers, permission should be sought from landowners to conduct on-site surveys. If surveys identify plover populations, landowners should be informed of their significance and offered incentives to continue current land uses that support species habitat. Appendix C, Table C-1 identifies 69 locations where landowner cooperation/cooperative agreements are occurring or are recommended to achieve management goals.

3.8 Identify and protect western snowy plover habitat available for acquisition. Federal, State, and private conservation organizations should protect western snowy plover habitat as it becomes available, through fee title or conservation easement, *etc.* We and other organizations should identify sites that may become available for acquisition, and we should continue to evaluate excess Federal lands for western snowy plover habitat and apply to acquire them as they become available. Each recovery unit working group should develop a list of priority properties for acquisition, and Federal, State, and nongovernmental organizations should work with land conservancy groups to implement land trades and acquisitions. Management plans for the western snowy plover should be developed during the land acquisition process.

3.9 Ensure that section 10(a)(1)(B) permits contribute to Pacific coast western snowy plover conservation. Recommendations contained in this recovery plan should guide the preparation of habitat conservation plans under section 10(a)(1)(B) of the Endangered Species Act for western snowy plovers on the Pacific coast by providing information to: (1) guide potential applicants in developing plans that minimize and mitigate the impacts of take and (2) assist us in evaluating the impacts of any proposed conservation plans on the recovery of the Pacific coast western snowy plover population. The section 10(a)(1)(B) permit process may be a valuable mechanism for developing the long-term protection agreements called for in Actions 3.3.2 and 3.4, especially where significant population growth has already occurred and productivity exceeds l.0 fledged chick per male.

3.10 Ensure that consultations conducted pursuant to section 7 of the Endangered Species Act contribute to Pacific coast western snowy plover conservation. The recovery plan should also guide the evaluation of impacts to western snowy plovers pursuant to section 7(a)(2) of the Endangered Species Act. In evaluating these impacts, we and other Federal agencies should consider each of the breeding and wintering locations listed in Appendix B as important for recovery, and should also refer to the management goal breeding numbers for applicable locations and determine how the proposed project will affect those goals. Coordination with military bases which have western snowy plover populations is important to ensure that military activities do not affect the western snowy plovers or their habitat. Appendix C, Table C-1 identifies 54 locations where military uses are either restricted or recommended for restriction to achieve management goals.

4 Undertake scientific investigations that facilitate recovery efforts. Major gaps remain in our understanding of useful protection measures and conservation efforts for the western snowy plover. These include effective methods for habitat restoration, predator control, and monitoring population numbers and demographic characteristics.

4.1 Investigate effective methods for habitat restoration.

4.1.1 Evaluate the effectiveness of past and ongoing methods for habitat restoration by removal of introduced beachgrass and identify and carry out additional investigations necessary. Land managers, in coordination with recovery unit working groups, should summarize methods used to date for removal of introduced beachgrass and review their effectiveness. They also should pursue any additional field studies necessary to determine the most effective and cost-efficient methods for habitat restoration through removal of introduced beachgrass. Controlled studies with improved monitoring would provide needed direction for management decisions.

4.1.2 Evaluate the impacts and potential benefits of past and ongoing beach nourishment activities and identify and carry out any additional studies necessary to determine effects of beach nourishment activities on western snowy plover habitat. Beach nourishment activities should be carefully evaluated to weigh the probable adverse and beneficial effects on plovers and on other sensitive coastal dune species. Pre- and post-deposition beach profiles and faunal studies (including invertebrates) should be conducted to determine effects on habitat suitability for western snowy plovers. Consideration should be given to whether the projected long-term benefits are likely to occur.

4.2 Develop and test new predator management techniques to protect western snowy plover nests and chicks. Because many of the techniques currently used to reduce predation have disadvantages or limitations in effectiveness, new predator management techniques should be investigated. Assistance from the U.S. Department of Agriculture, Wildlife Services Branch, from State wildlife agency furbearer biologists, and other predatory bird and mammal specialists should be sought on these matters.

4.2.1 Develop higher-efficiency nest exclosures. Because exclosures must be deployed quickly, and currently-designed exclosures are heavy and labor- and time-intensive to erect, new exclosure designs should be tested. Prototypes should include lightweight materials that are easier to transport and a design that is easy to assemble and install.

4.2.2 Develop California least tern exclosures that prevent harm to western snowy plovers. Resource managers should continue to investigate modified designs for California least tern enclosures to further minimize western snowy plover mortality.

4.2.3 Identify, prioritize, and carry out needed investigations on control of native and nonnative predators. Aspects of the

ecology of problematic avian predators (*e.g.*, ravens and shrikes) and native mammals (*e.g.*, coyotes and gray foxes) that could be used to gain an understanding of how to control their impact on western snowy plover nesting areas during the plover breeding season should be investigated. Information also is needed on the applicability and usefulness of other control methods, including aversive techniques for conditioning predators to avoid foraging in western snowy plover nesting areas or preying on western snowy plover eggs, chicks, or adults. Investigation is also needed to develop methods to discourage gull colonies. Aversive techniques may include taste aversions, displaying predator carcasses, or installing electric fences. Effective modifications of signs and fencing to prevent their use as predator perches also requires investigation. While in many cases there appear to be practical obstacles to development of effective aversion techniques that can be efficiently applied in the field, the goal of reducing predation with minimum disruption to native predator populations that are important to overall ecosystem balance is desirable and any methods that appear potentially practical and useful should be evaluated for success and cost-effectiveness. Initial study trials might be done at sites or seasons where western snowy plovers are not present in order to minimize unplanned adverse impacts. Recovery unit working groups should identify and prioritize studies needed and inform us of their recommendations.

4.2.4 Identify, prioritize, and carry out needed investigations on predator management at the landscape level. Resource managers should investigate landscape-level management of predators that inhabit western snowy plover nesting areas. This management could include removal of predator nest sites and other predator attractants or habitat on lands surrounding western snowy plover breeding areas. Recovery unit working groups should identify and prioritize studies needed and inform us of their recommendations.

4.2.5 Investigate techniques for identifying predators responsible for individual nest predation events. Techniques should be developed to identify predators responsible for nest predation events so that appropriate management measures can be applied. Such techniques could include installation of a remote video camera to monitor western snowy plover nests and exclosures and identify problematical predators.

4.3 Improve methods of monitoring population size and reproductive success of western snowy plovers. Methods used to monitor western snowy plover populations have differed over time and from site to site. To measure progress toward recovery reliably, standard monitoring guidelines have been developed (Appendix J). Logistical and financial constraints likely will preclude complete coverage of all areas, so sampling methods should be developed.

4.3.1 Improve methods of monitoring western snowy plover population size. Not all western snowy plovers at a given location are detected during a single survey, such as the annual breeding-season window survey. Consequently, correction factors are necessary to extrapolate population size from window surveys. Correction factors are determined on a site-specific basis. Intensive monitoring and/or color banding make it possible to know the number of western snowy plovers present at a site. When a window survey is completed, the ratio of the total number of western snowy plovers to the number of western snowy plovers counted provides a correction factor that may be used for future window surveys of the site and for other sites with window surveys but without intensive monitoring. Site-specific correction factors should be obtained for all major nesting locations. When correction factors have been determined for many sites, patterns may emerge that allow correction factors to be applied more broadly.

4.3.2 Develop sampling methods for annually estimating reproductive success within each recovery unit. While it is extremely valuable to monitor clutch hatching success and chick fledging success at each site as a measure of habitat quality, it is critical to determine the number of young fledged per male for each recovery unit to measure the potential for population stability and growth. Measuring the number of young fledged per male requires intensive monitoring, and at sites with large numbers of birds, some method of identifying individual males. Extensive color banding of adults and their young, enabling determination of young fledged per male, has been undertaken in large portions of coastal Oregon, the shoreline of Monterey Bay, and coastal San Diego County for the past several years. These efforts should continue. Since there are insufficient color band combinations to monitor all individuals in every recovery unit, sampling procedures should be developed to color band adequate samples of males, and if necessary their chicks, in the other recovery units to obtain estimates of the number of young fledged per male. Color banding for measuring reproductive success should be integrated with banding for estimating population size.

4.3.3 Develop methods to monitor western snowy plover survival rates within each recovery unit. Extensive color banding of adult plovers and their young in coastal Oregon, the shoreline of Monterey Bay, and coastal San Diego County has enabled survival rates of adults and young to be calculated for several years (see Population Status and Trends and Survival sections). These efforts should continue. Information on survival rates of birds from other recovery units can be derived from birds banded for monitoring reproductive success or estimating population size.

4.4 Conduct studies on western snowy plover habitat use and availability.

4.4.1 Identify western snowy plover brood habitat and map brood home ranges. Brood movements should be mapped and distances

quantified to identify how large an area must be protected for broods. Determine home ranges of western snowy plovers through radio telemetry studies. Traditionally used brood habitat should be identified and protected through actions 2 and 3.

4.4.2 Identify components of high-quality western snowy plover brood rearing habitat. The elements of high-quality brood habitat should be determined to facilitate creation and enhancement of suitable characteristics at other breeding locations.

4.4.3 Quantify wintering habitat needs of western snowy plovers along the Pacific coast. The amount of habitat needed to support wintering western snowy plovers along the Pacific coast should be determined. This effort should include estimating the numbers of western snowy plovers that can be supported at wintering locations listed in Appendix B and identifying important site characteristics. This action will require consideration of wintering habitat quality along the Pacific coast of the United States and Mexico, and quantifying the combined interior and coastal populations.

4.4.4 Identify any important migration stop-over areas used by migrating but not by breeding or wintering western snowy plovers. Additional information on western snowy plover migration patterns is needed because migration involves expenditure of energy that may affect survival or productivity. Although monitoring and protection of breeding and wintering locations are currently higher priorities than protection of migration sites, further investigations of, and protective measures for, migration sites should be undertaken when feasible. Threats and management needs of identified migration stop-over habitat should be evaluated and included in management monitoring, and protection tasks (see action 1.6).

4.5 Develop and implement a research program to determine causes of adult western snowy plover mortality, including investigation of possible causes, magnitude, and frequency of catastrophic mortality. Determine causes of mortality and the stage in the annual cycle (*e.g.*, post-breeding, migration, winter, pre-breeding, breeding) at which mortality occurs for each sex and age class. This assessment can be done through intensive, bi-weekly monitoring to determine relative health and potential for disease. Monitoring could include fat content and weight related to the season.

4.6 Improve techniques for banding western snowy plovers. Improve the technique for banding birds to reduce injuries. Because western snowy plover injuries are usually associated with Federal metal bands but not with plastic bands, removal of U.S. Fish and Wildlife Service lettering from the inside of the metal band should be investigated. Eliminating use of the U.S. Fish and Wildlife Service metal band also should be considered. Experimentation with new techniques must be conducted cautiously and may need to include pre-testing on nonlisted surrogate species.

4.6.1 Compile information regarding number and types of banding injuries to western snowy plovers to determine extent and causes of banding injuries. Several banding injuries to western snowy plovers have been reported. However, there is currently no consistent reporting of injuries to determine the extent or types of injuries. Working groups should compile information on banding injuries to use in determining the type and extent of the problem and in developing a course of action. Information collected should include number of injuries, type of injury (abrasion, foot loss, broken leg, *etc.*), probable cause of injuries (foreign object lodged between band and leg, wearing of band, *etc.*), effect of injuries on behavior (breeding, foraging, predator avoidance), type of bands (plastic or metal) associated with injuries, whether metal bands had writing on the inside or other rough areas likely to cause abrasion or lodging of foreign object.

4.6.2 Review compiled information and determine and implement a appropriate course of action to minimize banding injuries. The information complied in step 4.6.1 should be reviewed to determine the appropriate course of action to minimize banding injuries. Review may reveal that banding injuries are rare or have little impact on breeding success or survival, in which case no changes to banding procedures may be necessary. However, extensive numbers of injuries or impacts on breeding success and survival may require actions such as changing the location of metal bands from the tarsus to tibiotarsus, discontinuing use of metal bands, or using different band types. All decisions regarding changes to banding procedures should consider effects of such changes to the type, quantity, and quality of data that may be gathered from banding efforts, and whether such changes will affect the ability to determine population trends, monitor success of management actions, or otherwise affect recovery efforts. For example, discontinuing use of metal bands may affect the ability to gather information on survival, longevity, and dispersal useful in analyzing population viability.

4.7 Identify effects of oil spills on western snowy plovers. Research should be conducted on the direct and indirect effects of oil spills on western snowy plovers, including, but not limited to: (1) how oil spills affect the plover's prey base; (2) chronic effects of oiling; (3) transmission of oil on partially-oiled birds from the breast to the egg; (4) at what stage oiled plovers need to be captured or re-captured; (5) preferable methods to remove oil from soiled birds; and (6) impacts to plovers during oil clean-up and remediation activities.

4.8 Monitor levels of environmental contaminants in western snowy plovers. When abandoned eggs and/or dead chicks that are not needed for law enforcement investigations become available, they should be collected for potential contaminants assessment. Egg removal and salvage of dead chicks should only be done by individuals possessing proper Federal and State authorizations. Chemical analysis of salvaged specimens should be

coordinated through our Division of Environmental Contaminants. All salvaged eggs should be analyzed for organochlorine pesticides, total polychlorinated biphenyls (PCB's), selenium, mercury, and boron.

All sampling should be opportunistic, based on availability of eggs that are known to be abandoned. Eggs should never be removed from the beach as long as there is any realistic chance that they might hatch. In the case of unhatched eggs from a partially hatched clutch, eggs should not be collected until at least 36 hours after the known hatch date of the other eggs. Full clutches should not be collected unless it is known that 35 or more days have elapsed since the last egg was laid. When this opportunistic sampling of failed eggs indicates potential problems with contaminants, follow up studies should be carried out (see action 4.9).

4.9 Design and conduct contaminants studies if monitoring of contaminants in action 4.8 indicates potential contaminants effects. When opportunistic sampling of failed eggs (action 4.8) indicates potential problems with contaminants, additional studies should be carried out to evaluate the extent of contamination in western snowy plover diets, its effects on nest success and egg hatchability, and its effects on various life stages of snowy plovers (eggs vs. adults). Thresholds when management action is required should be identified. When the target threshold is exceeded research should be conducted to identify the source.

4.10 Identify, prioritize, and carry out needed investigations of the effects of human recreation on western snowy plovers. Many studies on the effects of recreational activities on western snowy plovers have already been conducted. To avoid duplicating previous or ongoing efforts, recovery unit working groups should evaluate and prioritize additional study needs to determine the effects of human recreation on western snowy plover. Western snowy plover should be monitored for effects from recreational activities such as off-road vehicle riding, horseback riding, walking, jogging, fishing, aircraft, ultralight aircraft, and kite-flying.

4.11 Revise the population viability analysis (Appendix D), if needed, when sufficient additional information on demographic characteristics (survival rates, reproductive success) is available from each recovery unit and information is obtained on the probability and magnitude of catastrophic mortality events. As new information on population numbers, survival rates, and reproductive success are acquired from monitoring (actions 1.1 and 1.2), monitoring techniques are improved (action 4.3), and mortality sources and rates of mortality are determined (action 4.5), the population viability analysis should be reviewed and revised if additional information differs significantly from that used to construct the original analysis.

5 Undertake public information and education programs. Expanded efforts are needed to increase public awareness of the needs of western snowy plovers, other rare beach species, and the beach and dune ecosystem. Public outreach efforts should be a major focus of each of the working groups for the six recovery units. Appendix C, Table C-1 identifies 84 locations where public information and education is either currently occurring or is recommended to achieve management goals.

5.1 Develop and implement public information and education programs. Millions of beach recreationists come in contact with western snowy plover nesting and wintering areas each year. Disregard to signs, symbolic fencing, and leash laws by beach users can directly affect the productivity and health of western snowy plovers on those beaches. Public information and education efforts play a key role in obtaining compliance of beach recreationists with plover protection measures that, in turn, affect the birds' recovery. Central messages to the beach-going public include: (1) respect areas fenced or posted for protection of plovers and other rare beach species; (2) do not approach or linger near western snowy plovers or their nests; (3) if pets are permitted on beaches used by plovers, keep the pets leashed; (4) don't leave or bury trash or food scraps on beaches, as garbage attracts predators that may prey upon plover eggs or chicks; and (5) do not build wood structures that can be used as predator perches.

Because of the importance of information and education for the western snowy plover recovery effort, as part of this recovery plan, we developed an Information and Education Plan for the Western Snowy Plover, Pacific coast population (Appendix K).

5.2 Inform Federal, State, and local resource/regulatory agencies and local planning departments of threats to breeding and wintering western snowy plovers and their habitats. Periodic meetings and/or workshops should be held to inform Federal, State, and local resource management and regulatory agencies, and city and county planning departments about threats, research, and management needs for plovers. A network of public agency staff from each of the six recovery unit working groups should develop a coordinated approach to present this information to these agencies periodically, or as needed.

5.3 Develop and maintain updated information and education materials on western snowy plovers. Members of the six recovery unit working groups should develop new western snowy plover information and education materials for target audiences to stimulate public interest and awareness. In addition, all materials should be kept reasonably current regarding the status of the species and protection efforts. These materials should also explain the need for conservation of the beach and dune ecosystem and the plight of other rare beach-dwelling species. Videos detailing needed western snowy plover recovery actions by location and recovery unit should be developed, and might be efficiently produced in conjunction with updated public service advertisements.

5.4 Alert landowners and beach-goers about access restrictions within western snowy plover habitats. Land managers should begin providing informational and educational outreach at least 2 weeks prior to the onset of the nesting season to provide beach-goers and interested landowners with advance notice of impending restrictions on publicly-owned western snowy plover breeding habitats. This outreach is particularly important for the first year of restrictions. If necessary,

follow-up publicity that includes information on citations issued to violators should be implemented to help reinforce the message.

5.5 Provide trained personnel to facilitate protective measures, provide public education, and respond to emergency situations. Biologists, docents, volunteers, and other personnel should be trained to patrol western snowy plover nesting areas to monitor birds, distribute educational materials, respond to emergency situations, and ensure that beach-goers stay out of fenced areas and adhere to other plover protection measures. Biologists engaged in monitoring, management, or research activities should also advance the public's understanding of plover management needs.

5.6 Develop protocols for handling sick, displaced, injured, oiled, and dead birds or salvaged eggs. Land managers within each recovery unit should develop protocols for all trained personnel identifying who should be contacted when injured, dead, oiled, or displaced birds are found, and who is permitted to handle these birds. Federal and State salvage permits are necessary for the disposal of dead birds and the transportation of injured birds. Federal and State endangered species permits are necessary for wildlife rehabilitators to accept and care for injured and sick birds. Coordination with biologists that are monitoring and banding western snowy plovers is essential for capture and release of injured/rehabilitated birds. Live chicks that are found should not be moved or taken for rehabilitation as these chicks are often not abandoned, even though plover adults may not be obvious at the time the chicks are seen. Protocols should also be developed on how to collect and preserve salvaged eggs used for contaminants analysis.

5.7 Establish a distribution system and repository for information and education materials. Land managers must distribute information and education materials to target audiences. To reach the large population of potential beach-goers within a few hours' drive of many major metropolitan areas, broad-scale information and education mechanisms should be implemented, including distribution by mass media such as

newspapers, radio and television announcements, and internet web sites. Land managers should also focus their information and education efforts on user groups at beach parking lot entry stations and kiosks, visitor centers, marinas, beach-front housing developments, equestrian and angler access points, and locations providing off-road vehicle permits. Public outreach efforts should be directed to groups within the geographical location of the managed beaches (*e.g.*, to private and commercial equestrian users) and to groups outside of the area who use the beaches on a regular or seasonal basis (*e.g.*, to off-road vehicle associations from out-of-state or inland locations). Land managers, with the help of docents and volunteers, should coordinate with local school teachers to develop and present environmental education lesson plans and participatory activities for elementary and middle school groups.

We will act as a central repository for current and new information and education materials received; upon request, we will make these materials available to recovery unit working groups and the general public. We will also maintain information on western snowy plovers at our website (http://www.fws.gov/arcata). Major distributional efforts should also continue by Federal, State, and local agencies, and private conservation organizations.

5.8 Establish a reporting and distribution system for annual monitoring data and management techniques. Our Arcata Fish and Wildlife Office should coordinate and produce an annual report of submitted breeding and wintering monitoring data and distribute it to recovery unit working groups. This report should describe results of monitoring throughout the western snowy plover population's range. A distribution system should also be established for sharing information on predator management techniques, nest protection, etc. among working groups.

6 Review progress towards recovery and revise recovery efforts as appropriate. Communication, evaluation, and coordination play a major role in western snowy plover recovery efforts. Land managers within each of the six recovery unit working groups should review the effectiveness of their

management activities in coordination with other members of their working group, and revise management measures as appropriate. They should also provide results of annual population monitoring and the effectiveness of management activities to their working group and to our Arcata Fish and Wildlife Office.

6.1 Develop and implement a tracking process for the completion of recovery actions and the achievement of delisting criteria. A tracking process should be developed to track the completion of recovery actions and progress toward delisting. Utilizing information from specific actions, the recovery criteria such as the implementation of management activities can be tracked. Information from the tracking process can be used in outreach and in helping identify when the western snowy plover can be delisted.

6.2 Review progress toward recovery annually within each recovery unit working group and revise site-specific recovery efforts as appropriate to meet recovery goals. Communication, evaluation, and coordination play a major role in western snowy plover recovery efforts. Land managers within each of the six recovery unit working groups should review the effectiveness of their management activities in coordination with other members of their working group, and revise management measures as appropriate. They should also provide results of annual population monitoring and the effectiveness of management activities to their working group and to our Arcata Fish and Wildlife Office.

Additionally, the working groups in conjunction with land managers should review success in meeting management goal breeding numbers recommended in Appendix B, and develop recommendations for any necessary revisions to those numbers based on site-specific conditions. Ongoing and needed management activities recommended in Appendix C also should be evaluated and revised according to site specific conditions. Revisions to management goals and management activities should be provided to our Arcata Fish and Wildlife Office.

6.3 Assess the applicability, value, and success of this recovery plan to the recovery of the western snowy plover every 5 years until the recovery criteria are achieved. Rather than revising the entire recovery plan, it is proposed that minor revisions, clarifications, and prioritization changes be made through an addendum, to be produced and distributed every 5 years. This addendum would address data gaps identified in this version of the recovery plan including recommended management prescriptions, specific habitat management recommendations, management goal breeding numbers, directed surveys; and necessary changes discussed in previous recovery actions. It would provide a summary of the recovery actions implemented to date, and it would be a forum to solicit comments from the Recovery Team, stakeholders, and others interested parties on any proposed major changes. Major changes, elimination, or addition of recovery actions may initiate a revision.

6.4 Prepare a delisting package for the Pacific coast population of the western snowy plover. If actions 6.1 through 6.3 indicate recovery criteria have been met, actions to ameliorate or eliminate threats have been implemented and determined to be effective, and analyses of threats demonstrate that threats identified during and since the listing process have been ameliorated or eliminated, prepare a delisting package.

6.5 Prepare and implement a post-delisting monitoring plan. If delisting is warranted, prepare a post-delisting monitoring plan. Section 4 of the Endangered Species Act requires, in cooperation with the States, monitoring for a minimum of five years all species that have been recovered (*i.e.*, delisted).

7 Dedicate sufficient U.S. Fish and Wildlife Service staff for coordination of western snowy plover recovery implementation. Our Arcata Fish and Wildlife Office holds lead responsibility for coordinating implementation of western snowy plover recovery. We should assure that the Arcata Fish and Wildlife Office has sufficient staff to handle the primary responsibility of

implementing the western snowy plover recovery plan. Duties should include coordination and distribution of monitoring information and educational materials; transmission of copies of annual population monitoring results to our field offices that are responsible for western snowy plover issues; compilation and distribution of annual population status updates to all working groups; coordination with our other field offices in CNO and Region 1 regarding western snowy plover conservation actions, consultations, habitat conservation plans, and permits; facilitating coordination among the working groups created for the six recovery units; and fund raising to support recovery implementation actions.

8 Establish an international conservation program with the government of Mexico to protect western snowy plovers and their breeding and wintering locations in Mexico. Meeting the recovery goals outlined in this recovery plan is dependent only on actions recommended for implementation along the Pacific coast of the United States. However, other actions are identified for Mexico to complement conservation efforts in the United States. Efforts should be made to establish an international conservation program between the U.S. Fish and Wildlife Service and Mexico's National Institute of Ecology, Ministry of Environment, Natural Resources and Fisheries. Programs to facilitate implementation of this conservation program should include Partners in Flight, North American Waterfowl Management Plan, and the Borderlands Initiative.

8.1 Develop a joint effort between the United States and Mexico to protect western snowy plover populations and their habitat. Joint efforts should be implemented to determine important habitat in Mexico and protect these breeding and wintering locations from human disturbance.

8.2 Encourage research and monitoring of breeding and wintering western snowy plovers in Baja California, Mexico, by universities and authorities of Mexico. Joint efforts should be made to develop and implement a long-term monitoring program for western snowy plover populations of Mexico. They should include developing methods for

consistent monitoring, coordination of banding and color-marking with banders from the United States, assessment of the population status of breeding and wintering birds, and assessment of environmental impacts that may adversely affect plover populations.

8.3 Encourage development and implementation of public information and conservation education in Mexico for western snowy plovers. Public information and educational efforts should be coordinated and implemented by the United States and Mexico. They should include development of bilingual pamphlets for distribution to anglers, tourists, and local communities, and construction and placement of bilingual signs alerting them of the presence of nesting western snowy plovers.

9 Coordinate with other survey, assessment, and recovery efforts for the western snowy plover throughout North America. Western snowy plovers range through much of North America, and many individuals of the Pacific Coast population of western snowy plovers may overwinter in areas that overlap with other populations. Participation and coordination with other groups working on survey, assessment, and recovery efforts may yield valuable information on the distribution, status, and management needs for the Pacific Coast population of the western snowy plover. This coordination effort should be included in establishment of an international conservation program with Mexico.

IV. IMPLEMENTATION SCHEDULE

The following Implementation Schedule outlines actions needed, responsible parties, and estimated costs to recover the United States portion of the Pacific coast population of the western snowy plover. Considering the recovery criteria, results of the population viability analysis (Appendix D), and fulfillment of the recommendations contained in the recovery plan, recovery of the western snowy plover could occur in approximately 40 years. This time estimate assumes dedicated, proactive efforts toward improvements in western snowy plover management in the near-term, and subsequent management at a maintenance level commensurate with fulfillment of the recovery criteria.

The total cost of implementing actions outlined in this recovery plan over 40 years is $149,946,000. However, this figure represents only a portion of the overall costs because the cost of many actions cannot be estimated at this time. For example, costs associated with intensive protection and management on Federal and State lands (Action 3.3) should be determined by members of each of the six recovery unit working groups because they are most familiar with their site-specific needs and constraints. Costs of many actions were estimated based on current management recommendations provided in Appendix C. However, coastal ecosystems are dynamic and necessary management actions may vary with time, as site conditions change. Improvements over time in methods for predator control, control of nonnative vegetation, and monitoring are also expected and may affect actual costs.

It should be recognized that expenditure of funds for recovery of the western snowy plover will provide far-reaching benefits beyond those gained for a single species. Allocation of these funds will also benefit many other sensitive fish and wildlife species, the coastal beach-dune ecosystem, public appreciation for natural habitats, and aesthetics. These estimated costs do not reflect a cost/benefit analysis that incorporates other values or economic effects with implementation of the recommendations contained in this recovery plan.

We believe that protection and management costs could be substantially reduced by selecting protection strategies that are more restrictive of other beach uses.

While we believe that it is neither feasible nor desirable to completely eliminate beach recreation in most western snowy plover habitat, we also recognize that management strategies that protect western snowy plovers on beaches where public use is also maintained require a continuing commitment of person-power, and are inherently expensive.

The Implementation Schedule lists and ranks actions that should be undertaken within the next 5 years. This schedule will be reviewed routinely until the recovery objective is met, and priorities and actions will be subject to revision.

Key to Acronyms used in the Implementation Schedule

Definition of action priorities:

Priority 1 - An action that must be taken to prevent extinction or prevent the species from declining irreversibly in the foreseeable future.

Priority 2 - An action that must be taken to prevent a significant decline in species population or habitat quality, or some other significant negative impact short of extinction.

Priority 3 - All other actions necessary to provide for full recovery of the species.

Definition of action durations and costs:

Annual - An action that will be implemented each year.

Continual - An action that will be implemented on a routine basis once begun.

Ongoing - An action that is currently being implemented and will continue until action is no longer necessary.

As needed - An action that will be implemented on an "as needed" basis.

Unknown - Either action duration or associated costs are not known at this time.

To Be Determined (TBD) - Costs to be determined at a later date.

Responsible parties*:

ARMY	U.S. Army
BLM	U.S. Bureau of Land Management
CCC	California State Coastal Commission
CDFG	California Department of Fish and Game
CDPR	California Department of Parks and Recreation
CE	U.S. Army Corps of Engineers
CI	Cities
CO	Counties
CON	California Coastal Conservancy
EBRPD	East Bay Regional Park District
ES	U.S. Fish and Wildlife Service, Division of Ecological Services (includes Endangered Species and Contaminants)
FAA	U.S. Department of Transportation, Federal Aviation Administration
HARD	Hayward Area Recreation and Park District
IA	U.S. Fish and Wildlife Service, Office of International Affairs
LE	U.S. Fish and Wildlife Service, Division of Law Enforcement
LMAO	Land Management Agencies and Organizations and other Cooperators. (This category includes Federal and local land management agencies listed above, private organizations and individuals that own and manage snowy plover breeding and wintering habitat, and private conservation groups that provide on-site protection of lands owned by others.)
MPOSD	Mid-Peninsula Open Space District
MPRPD	Monterey Peninsula Regional Park District
NASA	National Aeronautics and Space Administration-Ames Research Center
NAVY	U.S. Navy
NMFS	National Marine Fisheries Service
NPS	National Park Service
ODFW	Oregon Department of Fish and Wildlife
ODLCD	Oregon Department of Land Conservation and Development
OPRD	Oregon Parks and Recreation Department

P	Private landowners (except HARD, MPOSD, and TNC)
PA	U.S. Fish and Wildlife Service, Public Affairs
PGH	Port of Grays Harbor
PO	Port of Oakland
PRBO	Point Reyes Bird Observatory Conservation Science
PSL	Port of San Luis Harbor District
RSCH	Research institutions and agencies
RW	U.S. Fish and Wildlife Service, Division of Refuges and Wildlife (includes Realty)
SDRPJPA	San Dieguito River Park Joint Powers Authority
TNC	The Nature Conservancy
TPL	Trust for Public Land
USAF	U.S. Air Force
USCG	U.S. Coast Guard
USFS	U.S. Forest Service
USFWS	U.S. Fish and Wildlife Service
BBL	U.S. Geological Survey, Bird Banding Laboratory
BRD	U.S. Geological Survey, Biological Resources Division
USMC	U.S. Marine Corps
WDFW	Washington Department of Fish and Wildlife
WDNR	Washington Department of Natural Resources
WS	U.S. Department of Agriculture, Wildlife Services Branch
WSPRC	Washington State Parks and Recreation Commission

* All responsible parties listed for actions in Implementation Schedule are considered lead agencies for those actions.

IMPLEMENTATION SCHEDULE

Western Snowy Plover Pacific Coast Population Recovery Plan

Priority No.	Action Description	Action Number	Action Duration	Responsible Parties	Total Costs	Cost Estimate (in $1,000 units) FY1	FY2	FY3	FY4	FY5	Comments/Notes
1	Annually monitor abundance, population size and distribution at breeding and wintering locations.	1.1	annual	LMAO, CO, CI, RSCH	2,194	54.9	54.9	54.9	54.9	54.9	Assumes 157 window survey days, with 2 biologists per location at. Action needed to determine fulfillment of recovery criteria.
1	Develop and implement a program to monitor productivity and annual survival.	1.2	annual	LMAO, CO, CI. RSCH	TBD						Action needed to determine fulfillment of recovery criteria. Depends partly on completion of 4.3.2 and 4.3.3.
1	Develop and implement a program to monitor habitat condition and threats at all breeding and wintering sites.	1.3	annual	LMAO, RSCH	1,125	60	27	27	27	27	Assumes initial cost for development of standardized monitoring program and subsequent monitoring for 155 sites.
3	Develop and implement training and certification programs for western snowy plover survey coordinators and observers.	1.4	continual	ES, LMAO, RSCH	363.5	32	8.5	8.5	8.5	8.5	Assumes initial cost to develop program and subsequent implementation.

Priority No.	Action Description	Action Number	Action Duration	Responsible Parties	Cost Estimate (in $1,000 units) Total Costs	FY1	FY2	FY3	FY4	FY5	Comments/Notes
3	Improve submittal system for monitoring data to ensure consistent reporting.	1.5	continual	ES, LMAO, BBL, PRBO	346	32	8	8	8	8	Assumes initial cost to develop submittal and reporting system and subsequent annual review.
3	Assess and evaluate new breeding wintering and migration areas for threats and management needs and update lists as data become available.	1.6	continual	ES, LMAO, PRBO	TBD						Depends on results of annual surveys and monitoring.
3	Coordinate monitoring of snowy plovers and California least terns to minimize disturbances.	1.7	annual	ES, RW, NAVY, USMC, USAF, CDFG, CDPR, WS, BRD	1,020	25.5	25.5	25.5	25.5	25.5	Coordinate at biannual pre- and post-season California least tern monitoring meeting. Assumes 2 meetings at 2 days per meeting with 9 agency staff attending.
3	Develop a post-delisting monitoring plan.	1.8	TBD	ES, LMAO, CO, CI, RSCH	TBD						

Priority No.	Action Description	Action Number	Action Duration	Responsible Parties	Total Costs	Cost Estimate (in $1,000 units) FY1	FY2	FY3	FY4	FY5	Comments/Notes
1	Develop a prioritized list of wintering and breeding sites where natural coastal processes need protection and/or enhancement.	2.1.1	2 yrs	ES, LMAO, CO, CI, RSCH	59.65	59.65					Assumes time to evaluate sites and development of the prioritized list.
1	Identify and implement mechanisms to protect, enhance or restore natural coastal processes.	2.1.2	continual	ES, LMAO, CO, CI, RSCH	TBD						Incorporate into ongoing management in action 3. Costs will depend on mechanisms identified and carried out.
1	Develop and implement prioritized removal and control for introduced beachgrass and other non-native vegetation.	2.2.1.1	continual	CE, LMAO, CO, CI	TBD						App C identifies 86 sites. Costs range for mechanical, manual and/or chemical control: $1,000 to $87,000/hectare ($400 to $35,000 per acre).
2	Replace exotic dune plants with native dune vegetation where it is likely to improve habitat.	2.2.1.2	continual	CE, LMAO, CO, CI	TBD						Estimated cost of planting native vegetation: $30,000 per hectare ($12,000 per acre). Number of sites to be determined.

Priority No.	Action Description	Action Number	Action Duration	Responsible Parties	Total Costs	Cost Estimate (in $1,000 units) FY1	FY2	FY3	FY4	FY5	Comments/Notes
3	Evaluate breeding and wintering sites to determine whether dredged materials may be used to enhance or create nesting habitat.	2.2.2.1	2 yrs	CE, ES, LMAO, CO, CI	110	55	55				Assumes cost to evaluate each site.
3	Develop and implement plans to use dredged materials may be used to enhance or create nesting habitat.	2.2.2.2	ongoing	CE, ES, LMAO, CO, CI	TBD						Costs will depend on completion of acts on 2.2.2.1.
3	Identify sites where beach nourishment may be effective in creating and enhancing habitat.	2.2.3.1	2yrs	CE, ES, LMAO, CO, CI	110	55	55				Assumes cost to evaluate each site.
3	Develop and implement beach nourishment plans for site identified in action 2.2.3.1.	2.2.3.2	ongoing	CE, ES, LMAO, CO, CI	TBD						Cost dependent on number of sites identified in 2.2.3.1 and outcome of 4.1.1.

Priority No.	Action Description	Action Number	Action Duration	Responsible Parties	Cost Estimate (in $1,000 units) Total Costs	FY1	FY2	FY3	FY4	FY5	Comments/Notes
1	Create, manage, and enhance coastal ponds and playas for breeding habitat.	2.2.4	ongoing	ES, RW, CE, CDFG, NASA, HARD, LMAO	TBD						App C identifies 15 sites. Costs dependent on type and area of restoration.
1	Seasonally close areas used by breeding snowy plovers.	2.3.1.1.1	annual	LMAO, CO, CON, CI	559.2	13.98	13.98	13.98	13.98	13.98	App C identifies 81 sites. Assumes cost to close these sites.
1	Fence areas used by breeding snowy plovers	2.3.1.1.2	annual	LMAO, CO, CON, CI	14,840	371	371	371	371	371	App C identifies 64 sites. Cost assumes 1 kilometer fencing required per site at a cost of $5,900 per kilometer.
1	Post signs in areas used by breeding snowy plovers	2.3.1.1.3	annual	LMAO, CO, CON, CI	202	5	5	5	5	5	App C identifies 65 sites. Cost dependent on number of signs needed at each site, but assumes cost for installation and a minimum of 4 signs at $20 per sign.
1	Evaluate effects of existing and planned access at all breeding and wintering locations and any new locations identified.	2.3.1.2.1	1 year	LMAO, CO, CI	455	455					Appendix C identifies 81 sites. Assumes cost to conduct use survey for the identified sites.
1	Develop and implement plans to minimize adverse access effects.	2.3.1.2.2	continual	LMAO, CO, CI	TBD						Costs depend on outcome of 2.3.1.2.1.

Priority No.	Action Description	Action Number	Action Duration	Responsible Parties	Total Costs	Cost Estimate (in $1,000 units) FY1	FY2	FY3	FY4	FY5	Comments/Notes
3	Implement and enforce pet restrictions.	2.3.2	continual	LMAO, CO, CI	39,406	985	985	985	985	985	Appendix C identifies 120 sites Assumes staff time to implement and enforce restrictions at the identified sites.
1	Annually review recreational activities and develop and implement plans to prevent disturbance from disruptive recreational activities at breeding and wintering sites	2.3.3	annual	LMAO, CO, CI	21,948	549	549	549	549	549	Assumes staff cost to develop and implement plans at each site annually.
3	Prevent driftwood removal through posting of signs	2.3.4	continual	LMAO, CO, CI	1,805	50	45	45	45	45	Appendix C identifies 26 sites. Cost dependent on number of signs needed at each site, but assumes cost for installation and a minimum of 4 signs at $20 per sign.
1	Prevent disturbance, mortality, and habitat degradation by prohibiting or restricting off-road vehicles and beach-raking machines.	2.3.5	continual	LMAO, CO, CI	18,760	469	469	469	469	469	Appendix C identifies 101 sites. Assumes staff time for monitoring on weekends.
3	Implement restrictions on horseback riding through annual coordination.	2.3.6	annual	LMAO, CO, CI	1,033.7	25.8	25.8	25.8	25.8	25.8	Appendix C identifies 72 sites. Assumes staff time to implement restrictions.

Priority No.	Action Description	Action Number	Action Duration	Responsible Parties	Total Costs	Cost Estimate (in $1,000 units) FY1	FY2	FY3	FY4	FY5	Comments/Notes
3	Implement and enforce restrictions on livestock through annual coordination.	2.3.7	annual	LMAO, CO, CI	255	6.3	6.3	6.3	6.3	6.3	Appendix C identifies 18 sites. Assumes staff time to implement restrictions.
1	Determine enforcement needs and provide sufficient wardens, agents or officers to enforce protective measures in breeding and wintering habitat.	2.3.8.1	continual	LE, LMAO, CO, CI	TBD						Cost will depend on identified enforcement needs.
3	Develop and implement training programs for enforcement personnel to improve enforcement of regulations and minimize effects of enforcement.	2.3.8.2	continual	LE, LMAO, CO, CI	320	8	8	8	8	8	Annual training cost estimate $8,000 per year.
2	Develop and implement a program to annually coordinate with local airports, aircraft operations regarding minimum altitude requirementss.	2.3.9	annual	LMAO, CO, CI, FAA, LE	339.8	8.5	8.5	8.5	8.5	8.5	Assumes staff costs per recovery unit to compile list and notify aircraft operations and facilities.
3	Implement and enforce anti-littering regulations.	2.4.1.1	annual	LMAO, CO, CI	TBD						Incorporate into ongoing management and Action 3.

Priority No.	Action Description	Action Number	Action Duration	Responsible Parties	Total Costs	Cost Estimate (in $1,000 units) FY1	FY2	FY3	FY4	FY5	Comments/Notes
3	Evaluate the effects of current litter and garbage management on predation at breeding and wintering sites.	2.4.1.2	2 yrs	LMAO, CO, CI	110	55	55				Assumes evaluation time per site.
3	Develop and implement garbage and litter management plans where litter and garbage contribute to predation.	2.4.1.3	continual	LMAO, CO, CI	TBD						Costs will depend on 2.4.1.2 and plans developed.
3	Annually identify and remove predator perches and unnatural habitats attractive to predators.	2.4.2	continual	LMAO, CO, CI	375.2	9.4	9.4	9.4	9.4	9.4	Assumes staff time to complete action each year.
1	Erect predator exclosures to reduce egg predation and improve productivity.	2.4.3	annual	LMAO, CO, CI	18,266	456	456	456	456	456	App C identifies 53 sites. Assumes cost per unit installation.
1	Evaluate the need for predator removal and implement where warranted and feasible.	2.4.4	as needed	LMAO, CO, CI, WS, CDFG	TBD						App C identifies 61 sites for additional predator control. Costs dependent on assessment of needs and feasability.
3	Remove bird and mammal carcasses in nesting areas.	2.4.5	as needed	LMAO, CO, CI	TBD						

Priority No.	Action Description	Action Number	Action Duration	Responsible Parties	Total Costs	Cost Estimate (in $1,000 units) FY1	FY2	FY3	FY4	FY5	Comments/Notes
1	U.S. Fish and Wildlife Service biologists should participate in Area Committees responsible for maintaining the Area Contingency Plans for the Pacific Coast to facilitate the updating of spill response plans to include protection of western snowy plovers.	2.5.1	annual	ES	5,154	128.9	128.9	128.9	128.9	128.9	Assumes staff time from the six ES office responsible for coastlines of CA, OR, and WA.
1	Assign monitors to beaches that are inhabited by western snowy plovers to protect western snowy plovers from injury during spill responses.	2.5.2	as needed	ES, USCG, LMAO, CO, CI	1,984	49.6	49.6	49.6	49.6	49.6	Assumes cost of two weeks of monitoring for five incidents per year.
2	Compensate the loss of plover breeding and wintering habitat associated with recovery efforts for other sensitive species.	2.6	ongoing	ES, RW, CE, LMAO	TBD						Costs dependent on effectiveness of minimizing habitat loss.
3	Investigate feasibility and methods for discouraging pinniped use of nesting areas.	2.7.1	5 yrs	ES, NMFS, NAVY, LMAO	320	64	64	64	64	64	Assumes staff time to investigate.

Priority No.	Action Description	Action Number	Action Duration	Responsible Parties	Cost Estimate (in $1,000 units) Total Costs	FY1	FY2	FY3	FY4	FY5	Comments/Notes
3	Identify areas where pinniped use is negatively affecting nesting and implement any appropriate methods.	2.7.2	TBD	ES, NMFS, NAVY, LMAO	TBD						Costs dependent on number of sites identified and methods determined in 2.7.1.
1	Establish and maintain snowy plover working groups for each of the six recovery units.	3.1	continual	ES, LMAO, CO, C I, P	3,650	96	96	91	91	91	Essential mechanism to advance plover recovery. Includes biannual meeting costs and staff costs to establish new working groups.
2	Develop and implement regional participation plans for each of the six recovery units.	3.2	1 yr for develop-ment, continual thereafter	ES, LMAO	193		193				Assumes staff cost to develop and implement participation plans.
3	Develop and implement management plans for Federal lands.	3.3.1	ongoing	RW, ARMY, BLM, CE, NASA, NAVY, NPS, USAF, USMC, USFS	TBD						Implementation cost dependent on content of plans developed.

Priority No.	Action Description	Action Number	Action Duration	Responsible Parties	Total Costs	Cost Estimate (in $1,000 units) FY1	FY2	FY3	FY4	FY5	Comments/Notes
3	Develop and implement management plans and Habitat Conservation Plans on State wildlife areas, State ecological reserves, and State beaches.	3.3.2	5 years	CDFG, CDPR, ODFW, OPRD, WDFW, WDNR, WSPRC	966	193	193	193	193	193	Assumes cost for each recovery unit to assist in development. Implementation cost to be determined.
3	Develop and implement Habitat Conservation Plans or other management plans for sites owned by local governments or private landowners.	3.4	5 years	ES, LMAO, CO, CI, P, EBRPD, HARD, MPOSD, MPRPD, PGH, PO, SL, TNC, SDRPJPA	966	193	193	193	193	193	Assumes cost for each recovery unit to assist in development. Implementation cost to be determined.
2	Provide technical assistance to local governments in developing and implementing local land use protection measures through periodic workshops.	3.5	10 years	ES, CCC, CDFG, CDPR, CON, ODFW, ODLCD, OPRD, WDNR, WDFW, WSPRC, CO, CI	TBD						Estimated at 2 workshops per recovery unit at a cost of $ (Patty Carol in RO)

Priority No.	Action Description	Action Number	Action Duration	Responsible Parties	Total Costs	Cost Estimate (in $1,000 units) FY1	FY2	FY3	FY4	FY5	Comments/Notes
3	Develop and implement cooperative programs and partnerships with the California State Coastal Commission, the Oregon Department of Land Conservation and Development, the Washington State Parks and Recreation Commission, the Oregon Parks and Recreation Department, the California Department of Parks and Recreation, and the Oregon Department of Fish and Wildlife.	3.6	continual	ES, CCC, ODLCD, ODFW, OPRD, CDPR, WSPRC	TBD						Costs may vary from year to year based on identified program needs.
3	Obtain long-term agreements with private landowners.	3.7	12 years	ES, CDFG, P CDPR, ODFW, WDFW, WSPRC, LMAO	2,319	193	193	193	193	193	Assumes staff time to facilitate 6 agreements per year per recovery unit. Appendix C identifies 72 sites.
3	Identify and protect habitat available for acquisition.	3.8	ongoing	CON, ES, RW, LMAO	TBD						

Priority No.	Action Description	Action Number	Action Duration	Responsible Parties	Total Costs	Cost Estimate (in $1,000 units) FY1	FY2	FY3	FY4	FY5	Comments/Notes
3	Ensure that any section 10(a)(1)(B) and section 7(a)(2) permits contribute to Pacific coast western snowy plover conservation.	3.9	ongoing	ES, Federal agencies	1,288	32`32	32	32	32	32	Assumes staff time for annual evaluation.
3	Ensure that section 7 consultations contribute to Pacific coast western snowy plover conservation.	3.10	ongoing	ES, Federal agencies	1,288	32`32	32	32	32	32	Assumes staff time for annual evaluation.
2	Evaluate effectiveness of habitat restoration by removal of introduced beachgrass and identify additional studies necessary.	4.1.1	continual	CON, ES, LMAO, RSCH	TBD						Depends on the number and location of sites as well as the temporal duration of the restoration project.
3	Evaluate the impacts and potential benefits of past and ongoing beach nourishment activities and identify and carry out any additional studies necessary.	4.1.2	ongoing	ES, LMAO, RSCH, CE, CI, CO	TBD						
2	Develop higher-efficiency nest enclosures.	4.2.1	ongoing	ES, LMAO, RSCH	20	10	5	3	2	0	Compare new exclosures with current ones to determine effects on snowy plovers.

Priority No.	Action Description	Action Number	Action Duration	Responsible Parties	Cost Estimate (in $1,000 units) Total Costs	FY1	FY2	FY3	FY4	FY5	Comments/Notes
2	Develop California least tern enclosures that prevent harm to snowy plovers.	4.2.2	as needed	ES, USMC, CDFG, CDPR, LMAO, RSCH	TBD						Costs specific to sites with California least tern enclosures. Estimated cost for materials (fencing/posts): $7 per linear foot ($23 per meter).
3	Identify, prioritize and carry out investigations on control of predators.	4.2.3	as needed	ES, RW, LMAO, WS, CDFG, RSCH, CO, CI, P	TBD						Cost dependent on number and types of studies identified.
3	Investigate predator management at the landscape level.	4.2.4	as needed	ES, RW, LMAO, WS, RSCH, CO, CI, P	TBD						Costs dependent on number and types of studies identified.
3	Investigate techniques for identifying nest predators.	4.2.5	continual	LMAO, RSCH	TBD						
2	Improve methods of monitoring population size.	4.3.1	ongoing	ES, LMAO, RSCH	TBD						Dependent on costs of intensive monitoring of some sites.
2	Develop sampling methods for annually estimating reproductive success.	4.3.2	2 years	ES, RSCH	64	64					Assumes time to compile and review data and develop methodology.
3	Develop methods to monitor plover survival rates.	4.3.3	ongoing	ES, LMAO, RSCH	TBD						

Priority No.	Action Description	Action Number	Action Duration	Responsible Parties	Cost Estimate (in $1,000 units) Total Costs	FY1	FY2	FY3	FY4	FY5	Comments/Notes
3	Identify brood habitat and map brood home ranges.	4.4.1	ongoing continual	ES, LMAO, RSCH, CO, CI, P	TBD						Costs dependent on study design. May include radio telemetry.
3	Identify components of high-quality brood rearing habitat	4.4.2	1 year	ES, LMAO, RSCH, CO, CI, P	131	131					Assumes study at 6 geographically representative sites for duration of breeding season.
3	Quantify wintering habitat needs along the Pacific coast.	4.4.3	5 years	ES, RSCH, BRD, PRBO	75	75					Assumes study at 6 geographically representative sites during winter months.
3	Identify important migration stop-over habitat.	4.4.4	ongoing	ES, LMAO	TBD						
3	Develop and implement a research program to determine causes of adult mortality.	4.5	ongoing	LMAO, RSCH	TBD						Costs dependent on study design.
3	Compile information regarding number and types of banding injuries to plovers.	4.6.1	1 year	ES, RSCH, PRBO, BRD, BBL	32	32					Assumes staff time to develop, distribute and compile information requests.
3	Review compiled information (see 4.6.1) and determine and implement an appropriate course of action.	4.6.2	1 year	ES, RSCH, PRBO, BRD, BBL	32						Assumes staff time to review compiled information, distribution and coordination with other responsible parties.

Priority No.	Action Description	Action Number	Action Duration	Responsible Parties	Cost Estimate (in $1,000 units) Total Costs	FY1	FY2	FY3	FY4	FY5	Comments/Notes
3	Identify effects of oil spills on snowy plovers.	4.7	as needed	ES, RSCH, BRD, LMAO	TBD						Typical range of cost for study is estimated between $25,000 - $100,000.
3	Monitor levels of environmental contaminants in snowy plovers.	4.8	as needed	ES, RSCH, BRD, LMAO	TBD						Depends on number and type of samples. Cost estimate $700 per sample, but may vary depending on type of contaminant.
3	Design and conduct contaminants studies if monitoring of contaminants in action 4.8 indicates potential contaminants effects.	4.9	as needed	LMAO, ES, RSCH, BRD	TBD						Depends on number of sites and samples analyzed. Cost estimates for studies range from $25,000 to $50,000 per site.
3	Identify, prioritize and carry out studies on the effects of human recreation on western snowy plovers.	4.10	ongoing	LMAO, ES, RSCH, PRBO, BRD	TBD						Costs dependent on research needs identified.
3	Revise the population viability analysis when sufficient additional information is available	4.11	1 year	ES, RSCH, PRBO, BRD	25						Assumes cost to conduct modeling.
2	Develop and implement public information and education programs.	5.1	ongoing	ES, PA, LMAO	TBD	TBD	TBD	TBD	TBD	TBD	Depends on individual recovery unit strategies. See Appendix K (Information & Education Plan) for estimates of component expenses.

Priority No.	Action Description	Action Number	Action Duration	Responsible Parties	Cost Estimate (in $1,000 units) Total Costs	FY1	FY2	FY3	FY4	FY5	Comments/Notes
3	Inform Federal, State and local planning agencies and local planning departments of threats to breeding and wintering snowy plovers and their habitats.	5.2	continual	ES, LMAO, CCC, CDFG, CDPR, ODFW, ODLCD, OPRD, WDFW, WDNR, WSPRC, CO/CI	TBD						
3	Develop and maintain updated information and education materials on snowy plovers.	5.3	ongoing	ES, PA, LMAO, CO, CI	TBD						Incorporate into ongoing management and Action 3.1 through 3.10. See Appendix K
3	Alert landowners and beach-goers about access restrictions within snowy plover habitats.	5.4	ongoing	ES, PA, LMAO, CO, CI	TBD						Incorporate into ongoing management and Action 3.1 through 3.10. See Appendix K
3	Provide trained personnel to facilitate protective measures, provide public education, and respond to emergency situations.	5.5	continual	LMAO, CO, CI	TBD						Need to secure funds for volunteer coordinator and staff to train volunteers. Incorporate into Action 3.1 through 3.10. See Appendix K.
3	Develop protocols for handling sick, displaced, injured, oiled, and dead birds or salvaged eggs.	5.6	1 with periodic review	LMAO, CO, CI	32.2	32.2					Assumes staff time to develop protocol.

Priority No.	Action Description	Action Number	Action Duration	Responsible Parties	Cost Estimate (in $1,000 units) Total Costs	FY1	FY2	FY3	FY4	FY5	Comments/Notes
3	Establish a distribution system and repository for information and education materials.	5.7	continual	ES, LMAO, CO, CI	TBD						Incorporate into ongoing management and Action 3.1 through 3.10 and 7. See Appendix K.
3	Establish a reporting and distribution system for annual monitoring data.	5.8	annual	ES	644	16	16	16	16	16	Assumes time spent collecting and compiling data.
2	Develop and implement a tracking process for the completion of recovery actions and the achievement of delisting criteria.	6.1	continual	ES, RW, ARMY, BLM, CE, NASA, NAVY, NPS, USAF, USFS, USMC, CDFG, CDPR, ODFW, OPRD, WDFW, WDNR, WSPRC, LMAO	688	64	16	16	16	16	Assumes staff time to develop and implement tracking process.
3	Review progress toward recovery annually.	6.2	annual	ES, LMAO	566	14	14	14	14	14	Assumes staff time to compile and review data.
3	Assess the applicability, value and success of this plan to the recovery of the western snowy plover every 5 years.	6.3	every 5 years		258					32.2	Assumes staff time to review every 5 years.
3	Prepare a delisting package for the Pacific coast population of the western snowy plover.	6.4	6 months	ES	64	64					Assumes staff time to prepare delisting package.

Priority No.	Action Description	Action Number	Action Duration	Responsible Parties	Total Costs	Cost Estimate (in $1,000 units) FY1	FY2	FY3	FY4	FY5	Comments/Notes
3	Prepare and implement a post-delisting monitoring plan.	6.5	6 months	ES	64	64					Assumes staff time to prepare and implement post-delisting monitoring plan.
1	Dedicate sufficient U.S. Fish and Wildlife Service staff for coordination of western snowy plover recovery implementation.	7	continual	ES	5,152	128.8	128.8	128.8	128.8	128.8	Assumes staff time to coordinate recovery implementation
3	Develop a joint United States and Mexico effort to protect snowy plover populations and their habitat.	8.1	continual	ES, IA	TBD						
3	Encourage research and monitoring of breeding and wintering snowy plovers in Baja California, Mexico by universities and authorities of Mexico.	8.2	continual	ES, IA, RSCH, BRD	TBD						
3	Encourage development and implementation of public information and conservation education in Mexico.	8.3	continual	ES, IA, PA	TBD						

Priority No.	Action Description	Action Number	Action Duration	Responsible Parties	Cost Estimate (in $1,000 units) Total Costs	FY1	FY2	FY3	FY4	FY5	Comments/Notes
3	Coordinate with other survey, assessment, and recovery efforts for the western snowy plover throughout North America.	9	continual	ES, IA, RSCH, BRD	TBD						

Total Cost of Recovery through 2046: $149,946,000 plus additional costs that cannot be estimated at this time.

V. REFERENCES

A. Literature Cited

Albers, P.H. 1977. Effects of external applications of fuel oil on hatchability of mallard eggs. Pages 158-163 *in* D. A. Wolfe, editor. Fate and effects of petroleum hydrocarbons in marine organisms and ecosystems. Pergamon Press, New York, N.Y.

Albers, P.H. and R.C. Szaro. 1978. Effects of No. 2 fuel oil on common eider eggs. Marine Pollution Bulletin 9:138-139.

Ainley, D.G., C.R. Grau, T.E. Roudybush, S.H. Morrell and J.M. Utts. 1981. Petroleum ingestion reduces reproduction in Cassin's auklets. Marine Pollution Bulletin 12:314-317.

American Ornithologists' Union. 1957. The A.O.U. checklist of North American birds. Fifth edition. 168 pp.

Anthony, J.L. 1985. A report on the distribution, numbers and human disturbance of snowy plovers at Damon Point, Washington. Report to the Washington Dept. of Game. Evergreen State College, WA. 24 pp.

Anthony, J.L. 1987. The snowy plover and biopolitics at Damon Point, Washington. M.E.S. Thesis, The Evergreen State College, Olympia, WA. 106 pp.

Applegate, T.E. 1998. Vandenberg Air Force Base snowy plover monitoring, Torch/Platform Irene Pipeline Oil Spill. Pp C1-C5 In: Preliminary Bird Injury Assessment for the Torch/Platform Irene Pipeline Oil Spill, R.G. Ford, editor. Unpublished report to California Department of Fish and Game, Office of Spill Prevention and Response.

Avery, M.L., M. A. Pavelka, D. L. Bergman, D. G. Decker, C. E. Knittle, and G. M. Linz. 1995. Aversive conditioning to reduce raven predation on California least tern eggs. Colonial Waterbirds 18(2):131-138.

Barbour, M.G. and J. Major. 1990. Terrestrial vegetation of California. California Native Plant Society. Special Publication Number 9, University of Davis, CA. 1028 pp.

Binford, L.C. 1989. A distributional survey of the birds of the Mexican State of Oaxaca. Ornithological Monographs 43. 418 pp.

Blus, L.J. 1982. Further interpretation of the relation of organochlorine residues in brown pelican eggs to reproductive success. Environmental Pollution (Series A) 28:15-33.

Brennan, K. 2003. Snowy plovers at Leadbetter Point, Washington, 2002 Annual Report. U.S. Fish and Wildlife Service, Willapa National Wildlife Refuge. 14 pp.

Brennan, K. and M. Fernandez. 2004*a*. Snowy Plovers at Leadbetter Point, Washington, 2003 Annual Report. U.S. Fish and Wildlife Service, Willapa National Wildlife Refuge. 16 pp.

Brennan, K and M. Fernandez. 2004*b*. Snowy Plovers at Leadbetter Point, Washington, 2004 Annual Report. U.S. Fish and Wildlife Service, Willapa National Wildlife Refuge.

Brennan, K., and M. Fernandez. 2006. Snowy plovers at Leadbetter Point, Washington, 2005 Annual Report. U.S. Fish and Wildlife Service, Willapa National Wildlife Refuge. 14 pp.

Brennan, K. and D. Jaques. 2002. Snowy Plovers at Leadbetter Point, Washington, 2001 Annual Report. U.S. Fish and Wildlife Service, Willapa National Wildlife Refuge.

Brittell, J.D., J.M. Brown, and R.L. Eaton. 1976. Marine shoreline fauna of Washington, Vol. II. Washington Dept. of Game and Ecology, Olympia. 341 pp.

Buick, A.M. and D.C. Paton. 1989. Impact of off-road vehicles on the nesting success of hooded plovers (*Charadrius rubricollis*) in the Coorong region of South Australia. Emu 89:159-172.

Burger, J. 1986. The effect of human activity on shorebirds in two coastal bays in the northeastern United States. Environmental Conservation 13:123-130.

Burger, J. 1993. Shorebird squeeze. Natural History 102(5):8-14.

Burger, J. 1997. Effects of oiling on feeding behavior of sanderlings and semipalmated plovers in New Jersey. Condor 99:290-298.

Burger, A.E. and D.M. Fry. 1993. Effects of oil pollution on seabirds in the northeast Pacific. Pages 254-263 *in* K. Vermeer, K.T. Briggs, K.H. Morgan, and D. Siegel-Causey, eds. The status, ecology, and conservation of marine birds of the North Pacific. Canadian Wildlife Service Special Publication, Ottawa.

Caffrey, C. 1993. California least tern breeding survey, 1992 season. California Department of Fish and Game, Wildlife Management Division, Nongame Bird and Mammal Section Report 93-11, Sacramento, California. Unpublished final report.

Cairns, W.E. and I.A. MacLaren. 1980. Status of the piping plover on the east coast of North America. American Birds 34(2):206-208.

California Coastal Commission. 1995. Regional cumulative assessment project. ReCAP pilot project, findings and recommendations: Monterey Bay Region. 162 pp. plus appendices.

California Department of Fish and Game. Undated [1994]. Managing non-native species in California: The red fox. The Resources Agency, California Department of Fish and Game, Sacramento, CA. 8 pp.

California Department of Parks and Recreation, Off-Road Vehicle Division. 2005. Nesting season management plan for 2005. San Luis Obispo County, California.

California Native Plant Society. 1996. Policy on invasive exotic plants. http://www.cnps.org/cnps/archive/exotics.php

California Natural Diversity Database. 2001. Special Animals. California Department of Fish and Game, Wildlife and Habitat Data Analysis Branch. January 2001. 52 pp.

Cape Mohican Trustee Council. 2002. SS Cape Mohican Oil Spill Restoration Plan and Environmental Assessment. Unpublished report, National Park Service, U.S. Fish and Wildlife Service, National Oceanic and Atmospheric Administration, California Dept. of Fish and Game, California Dept. of Parks and Recreation. 99 pp.

Carter, R.W.G. 1988. Coastal environments, an introduction to the physical, ecological and cultural systems of coastlines. 617 pp.

Carter, H.R. and R.T. Golightly, editors. 2003. Seabird injury from the 1997-98 Point Reyes Tarball Spill Incidents. Unpublished draft report, Humboldt State University, Dept. of Wildlife, Arcata, California.

Casler, B.R., C.E. Hallett, M.A. Stern, and G.A. Rosenberg. 1993. Unpublished report submitted to Oregon Department of Fish and Wildlife; Coos Bay District, Bureau of Land Management; and Oregon Dunes National Recreation Area, Suislaw National Forest. 26 pp.

Castelein, K.A., D.J. Lauten, S.R. Pixley, L.N. Renan, M.A. Stern, and C. Grinnell. 2002. The distribution and reproductive success of the western snowy plover along the Oregon coast- 2002. Page 54. The Oregon Natural Heritage Program, Portland.

Center for Disease Control. 2004. West Nile Virus. Retrieved from: http://www.cdc.gov/ncidod/dvbid/westnile/birdspecies.htm/ 9-20-04

Center for Marine Conservation. 1995. National Marine Debris Monitoring Program. Volunteer Handbook. 12 pp.

Chestnut, J. 1997. The distribution of rare species and the distribution and trend of invasive weeds on the Mobil Coastal preserve, Guadalupe-Nipomo Dunes, California. Unpublished document, The Nature Conservancy, San Francisco, CA. 135 pp.

Colwell, M.A., J. Hall, C.B. Millett, J.J. Meyer, R.R. LeValley, S.E. McAllister, A.N. Transou, D. LeValley. 2002. Final Report: 2002 Snowy Plover breeding in northern California, with emphasis on Humboldt County. Unpublished Report, Mad River Biologists, Inc., McKinleyville, CA and Humboldt State University Wildlife Department, Arcata, CA. 14 pp.

Colwell, M.A., Z. Nelson, S. Mullin, C. Wilson, S.E. McAllister, K.G. Ross, and R.R. LeValley. 2005. Final Report: 2005 Snowy Plover breeding in coastal northern California, Recovery Unit 2. Unpublished report, Mad River Biologists, Inc., and Humboldt State University Wildlife Department, Arcata, CA. 11 pp.

Colwell, M.A., S.M. Mullin, Z.J. Nelson, C.A. Wilson, J.M. Muir, W.P. Goldenberg, S.E. McAllister, and K.G. Ross. 2006. Final Report: 2006 Snowy Plover breeding in coastal northern California, Recovery Unit 2. Unpublished report, Mad River Biologists, Inc., and Humboldt State University Wildlife Department, Arcata, CA.

Connors, P.G., V.C. Anderlini, R.W. Risebrough, M. Gilbertson, and H. Hays. 1975. Investigations of heavy metals in common tern populations. The Canadian Field-Naturalist 89:157-162.

Cooch, E., R. Pradel and N. Nur. 1996. A practical guide to capture/recapture analysis using SURGE. CNRS, Montpellier, France. Approx. 130 pages.

Craig, D.P., M.A. Stern, K.A. Mingo, D.M. Craig, and G.A. Rosenberg. 1992. Reproductive ecology of the western snowy plover on the south coast of Oregon. Submitted to Oregon Department of Fish and Wildlife, Roseburg, OR, and Coos Bay District, Bureau of Land Management, North Bend, OR. 13 pp plus tables and maps.

Davis, W.A. and S.M. Russell. 1984. Birds in southeastern Arizona. Tucson Audubon Society, Tucson, AZ. 169 pp.

Dugan, J.E., H.M. Page, and R. Castelli. 1997. Assays of fertilization success, embryonic development and larval viability in a brooding invertebrate as indicators of ecosystem condition, with emphasis on the intertidal sand crab, Emerita analoga. Research abstracts, University of California Toxic Substances Research and Teaching Program Annual Report. 4 pp.

Evans, M.I. and G.O. Keijl. 1993. Impact of Gulf War oil spills on the wader populations of the Saudi Arabian Gulf Coast. Sandgrouse 15:85-105.

Evans, M.I., P. Symens, and C.W.T. Pilcher. 1993. Short-term damage to coastal bird populations in Saudi Arabia and Kuwait following the 1991 Gulf War. Marine Pollution Bulletin 27:157-161.

Fancher, J., R. Zembal, L. Hays, and P. Knapp. 1998. Western snowy plover nesting at Bolsa Chica, Orange County, California, 1998. Fish and Wildlife Service, Carlsbad Office, October 1998.

Fancher, J., L. Hays, and P. Knapp. 2002. Western snowy plover nesting at Bolsa Chica, Orange County, California, 2002. Fish and Wildlife Service, Carlsbad Office, December 2002.

Fahy, K.A. and C.D. Woodhouse. 1995. 1995 snowy plover linear restriction monitoring project, Vandenberg Air Force Base. Prepared for Natural Resources, Vandenberg Air Force Base, CA. 37 pp.

Feeney, L.R. and W.A. Maffei. 1991. Snowy plovers and their habitat at the Baumberg area and Oliver salt ponds, Hayward, California, March 1989 through May 1990. City of Hayward, Hayward, CA. 162 pp.

Flemming, S.P., R.D. Chiasson, P.C. Smith, P.J. Austin-Smith, and R.P. Bancroft. 1988. Piping plover status in Nova Scotia related to its reproductive and behavioral responses to human disturbance. Journal of Field Ornithology 59(4):321-330.

Ford, R.G. 1998. Preliminary bird injury assessment for the Torch/Platform Irene Pipeline Oil Spill. Unpublished report to California Department of Fish and Game, Office of Spill Prevention and Response.

Foster, B. 2005. Breeding status of the western snowy plover at Marine Corps Base, Camp Pendleton, California, 2003. Unpublished report prepared for the Environmental Core, Natural and Cultural Resources Team, Naval Facilities Engineering Command, Southwest, San Diego, California. 106 pp.

Fox, R. 1990. Snowy plover distribution and nesting success and human activity during summer, 1990, on Damon Point, Washington. Unpublished report., Washington Department of Wildlife, Olympia, WA. 9 pp.

Funk, W.C., T.D. Mullins, and S.M. Haig. 2006. Conservation genetics of North American and Caribbean Snowy Plovers (Charadrius alexandrinus): population genetic structure and delineation of subspecies. Final Report. USGS Forest and Rangeland Ecosystem Science Center, Corvallis, Oregon.

Fry, D.M. and L.J. Lowenstine. 1985. Pathology of common murres and Cassin's auklets exposed to oil. Archives of Environmental Contamination and Toxicology 14:725-737.

Fry, D.M., J. Swenson, L.A. Addiego, C.R. Grau, and A. Kang. 1986. Reduced reproduction of wedge-tailed shearwaters exposed to weathered Santa Barbara crude oil. Archives of Environmental Contamination and Toxicology 15:453-463.

George, D.E. 1997. Nesting success of snowy plovers at Wilder, Laguna, Scott Creek and Waddell Beaches, Santa Cruz County, California, in 1997. Report of Point Reyes Bird Observatory, Stinson Beach, CA. 12 pp.

Golightly, R.T., Jr., M.R. Faulhaber, K.L. Sallee, and J.C. Lewis. 1994. Food habits and management of introduced red fox in southern California. Pages 15-20 *in* Halverson, W.S., and A. C. Crabb, Eds. Proceedings of the 16th Vertebrate Pest Conference. Published at University of California, Davis.

Gorman, L. R. 2000. Population differentiation among Snowy Plovers (*Charadrius alexandrinus*) in North America. M.S. thesis. Oregon State University, Corvallis, OR. 23pp.

Grinnell, J., H.D. Bryant, and T.I. Storer. 1918. The game birds of California. University of California Museum of Vertebrate Zoology, Berkeley, CA. pp. 473-478.

Guinon, M. 1988. Dune restoration at Spanish Bay. Fremontia. October 1988. pp. 8-11.

Hallett, C.E., B.R. Casler, M.A. Platt, and M.A. Stern. 1995. Snowy plover distribution and reproductive success along the Oregon coast. Submitted to Oregon Department of Fish and Wildlife, Portland, OR; Coos Bay District, Bureau of Land Management, North Bend, OR; and Oregon Dunes National Recreation Area, Reedsport, OR. 40 pp.

Hamilton, R.A., and D.R. Willick. 1996. The birds of Orange County, California. Sea and Sage Press. Sea and Sage Audubon Society, Irvine, California.

Harding, E.K., D.F. Doak, J. Albertson, and J.E. Takekawa. 1998. Predator management in San Francisco Bay wetlands: past trends and future strategies. Prepared for U.S. Fish and Wildlife Service, Sacramento, CA. 41 pp.

Hatch, D. 1997. Draft snowy plover management plan for Ocean Beach, Golden Gate National Recreation Area. 58 pp. plus tables and appendices.

Hayman, P., J. Marchant, and T. Prater. 1986. Shorebirds: an identification guide to the waders of the world. Houghton Mifflin Co., Boston. 412 pp.

Heintz, G.H., D.J. Hoffman, A.J. Krynitsky, and D.M.G. Weller. 1987. Reproduction in mallards fed selenium. Environmental Toxicology and Chemistry 6:423-433.

Hickey, C.M., G.W. Page, and K. Wilson. 1995. Nesting success of snowy plovers at Point Reyes National Seashore in 1995. Report of Point Reyes Bird Observatory, Stinson Beach, CA. 10 pp.

Hoopes, E.M., C.R. Griffin, and S.M. Melvin. 1992. Relationships between human recreation and piping plover foraging ecology and chick survival. Unpublished report. University of Massachusetts, Amherst, MA. 77 pp.

Hothem, R.L. and A.N. Powell. 2000. Contaminants in western snowy plovers and California least terns: Is there a link to population decline? Bulletin of Environmental Contamination and Toxicology 65:42-50.

Howard, J.M., R.J. Safran, and S.M. Melvin. 1993. Biology and conservation of piping plovers at Breezy Point, New York. Unpublished report. Department of Forestry and Wildlife Management, University of Massachusetts, Amherst. 34 pp.

Hughes, R. 2003. SS Jacob Luckenbach oil removal project completed. The OSPR News 10:1-5.

Hutchinson, E.S., G.W. Page, and P.E. Persons. 1987. The nesting of snowy plovers on Morro Bay sand spit during and after the 1987 maintenance dredging of Morro Bay harbor. Report of Point Reyes Bird Observatory, Stinson Beach, CA. 16 pp.

Jacobs, R.A. 1986. Snowy plover (*Charadrius alexandrinus*). Section 4.4.1, U.S. Army Corps of Engineers Wildlife Resources Management Manual, Technical Report EL-86-54, Portland, OR. 25 pp.

Jaques, D. 2001. Snowy plovers at Leadbetter Point, Washington- 2000 Annual Report. U.S. Fish and Wildlife Service, Willapa National Wildlife Refuge. 19 pp.

Jurek, R.M. 1992. Non-native red foxes in California. California Department of Fish and Game, Wildlife Management Division, Nongame Bird and Mammal Section Report 92-04. Sacramento, CA. 16 pp.

Kelly, P. and J. Rotenberry. 1996/1997. Buffer zones for ecological reserves in California: replacing guesswork with science. Native Species Network. Winter 1996/97.

Khan, R.A. and P. Ryan. 1991. Long-term effects of crude oil on common murres (Uria aalge) following rehabilitation. Bulletin of Environmental Contamination and Toxicology 46:216-222.

Kindinger, M.E. 1981. Impact of the Ixtoc I oil spill on the community structure of intertidal and subtidal infauna along south Texas beaches. M.S. Thesis, Corpus Christi State University, Corpus Christi, TX. 91 pp.

King, K.A. and C.A. Lefever. 1979. Effects of oil transferred from incubating gulls to their eggs. Marine Pollution Bulletin 10:319-321.

Kloempken, D. and S.A. Richardson. 1995. Snowy plovers and human activity at Leadbetter Point in April 1995. 8 pp. plus survey form.

Lafferty, K. 2001. Disturbance to wintering western snowy plovers. Biological Conservation 101:315-325.

Larsen, E.M. and S.A. Richardson. 1990. Some effects of a major oil spill on wintering shorebirds at Grays Harbor, Washington. Northwestern Naturalist 71:88-92.

Lauten, D.J., K. A. Castelein, E. Seckinger, and E. P. Gaines. 2006*a*. The distribution and reproductive success of the western snowy plover along the Oregon coast - 2005. The Oregon Natural Heritage Information Center Institute for Natural Resources, Portland, OR.

Lauten, D.J., K. A. Castelein, S. Weston, K. Eucken, and E. P. Gaines. 2006*b*. The distribution and reproductive success of the western snowy plover along the Oregon coast - 2006. The Oregon Natural Heritage Information Center Institute for Natural Resources, Portland, OR.

Lebreton, J.-D., K.P. Burnham, J. Clobert, and D.R. Anderson. 1992. Modeling survival and testing biological hypotheses using marked animals: a unified approach with case studies. Ecological Monographs 62:67-118.

Leibezet, J.R., and T. L. George. 2002. A summary of predation by corvids on threatened and endangered species in California and management recommendations to reduce corvid predation. California Department of Fish and Game, Species Conservation and Recovery Program Report 2002-02, Sacramento, CA. 103 pp.

Leighton, F.A. 1991. The toxicity of petroleum oils to seabirds: an overview. Pages 43-57 *in* White, J. (Ed.). The effects of oil on wildlife. Sheridan Press, Hanover, PA.

LeValley, R., S. McAllister, and A. Transou. 2001. Effects of the Stuyvesant spill on reproductive success of the western snowy plover at Clam Beach, Humboldt County, California, Year 2000 Season. Unpublished draft report to California Dept. of Fish and Game. 19 pp.

Lewis, J.C., R.T. Golightly, and R.M. Jurek. 1995. Introduction of non-native red foxes in California: Implications for the Sierra Nevada red fox. Transactions of the Western Section of the Wildlife Society 31:29-32.

Lewis, J.C., K.L. Sallee, and R.T. Golightly. 1993. Introduced red fox in California. California Department of Fish and Game, Nongame Bird and Mammal Section Report 93-10. Sacramento, CA. 56 pp plus appendices.

Lingle, G.R., J.G. Sidle, A. Hecht, and E.M. Kirsch. 1999. Observations of banding-related leg injuries in the piping plover. Pp. 118-123 *in* Higgins, K.F., M.R. Brashier, and C.D. Kruse (eds). Proceedings, Piping plovers and least terns of the Great Plains and nearby. Brookings: South Dakota State University.

Lockyer, B., R.M. Frank, T. Berger, B. Hembacher, G.R. Overton, T.L. Samsonetti, S. O'Rourke, J.S. Gordon, L.W. Weidman, and M.L.Miller. 2002. Consent Decree, United States of America and State of California v. Torch Energy Services, Inc., CV-02-3977. U.S. District Court, Central District of California, Western Division.

Mabee, T.J. and V.B. Estelle. 2000. Assessing the effectiveness of predator exclosures for plovers. Wilson Bulletin 112(1):14-20.

Mangel, M., and C. Tier. 1994. Four facts every conservation biologist should know about persistence. Ecology 75:607-614.

Marshall, D.B., M.G. Hunter, and A.L. Contreras, Eds. 2003. Birds of Oregon: a general reference. Oregon State University Press, Corvallis, OR. 768pp.

Marriott, M. 2001. Pacific coast western snowy plover monitoring program at the Don Edwards San Francisco Bay NWR and Eden Landing Ecological Reserve and selected Cargill Salt Division properties. Unpublished report to San Francisco Bay NWR Complex; Fremont, CA.

McCaskie, G., and K.L. Garrett. 2005. Southern California summary. North American Birds 59:493-497.

McGrath Oil Spill Restoration Scoping Document (Berry Petroleum). 1995. 6 pp.

McIvor, L.H. 1991. Research proposal: Conditioned taste aversion: A technique to reduce red fox predation at piping plover nests on Assateague Island National Seashore. Maryland Department of Natural Resources, Annapolis, MD. 8 pp.

Meffe, G., and C. Carroll. 1994. Principles of conservation biology. Sinauer Associates, New York.

Mills, L.S., and F.W. Allendorf. 1996. The one-migrant-per-generation rule in conservation and management. Conservation Biology 10:1509-1518.

Molina, K.C., and M. Erwin. 2006. The distribution and conservation status of the gull-billed tern (*Gelochelidon nilotica*) in North America. Waterbirds 29:271-295.

Monson, G. and A.R. Phillips. 1981. Annotated checklist of the birds of Arizona, 2d ed. University of Arizona Press, Tucson. 240 pp.

National Audubon Society. 2006. West Nile virus. Retrieved from: http://www.audubon.org/bird/wnv/ Accessed July 25, 2006.

National Research Council. 1985. Oil in the sea: inputs, fates and effects. National Academy Press, Washington, D.C. 601 pp.

National Research Council. 1995. Science and the Endangered Species Act. National Academy Press; Washington, DC. 271 pages.

Neuman, K.K., G.W. Page, L.E. Stenzel, J.C. Warriner, and J.S. Warriner. 2004. Effect of mammalian predator management on snowy plover breeding success. Waterbirds 27:257-263.

Ohlendorf, H.M., R.L. Hothem, C.M. Bunck, T.W. Aldrich, and J.F. Moore. 1986. Relationships between selenium concentrations and avian reproduction. Transactions of the Fifty-first North American Wildlife and Natural Resources Conference 51:330-342.

Oregon Department of Fish and Wildlife. 1994. Final Draft. Oregon conservation program for the western snowy plover (*Charadrius alexandrinus nivosus*). Portland, OR. 56 pp.

Oregon Department of Fish and Wildlife. 1996. Snowy plover habitat restoration on the Coos Bay North Spit using inmate labor. 21 pp.

Page, G.W. 1988. Nesting success of snowy plovers in central coastal California in 1988. Report of the Point Reyes Bird Observatory, Stinson Beach, CA. 7 pp.

Page, G.W. 1990. Nesting success of snowy plovers in central coastal California in 1989 and 1990. Report of the Point Reyes Bird Observatory, Stinson Beach, CA. 13 pp.

Page, G.W. and P.E. Persons. 1995. The snowy plover at Vandenberg Air Force Base: population size, reproductive success, and management. Point Reyes Bird Observatory, Stinson Beach, CA. 24 pp. plus appendices.

Page, G.W. and L.E. Stenzel (eds.). 1981. The breeding status of the snowy plover in California. Western Birds 12(1):1-40.

Page, G.W., J.S. Warriner, and L.E. Stenzel. 1997. Nesting success of snowy plovers on Monterey Bay in 1997. Unpublished report of Point Reyes Bird Observatory, Stinson Beach, CA. 10 pp.

Page, G.W., F.C. Bidstrup, R.J. Ramer, and L.E. Stenzel. 1986. Distribution of wintering snowy plovers in California and adjacent states. Western Birds 17(4):145-170.

Page, G.W., L.E. Stenzel, W.D. Shuford, and C.R. Bruce. 1991. Distribution and abundance of the snowy plover on its western North American breeding grounds. Journal of Field Ornithology 62(2):245-255.

Page, G.W., L.E. Stenzel, D.W. Winkler, and C.W. Swarth. 1983. Spacing out at Mono Lake: breeding success, nest density, and predation in the snowy plover. The Auk 100:13-24.

Page, G.W., J.S. Warriner, J.C. Warriner, and R.M. Halbeisen. 1977. Status of the snowy plover on the northern California coast. Part I: Reproductive timing and success. California Department of Fish and Game Nongame Wildlife Investigations, Sacramento, CA. 6 pp.

Page, G.W., J.S. Warriner, J.C. Warriner, and P.W.C. Paton. 1995*a*. Snowy plover (*Charadrius alexandrinus*). *In* The Birds of North America, No. 154 (A. Poole and F. Gill, eds.). The Academy of Natural Sciences, Philadelphia, PA, and The American Ornithologists' Union, Washington, D.C. 24 pp.

Page, G.W., M.A. Stern, and P.W. Paton. 1995*b*. Differences in wintering areas of snowy plovers from inland breeding sites in western North America. The Condor 97:258-262.

Page, G.W., J.S. Warriner, J.C. Warriner, D.E. George, and L.E. Stenzel. 1997. Nesting success of snowy plovers at Monterey Bay and northern Santa Cruz County pocket beaches in 1996. Report of Point Reyes Bird Observatory, Stinson Beach, CA. 12 pp.

Page, G.W., D. Dixon, C. Eyster, D.E. George, K. Neuman, L.E. Stenzel, J.C. Warriner, and J.S. Warriner. 1998. Reproduction of snowy plovers at Monterey Bay and pocket beaches of northern Santa Cruz County in 1998. Unpubl. report of Point Reyes Bird Observatory, Stinson Beach, CA. 12 pp.

Palacios, E.P., L. Alfaro, and G.W. Page. 1994. Distribution and abundance of breeding snowy plovers on the Pacific coast of Baja California. Journal of Field Ornithology 65(4):490-497.

Patton, R. 2006a. The status of western gull-billed terns at South San Diego Bay National Wildlife Refuge. Unpublished draft report for the U.S. Fish and Wildlife Service, San Diego National Wildlife Refuge Complex, Carlsbad, California. November 2006. 16 pp. plus tables and figures.

Patton, R. 2006b. Foraging by western gull-billed terns at Tijuana Slough National Wildlife Refuge and Borderfield State Park in 2006. Unpublished final report for the U.S. Fish and Wildlife Service, San Diego National Wildlife Refuge Complex, Carlsbad, California. November 2006. 10 pp. plus tables and figures.

Pearson, S.F, C. Sundstrom, K. Brennan, and M. Fernandez. 2006. Snowy plover distribution, abundance, and reproductive success: 2006 research progress report. Washington Department of Fish and Wildlife, Olympia, WA.

Persons, P.E. 1994. Western snowy plover monitoring in 1993 at Vandenberg Air Force Base, California. Unpublished report of U.S. Fish and Wildlife Service, Ventura, CA. 22 pp.

Persons, P.E. 1995. Western snowy plover population size and nesting success in 1994 at Vandenberg Air Force Base, California. A monitoring report prepared for U.S. Fish and Wildlife Service, Ventura, CA. 21 pp.

Persons, P.E. and T.E. Applegate. 1996. Western snowy plover population size and reproductive success in 1996 at Vandenberg Air Force Base, California. Point Reyes Bird Observatory, Stinson Beach, CA. 35 pp. plus maps.

Persons, P.E. and T.E. Applegate. 1997. Monitoring of the western snowy plover at Vandenberg Air Force Base in 1997: population size, reproductive success, and management. Point Reyes Bird Observatory, Stinson Beach, CA. 30 pp. plus map.

Persons, P.E. and J.A. Ellison. 2001. Nesting of the Western Snowy Plover at Morro Bay Sandspit in San Luis Obispo County, California in 2000.

Peterlein, C., and D. Roth. 2003. Distribution, protection and reproductive success of snowy plovers at Point Reyes National Seashore in 2003. Report to the National Park Service. PRBO contribution number 1071.

Pethick, J. 1984. An introduction to coastal geomorphology. 260 pp.

Pfister, C., B. Harington, and M. Lavine. 1992. The impact of human disturbance on shorebirds at a migration staging area. Biological Conservation 60:115-126.

Philip Williams & Associates, EDAW, H.T. Harvey & Associates, and Brown & Caldwell. 2006. South Bay Salt Pond Restoration Project: Final Alternatives Report. Report submitted to California State Coastal Conservancy, U.S. Fish and Wildlife Service, and California Department of Fish and Game. 122 pp.

Pickart, A.J. 1997. Control of European beachgrass (*Ammophila arenaria*) on the west coast of the United States. Proceedings, 1997, California Exotic Plant Council Annual Meeting, Concord, CA. 13 pp.

Pickart, A.J. and J.O. Sawyer. 1998. Ecology and restoration of northern California coastal dunes. California Native Plant Society, Sacramento, CA. 172 pp.

Powell, A.N. 1996. Western snowy plover use of state-managed lands in southern California, 1995. California Department of Fish and Game, Wildlife Management Division, Bird and Mammal Conservation Program Report 96-103, Sacramento, CA. 14 pp.

Powell, A.N. 2001. Habitat characteristics and nest success of snowy plovers associated with California least tern colonies. The Condor 103:785-792.

Powell, J.A. 1981. Endangered habitats for insects: California coastal sand dunes. Atala 6:41-55.

Powell, A.N. and C.L. Collier. 1994. The status of western snowy plovers (*Charadrius alexandrinus nivosus*) in San Diego County, 1994. Report to California Department of Fish and Game, Sacramento, CA, and U.S. Fish and Wildlife Service, Portland, OR. 28 pp.

Powell, A.N. and C.L. Collier. 1995. The status of western snowy plovers (*Charadrius alexandrinus nivosus*) at Camp Pendleton, 1995. Annual breeding season interim summary report to the Assistant Chief of Staff, Environmental Security, Marine Corps Base, Camp Pendleton, CA. 32 pp.

Powell, A.N., B.L. Peterson, and J.M. Terp. 1996. The status of western snowy plovers (*Charadrius alexandrinus nivosus*) in San Diego County, 1996. Report to the California Department of Fish and Game, Sacramento, CA, and U.S. Fish and Wildlife Service, Carlsbad, CA, and Portland, OR. 25 pp.

Powell, A.N., J.M. Terp, C.L. Collier, and B.L. Peterson. 1995. The status of western snowy plovers (*Charadrius alexandrinus nivosus*) in San Diego County, 1995. Report to the California Department of Fish and Game, Sacramento, CA, and U.S. Fish and Wildlife Service, Carlsbad, CA, and Portland, OR. 24 pp.

Powell, A.N., J.M. Terp, C.L. Collier, and B.L. Peterson. 1997. The status of western snowy plovers (*Charadrius alexandrinus nivosus*) in San Diego County, 1997. Report to the California Department of Fish and Game, Sacramento, CA, and U.S. Fish and Wildlife Service, Carlsbad, CA, and Portland, OR. 34 pp.

Powell, A.N., C.L. Fritz, B.L. Peterson, J.M. Terp. 2002. Status of breeding and wintering Snowy Plovers in San Diego County, California, 1994-1999. Journal of Field Ornithology 73(2):156-165.

Raup, D.M. 1991. Extinction: bad genes or bad luck? W. W. Norton & Company, New York.

Remsen, J.V., Jr. 1978. Bird species of special concern in California. California Department of Fish and Game, Sacramento, CA. 54 pp.

Richardson, S.A., P.J. Doran, W.A. Michaelis, C.S. Sundstrom-Bagley, J.L. Anthony and H.M. Bahn. 2000. A new snowy plover nesting area in Washington: Midway Beach, Pacific County. Washington Birds 7:25-35.

Roletto, J., J. Martinson, L. Grella, and L. Culp. 2000. Beach Watch Annual Report: 2000. Unpublished report, Gulf of the Farallones National Marine Sanctuary, San Francisco, California. 61 pp.

Ruhlen, T.D., S. Abbott, L.E. Stenzel, G.W. Page. 2003. Evidence that human disturbance reduces snowy plover chick survival. Journal of Field Ornithology 74(3):300-304.

Ryan, T.P. and J.L. Parkin. 1998. The western snowy plover (*Charadrius alexandrinus nivosus*) in southern San Francisco Bay. Summary of detections made during colonial waterbird monitoring surveys from 1981 to 1997. Prepared for Santa Clara Valley Water District, San Jose, CA. 19 pp.

Saul, S.M. 1982. Clam diggers and snowy plovers. Washington Wildlife 32(1):28-30.

Schultz, R. and M. Stock. 1993. Kentish plovers and tourists: competitors on sandy coasts? Wader Study Group Bulletin 68:83-91.

Schwarzbach, S.E., M. Stephenson, T. Adelsbach, T. Ruhlen, S. Abbott, L.E. Stenzel, and G.W. Page. 2003. Elevated mercury concentrations in failed eggs of snowy plovers at Point Reyes National Seashore. Unpublished manuscript. 17 pp.

Schwendiman, J. L. 1975. Coastal dune stabilization in the Pacific Northwest. International Journal of Biometeorology 21:281-289.

Seabloom, E.W. and A.M. Wiedemann. 1994. Distribution and effects of *Ammophila breviligulata* Fern. (American beachgrass) on the foredunes of the Washington coast. Journal of Coastal Research 10(2):178-188.

Shuford, W.D., G.W. Page, and C.M. Hickey. 1995. Distribution and abundance of snowy plovers wintering in the interior of California and adjacent states. Western Birds 26:82-98.

Sibley, C.G. and B. L. Monroe, Jr. 1990. Distribution and taxonomy of birds of the world. Yale University Press, New Haven and London. 1111 pp.

Slobodchikoff, C.N. and J.T. Doyen. 1977. Effects of *Ammophila arenaria* on sand dune arthropod communities. Ecology 58:1171-1175.

Smith, G.J. and V.P. Anders. 1989. Toxic effects of boron on mallard reproduction. Environmental Toxicology and Chemistry 8:943-950.

Stanley, T.R., Jr., J.W. Spann, G.J. Smith, and R. Roscoe. 1994. Main and interactive effects of arsenic and selenium on mallard reproduction and duckling growth and survival. Archives of Environmental Contamination and Toxicology 26:444-451.

Stein, R. 1993. Population size and reproductive success of snowy plovers at Skunk Point, Santa Rosa Island. Point Reyes Bird Observatory, Stinson Beach, CA. 38 pp.

Stenzel, L.E., S.C. Peaslee, and G.W. Page. 1981. II. Mainland Coast. Pages 6-16 in Page, G.W. and L.E. Stenzel, (eds.). The breeding status of the snowy plover in California. Western Birds 12(1):1-40.

Stenzel, L.E., J.C. Warriner, J.S. Warriner, K.S. Wilson, F.C. Bidstrup, and G.W. Page. 1994. Long-distance breeding dispersal of snowy plovers in western North America. Journal of Animal Ecology 63:887-902.

Stern, M.A., J.S. McIver, and G.A. Rosenberg. 1990. Investigations of the western snowy plover at the Coos Bay North Spit and adjacent sites in Coos and Curry Counties, Oregon, 1990. Report to Oregon Department of Fish and Wildlife Nongame Program. 33 pp.

Stern, M.A., J.S. McIver, and G.A. Rosenberg. 1991. Nesting and reproductive success of snowy plovers along the south Oregon coast, 1991. Report to Oregon Department of Fish and Wildlife-Nongame, Roseburg, OR, and Coos Bay District, Bureau of Land Management, North Bend, OR. 18 pp.

Stern, M.A., D.J. Lauten, K.A. Castelein, K.J. Popper, and J.A. Fukuda. 2000. Impact assessment of oil spilled from the New Carissa on the Western Snowy Plover along the Oregon Coast. Unpublished report by the Oregon Natural Heritage Program and The Nature Conservancy to TMM Co, Ltd; Coos Bay District Bureau of Land Management; Oregon Dept. of Fish and Wildlife; Dunes National Recreation Area; U.S. Fish and Wildlife Service. 32 pp.

Strong, C., and R. Dakin. 2004. Western snowy plover breeding season surveys for 2003. San Francisco Bird Observatory, Alviso, CA. 28 pp.

Strong, C., N. R. Wilson, and J. D. Albertson. 2004. Western snowy plover numbers, nesting success, and avian predator surveys in the San Francisco Bay, 2004. San Francisco Bay Bird Observatory, Alviso, CA, and Don Edwards San Francisco Bay National Wildlife Refuge, Newark, CA. 41 pp.

Sundstrom, C. 2001. 2000 Snowy plover surveys in coastal Washington (Moclips-Tokeland). Washington Department of Fish and Wildlife, Region 6, Montesano, WA. 8 pp.

Sundstrom, C. 2002*a*. 2001 Snowy plover surveys in coastal Washington. Washington Department of Fish and Wildlife, Region 6, Montesano, WA. 8 pp.

Sundstrom, C. 2002*b*. 2002 Snowy plover surveys in coastal Washington. Washington Department of Fish and Wildlife, Region 6, Montesano, WA. 13 pp.

Sundstrom, C. 2003. 2003 Snowy plover surveys in coastal Washington. Washington Department of Fish and Wildlife, Region 6, Montesano, WA. 11 pp.

Sundstrom, C. 2004. 2004 Snowy plover surveys in coastal Washington. Washington Department of Fish and Wildlife, Region 6, Montesano, WA. 14 pp.

Sundstrom, C. 2005. 2005 snowy plover surveys in coastal Washington. Washington Department of Fish and Wildlife, Region 6, Montesano, WA.

Sundstrom-Bagley, C., W. Michaelis, J. Anthony, M. Bahn. 2000. Snowy plover distribution and nesting success in coastal Washington (1999). Washington Department of Fish and Wildlife, Region 6, Montesano, WA. 27 pp.

Tear, T.H., J.M. Scott, P.H. Hayward, and B. Griffith. 1993. Status and prospects for success of the Endangered Species Act: A look at recovery plans. Science 262:976-977.

Trivelpiece, W.Z., R.G. Butler, D.S. Miller, and D.P. Peakall. 1984. Reduced survival of chicks of oil-dosed adult Leach's storm-petrels. Condor 86:81-82.

Tucker, M.A. and A.N. Powell. 1999. Snowy plover diets in 1995 at a coastal southern California breeding site. Western Birds 30:44-48.

Tucci, L., C. Strong, and J. Albertson. 2006. Western snowy plover numbers, nesting success, and predator surveys in the San Francisco Bay - 2005 breeding season. San Francisco Bay Bird Observatory, Alviso, CA, and Don Edwards San Francisco Bay National Wildlife Refuge, Newark, CA. 33 pp.

Tuttle, D.C., R. Stein, and G. Lester. 1997. Snowy plover nesting on Eel River gravel bars, Humboldt County. Western Birds 28:174-176.

U.S. Bureau of Land Management. 1995*a*. Coos Bay Shorelands, Final Management Plan. 25 pp. plus map.

U.S. Bureau of Land Management. 1995*b*. The New River Area of Critical Concern Management Plan. 149 pp

U.S. Bureau of Land Management. 2001. Notice of Intent to Conduct Restoration Planning, M/V New Carissa Natural Resource Damage Assessment. Federal Register 66:56339-56340.

U.S. Coast Guard. 2001. Marine Casualty Investigation Report, Case Number MC99011413. 13 pp.

U.S. Department of Agriculture, Forest Service, Pacific Northwest Region. 1994. Management Plan, Oregon Dunes National Recreation Area, Siuslaw National Forest. 157 pp.

U.S. Department of Agriculture. 2002. Environmental Assessment: Predator damage management to protect the federally threatened Pacific coast population of the western snowy plover in Lane, Douglas, Coos, Curry, Clatsop, Tillamook, and Lincoln Counties, Oregon. Prepared by APHIS-WS Program, Western Region, for USFWS, BLM, USFS, in cooperation with ODFW, OPRD.

U.S. Department of Transportation, Federal Aviation Administration. 1997. Federal Aviation Regulations, Part 91, General Operating and Flight Rules.

U.S. District Court, Central District of California. 1995. Order. Environmental Defense Center, Plaintiff, v. Bruce Babbitt, Secretary of the Interior, *et al.*, Defendants. No. CV 94-5561 ER (Shx). 2 pp.

U.S. District Court, Central District of California. 1998. Order and Judgment. Environmental Defense Center *et al.*, Plaintiffs, v. Babbitt, *et al.,* Defendants. Case No. CV 94-5561 ER (Shx). 4 pp.

U.S. District Court, District of Massachusetts. 1998. Memorandum and Order. United States of America, plaintiff, v. Town of Plymouth, Massachusetts, defendant. Civil Action No. 98-10566-PBS. 29 pp.

U.S. Fish and Wildlife Service. 1983. Endangered and threatened species listing and recovery priority guidelines. Federal Register 48:43098-43105. September 21, 1983.

U.S. Fish and Wildlife Service. 1985. Light-footed clapper rail recovery plan, Portland, OR. 121 pp.

U.S. Fish and Wildlife Service. 1992. Status and trends report on wildlife of the San Francisco Estuary. San Francisco Estuary Project. 315 pp.

U.S. Fish and Wildlife Service. 1993*a*. Endangered and threatened wildlife and plants; determination of threatened status for the Pacific coast population of the western snowy plover; final rule. Federal Register 58(42):12864-12874.

U.S. Fish and Wildlife Service. 1993*b*. Intra-Service Formal Consultation on take of the threatened Pacific coast population of the western snowy plover for scientific purposes and/or enhancement of propagation or survival. Portland, Oregon. 9 pp.

U.S. Fish and Wildlife Service. 1993*c*. Endangered Species Technical Bulletin, Vol. XVIII, No. 2, pp. 7-9.

U.S. Fish and Wildlife Service. 1995*a*. Endangered Species Bulletin, Vol. XX, No. 5. 28 pp.

U.S. Fish and Wildlife Service. 1995*b*. Endangered and threatened wildlife and plants; proposed designation of critical habitat for the Pacific coast population of the western snowy plover; proposed rule. Federal Register 60(41):11768-11809.

U.S. Fish and Wildlife Service. 1996*a*. Piping Plover (*Charadrius melodus*), Atlantic Coast Population, Revised Recovery Plan. Hadley, Massachusetts. 258 pp.

U.S. Fish and Wildlife Service. 1996*b*. Endangered and threatened wildlife and plants; final listing priority guidance for fiscal year 1997; final rule. Federal Register 61:64475-64481.

U.S. Fish and Wildlife Service. 1998. Endangered and threatened wildlife and plants; proposed listing priority guidance for fiscal years 1998 and 1999; proposed rule. Federal Register 63:10931-10935.

U.S. Fish and Wildlife Service. 1999. Endangered and threatened wildlife and plants; Designation of critical habitat for the Pacific coast population of the western snowy plover; final rule. Federal Register 64:68508-68544. December 7, 1999.

U.S. Fish and Wildlife Service. 2002. Draft Recovery Plan for the Great Lakes Piping Plover (*Charadrius melodus*). Fort Snelling, Minnesota. 121 pp.

U.S. Fish and Wildlife Service. 2004*a*. Endangered and threatened wildlife and plants; 90-day finding on a petition to delist the Pacific coast population of the western snowy plover and initiation of a 5-year review. Federal Register 69:13326-13329. March 22, 2004.

U.S. Fish and Wildlife Service. 2004*b*. Endangered and threatened wildlife and plants; Proposed designation of critical habitat for the Pacific coast population of the western snowy plover. Federal Register 69:75608-75771. December 17, 2004.

U.S. Fish and Wildlife Service. 2005. Endangered and threatened wildlife and plants; Designation of critical habitat for the Pacific coast population of the western snowy plover. Federal Register 70:56970-57018. September 29, 2005.

U.S. Fish and Wildlife Service. 2006*a*. Endangered and threatened wildlife and plants; 12-month finding on a petition to delist the Pacific coast population of the western snowy plover. Federal Register 71:20607-20624. April 21, 2006.

U.S. Fish and Wildlife Service. 2006*b*. Endangered and threatened wildlife and plants; Proposed special rule pursuant to section 4(d) of the Endangered Species Act for the Pacific coast distinct population segment of the western snowy plover. Federal Register 71:20625-20636. April 21, 2006.

U.S. Fish and Wildlife Service. 2006*c*. San Diego Bay National Wildlife Refuge Sweetwater Marsh and South San Diego Bay Units Final Comprehensive Conservation Plan and Environmental Impact Statement – August 2006.

U.S. Fish and Wildlife Service. In preparation. Recovery Plan for Tidal Marsh Ecosystems of Northern and Central California.

U.S. Fish and Wildlife Service and National Oceanic and Atmospheric Administration. 1994. Endangered and threatened wildlife and plants: Notice of interagency cooperative policy on recovery plan participation and implementation under the Endangered Species Act. Federal Register 59(126):34272-34273.

U.S. Geological Survey. 2006. West Nile virus maps. Retrieved from: http://diseasemaps.usgs.gov/wnv_us_bird.html Accessed December 27, 2006.

U.S. Navy. 2001. Integrated Natural Resources Management Plan, Naval Base Coronado.

U.S. Marine Corps. 2006. Integrated Natural Resources Management Plan, Marine Corps Base Camp Pendleton. August 2006 draft revision.

Unitt, P. 2004. San Diego County Bird Atlas. San Diego Natural History Museum and Ibis Publishing Company. San Diego, California.

Warriner, J.S., J.C. Warriner, G.W. Page, and L.E. Stenzel. 1986. Mating system and reproductive success of a small population of polygamous snowy plovers. Wilson Bulletin 98(1):15-37.

Washington Department of Fish and Wildlife. 1995. Washington State recovery plan for the snowy plover. Olympia, WA. 87 pp.

White, J.D., and S.G. Allen. 1999. Western snowy plover management plan. Report to the National Park Service, Point Reyes National Seashore, Point Reyes, CA.

White, J.D., and C.M. Hickey. 1997. Distribution, protection and nest success of snowy plovers at Point Reyes National Seashore. A report of Point Reyes Bird Observatory, Stinson Beach, CA. 11 pp.

Widrig, R.S. 1980. Snowy plovers at Leadbetter Point: An opportunity for wildlife management? Prepared for the U.S. Fish and Wildlife Service, Willapa NWR, Ilwaco, WA. 14 pp.

Widrig, R.S. 1981. Snowy plovers at Leadbetter Point. Prepared for the U.S. Fish and Wildlife Service, Willapa NWR, Ilwaco, WA. 13 pp.

Wiedemann, A.M. 1987. The ecology of European beachgrass (*Ammophila arenaria* (L.) Link). A review of the literature. Oregon Department of Fish and Wildlife Nongame Wildlife Program Technical Report #87-1-01. 18 pp.

Wiedemann, A.M., L.J. Dennis, and F.H. Smith. 1969. Plants of the Oregon coastal dunes. Department of Botany, Oregon State University. Oregon State University Book Stores, Inc., Corvallis, OR. 117 pp.

Willapa National Wildlife Refuge. 1988. Willapa National Wildlife Refuge, 1988 annual narrative report. Ilwaco, WA. 46 pp.

Williamson, D.A. 1995. Snowy plovers at Willapa Bay, Washington, 1995. Willapa National Wildlife Refuge Complex, Ilwaco, WA. 13pp.

Williamson, D.A. 1996. Snowy plovers at Willapa Bay, Washington, 1996. Willapa National Wildlife Refuge Complex, Ilwaco, WA. 13 pp.

Williamson, D.A. 1997. Snowy plovers at Willapa Bay, Washington, 1997. Willapa National Wildlife Refuge Complex, Ilwaco, WA. 15 pp.

Wilson, R.A. 1980. Snowy plover nesting ecology on the Oregon coast. MS Thesis, Oregon State University, Corvallis. 41 pp.

Wilson-Jacobs, R., and G.L. Dorsey. 1985. Snowy plover use of Coos Bay north spit, Oregon. Murrelet 66(3):75-81.

Wilson-Jacobs, R., and E.C. Meslow. 1984. Distribution, abundance, and nesting characteristics of snowy plovers on the Oregon coast. Northwest Science 58(1):40-48.

Woolington, M.C. 1985. A preliminary investigation of the effect of recreational use on nesting snowy plovers at Sutton and Siltcoos beach areas, Oregon. Oregon Department of Fish and Wildlife Nongame program. 37 pp.

B. Personal Communications

Albertson, J. 2005. Don Edwards - San Francisco Bay National Wildlife Refuge, U.S. Fish and Wildlife Service, Newark, CA.

Applegate, T. 1996, 1999. Bioresources, Los Osos, CA.

Baye, P. 1997. Sacramento Fish and Wildlife Office, U.S. Fish and Wildlife Service, Sacramento, CA.

Burns, C. U.S. Forest Service, Siuslaw National Forest, Mapleton Ranger District, Florence, OR.

Dorsey, G. 1997. U.S. Army Corps of Engineers, Portland, OR.

Fernandez, E. 1998. Don Edwards-San Francisco Bay National Wildlife Refuge, U.S. Fish and Wildlife Service, Newark, CA.

George, D. 1998. Point Reyes Bird Observatory, Stinson Beach, CA.

Grettenberger, J. 2004. Western Washington Fish and Wildlife Office, U.S. Fish and Wildlife Service, Lacey, WA.

Heaney, J. 2003. Bureau of Land Management, Coos Bay District, North Bend, OR. June 27, 2003.

Hecht, A. 1996. U.S. Fish and Wildlife Service, Hadbury, MA.

Klinger, R. 1998. The Nature Conservancy, Santa Barbara, CA.

Kolar, M. 2004. Manager, San Francisco Bay National Wildlife Refuge Complex, U.S. Fish and Wildlife Service, Newark, CA.

Mangan, L. 2003. Bureau of Land Management, Coos Bay District, North Bend, OR.

Neuman, K. 1997. California Department of Parks and Recreation, Monterey, CA.

Page, G. 1997, 1998. Point Reyes Bird Observatory, Stinson Beach, CA.

Palermo, K. 1998. U.S. Forest Service, Oregon Dunes National Recreation Area, Siuslaw National Forest, Reedsport, OR.

Pearson, D. 1996. Southern California Edison, Rosemead, CA.

Pickart, A. 1997. The Nature Conservancy, Arcata, CA.

Powell, A.N. 1998. U.S. Geological Survey, Biological Resources Division, Fayetteville, AR.

Read, N. 1998. U.S. Department of the Air Force, Vandenberg Air Force Base, California.

Sandoval, C. 2005. University of California, Santa Barbara, CA.

Stadtlander, D. 1999. Carlsbad Fish and Wildlife Office, U.S. Fish and Wildlife Service, Carlsbad, CA.

Richardson, S. 1998. Washington Department of Fish and Wildlife, Olympia, WA.

Stern, M. 1999. The Nature Conservancy, Oregon Natural Heritage Program, Portland, OR.

VanderHeyden, M. Bureau of Land Management, Coos Bay District, North Bend, OR.

Vissman, S. 2007. U.S. Fish and Wildlife Service, Carlsbad, CA.

Walton, B. 1998. Predatory Bird Research Group, University of California at Santa Cruz, Santa Cruz, CA.

Watkins, J. 2001, 2006. Arcata Fish and Wildlife Office, U.S. Fish and Wildlife Service, Arcata, California.

C. *In Litt.* References

Albertson, J. 1999. Don Edwards-San Francisco Bay National Wildlife Refuge, U.S. Fish and Wildlife Service, Newark, California. Electronic message to U.S. Fish and Wildlife Service, Sacramento, CA, on working draft of the Western Snowy Plover Recovery Plan. 4 pp.

Allen, S. 2004. Point Reyes National Seashore, Point Reyes, CA. Electronic message to Valary Bloom, U.S. Fish and Wildlife Service, Sacramento, CA, providing comments on the Western Snowy Plover Draft Recovery Plan. 3 pp.

Bloom, V. 2005. U.S. Fish and Wildlife Service, Sacramento, CA. Electronic message to Cay Goude, U.S. Fish and Wildlife Service, Sacramento, CA, regarding snowy plover management in San Francisco Bay. 1 p.

Buffa, J. 2004. Don Edwards-San Francisco Bay National Wildlife Refuge, U.S. Fish and Wildlife Service, Newark, California. Electronic message to Valary Bloom, U.S. Fish and Wildlife Service, Sacramento, CA, providing comments on the Western Snowy Plover Draft Recovery Plan. 4 pp.

Copper, E., and B. Foster. 2001. Letter to U.S. Fish and Wildlife Service, Sacramento, CA, providing comments and additional information on the Western Snowy Plover Pacific Coast Population Draft Recovery Plan.

Didion, J. 1999. California Department of Parks and Recreation, Sacramento, CA. Letter to U.S. Fish and Wildlife Service, Sacramento, CA, on working draft of the Western Snowy Plover Recovery Plan. 6 pp.

Dixon, D. 1998. California Department of Parks and Recreation, Monterey, CA. Information provided to U.S. Fish and Wildlife Service, Sacramento, CA, regarding European beachgrass control and snowy plover guardian program. 5 pp.

Dyste, R. 2004. Comprehensive Planning Division, County of Santa Barbara, Santa Barbara, CA. Electronic message to Valary Bloom, U.S. Fish and Wildlife Service, Sacramento, CA, providing comments on the Western Snowy Plover Draft Recovery Plan. 3 pp.

George, D. 2001. Letter to U.S. Fish and Wildlife Service, Sacramento, CA, on working draft of the Western Snowy Plover Recovery Plan. 1 pp.+ attachment.

Goldsmith, G. 2004. U.S. Fish and Wildlife Service, Arcata, CA. Electronic message to Jim Watkins, U.S. Fish and Wildlife Service, Arcata, CA.

Henry, S. 1998. U.S. Fish and Wildlife Service, Ventura, CA. Electronic message to U.S. Fish and Wildlife Service, Sacramento, CA. 2 pp.

Jensen, M. 2006a. U.S. Fish and Wildlife Service, Lacey, WA. Table of window survey data. 1 p.

Jensen, M. 2006b. U.S. Fish and Wildlife Service, Lacey, WA. Electronic message to Grant Canterbury, U.S. Fish and Wildlife Service, Portland OR. 1 p.

Kelly, L. 2005. U.S. Fish and Wildlife Service, Newport, OR. Comparison of the 2005 winter and 2005 summer Snowy Plover surveys of the Oregon/Washington coast. (Recovery Unit 1). 2 pp.

Long, M. 2006. U.S. Fish and Wildlife Service, Arcata, CA. Botulism antitoxin treatment for federally threatened Pacific coast population of western snowy plover in southern California. Memo to Rex Sohn, U.S.G.S. National Wildlife Health Center, Madison, WI. 2 pp.

Mesta, R. 1998. U.S. Fish and Wildlife Service, Ventura, CA. Comments provided to U.S. Fish and Wildlife Service, Sacramento, CA, on the working draft of the Western Snowy Plover Recovery Plan. 3 pp.

Moulton, C. 1997. Manager, Eureka Fisheries, Inc., Fields Landing, CA. Letter to Field Supervisor, U.S. Fish and Wildlife Service, Sacramento, CA. 2 pp.

Myers, J.P. 1988. Senior Vice President, Science and Sanctuaries, National Audubon Society, New York, N.Y. Letter to Director, U.S. Fish and Wildlife Service, Washington, D.C. 19 pp.

Page, G.W. 1988. Point Reyes Bird Observatory, Stinson Beach, CA. Letter to U.S. Fish and Wildlife Service, Portland, OR. 1 p.

Page, G.W. 2004*a*. Point Reyes Bird Observatory, Stinson Beach, CA. Letter to Field Supervisor, Sacramento Fish and Wildlife Office, U.S. Fish and Wildlife Service, Sacramento, CA, providing comments on reassessment of snowy plover listing status. 20 pp.

Page, G.W. 2004*b*. Point Reyes Bird Observatory, Stinson Beach, CA. Letter to Glen Tarr, Sacramento Fish and Wildlife Office, U.S. Fish and Wildlife Service, Sacramento, CA, providing comments on western snowy plover management in Monterey Bay area. 3 pp.

Page, G.W. 2005*a*. Point Reyes Bird Observatory, Stinson Beach, CA. Year 2005 breeding season snowy plover survey of California coast. Electronic file sent to U.S. Fish and Wildlife Service. 3 pp.

Page, G.W. 2005*b*. Point Reyes Bird Observatory, Stinson Beach, CA. Snowy plover banding study proposal. Electronic message to U.S. Fish and Wildlife Service, Arcata, CA. 6 pp.

Page, G. W. 2006. Point Reyes Bird Observatory, Stinson Beach, CA. Comparison of the 2005 and 2006 snowy plover surveys of the Pacific coast. Electronic file sent to U.S. Fish and Wildlife Service. 8 pp.

Palermo, K. 1998*a*. U.S. Forest Service, Oregon Dunes National Recreation Area, Siuslaw National Forest, Reedsport, OR. Letter to U.S. Fish and Wildlife Service, Sacramento, CA, on the working draft of the Western Snowy Plover Recovery Plan. 2 pp.

Pearson, S. 2006. Washington Department of Fish and Wildlife, Olympia, WA. Electronic message to Martha Jensen, U.S. Fish and Wildlife Service, Lacey, WA. 2 pp.

Palermo, K. 1998*b*. U.S. Forest Service, Oregon Dunes National Recreation Area, Siuslaw National Forest, Reedsport, OR. Electronic message to U.S. Fish and Wildlife Service, Sacramento, CA, on European beachgrass control. 1 p.

Pickart, A. 1996. The Nature Conservancy, Arcata, CA. Information provided to U.S. Fish and Wildlife Service, Sacramento, CA, regarding the status of European beachgrass in California. 2 pp.

Price, J.B. 1992. Chief Ranger, Department of Parks and Recreation, Ventura, CA. Memorandum to Oxnard Police Department *et al.* 1 p.

Read, N. 1998. Vandenberg Air Force Base, CA. Electronic message to U.S. Fish and Wildlife Service, Sacramento, CA, on the working draft of the Western Snowy Plover Recovery Plan. 2 pp.

Read Francine, N. 2001. Department of the Air Force, Vandenberg Air Force Base, CA. Letter to U.S. Fish and Wildlife Service, Sacramento, CA, providing comments and additional information on the Western Snowy Plover Pacific Coast Population Draft Recovery Plan. 8 pp.

Richardson, S. 2001. Bureau of Reclamation. Letter to U.S. Fish and Wildlife Service, Sacramento, CA, providing comments and additional information on the Western Snowy Plover Pacific Coast Population Draft Recovery Plan.

Trulio, L. 2007. Electronic message to Valary Bloom, U.S. Fish and Wildlife Service, Sacramento, CA, on snowy plover habitat in San Francisco Bay.

U.S. Department of the Navy. 2001. Letter from U.S. Department of the Navy, China Lake, CA, to the U.S. Fish and Wildlife Service, Sacramento, CA, on increasing pinniped populations on San Nicholas Island.

U.S. Department of the Navy. 2007. Letter to U.S. Fish and Wildlife Service, Carlsbad, CA, requesting re-initiation of consultation at Naval Base Coronado. 13 pp.

U.S. Fish and Wildlife Service. 1995. Memorandum from Acting Field Supervisor, Ventura Field Office, Ecological Services, Ventura, CA, to Superintendent, Channel Islands National Park, National Park Service, Ventura, CA. 26 pp.

Watkins, J. 1999. U.S. Fish and Wildlife Service, Arcata, CA. Electronic message to U.S. Fish and Wildlife Service, Sacramento, CA, on the working draft of the Western Snowy Plover Recovery Plan. 11 pp.

Zielinski, E.Y. 1999. U.S. Bureau of Land Management, Portland, OR, and R.W. Williams, U.S. Forest Service, Portland, OR. Letter to U.S. Fish and Wildlife Service, Sacramento, CA, on the working draft of the Western Snowy Plover Recovery Plan. 15 pp. plus enclosures.

circle of blue
where water speaks

WaterNews

Peter Gleick: Clarifying the Discussion about California Drought and Climate Change

March 7, 2014 / in California, California Drought, In the Circle / by Dr. Peter Gleick

In the last few months, as the severe California drought has garnered attention among scientists, policymakers, and media, there has been a growing debate about the links between the drought and climate change. The debate has been marked by considerable controversy, confusion, and opaqueness.

The confusion stems from the failure of some scientists, bloggers, reporters, and others to distinguish among three separate questions. All three questions are scientifically interesting. But the three are different in their nuance, their importance to policy, and their interest to politicians and water managers. Here are the three different questions:

1. Is the California drought caused by climate change?
2. Is the California drought, no matter the cause, influenced or affected by climate changes already occurring?
3. How will climate changes affect future drought risks in California?

These questions are not the same thing. Yet repeatedly, some have asked one question when they thought they were asking a different one. Some have been asked one

Recent Posts

UN Conference on Water Aims to Rally Support for Ambitious Goals
Federal Water Tap, March 20: EPA Draft Proposal Expands Number of PFAS Regulated in Drinking Water
Majority Say Water Supply and Pollution 'Very Serious' Problems
Perspective | How Water Partnerships Can Yield Better Results
The Stream, March 15, 2023: Another Round of Flooding Expected in

question and intentionally or accidentally answered a different one. Some have confused an answer to one question as an answer to a different question.

Frankly, the entire discussion has been frustrating for all involved. For the sake of clarity in the future, people involved in this discussion should be clear whether they are asking or answering question #1, #2, or #3, or even another question entirely. In addition, the answers for California may be different than other regions of the U.S. or world.

ABOUT THE AUTHOR:

Dr. Peter Gleick is president of the Pacific Institute, an internationally recognized water expert, and a MacArthur Fellow.

And to prime the pump, so to speak, here are my thoughts about the answers, with some relevant recent scientific papers listed at the end.

1. Is the California drought caused by climate change?

The "causality" question is a bad question to ask and I wish journalists would stop asking it, but without a doubt it is the most common one I receive. From a scientific perspective, the influence of climate change on current extreme events is a very exciting research topic and there is evidence linking climate change with some specific recent extremes. Nevertheless, I think the current scientific answer to this question for the California drought is neither "yes" nor "no." We do not know. The current drought, measured by hydrologic variables of precipitation amount, form, location, and timing, is certainly severe, but it does not appear to be statistically significantly different from past droughts in the instrumental or longer-term

California as Atmospheric Rivers Persist

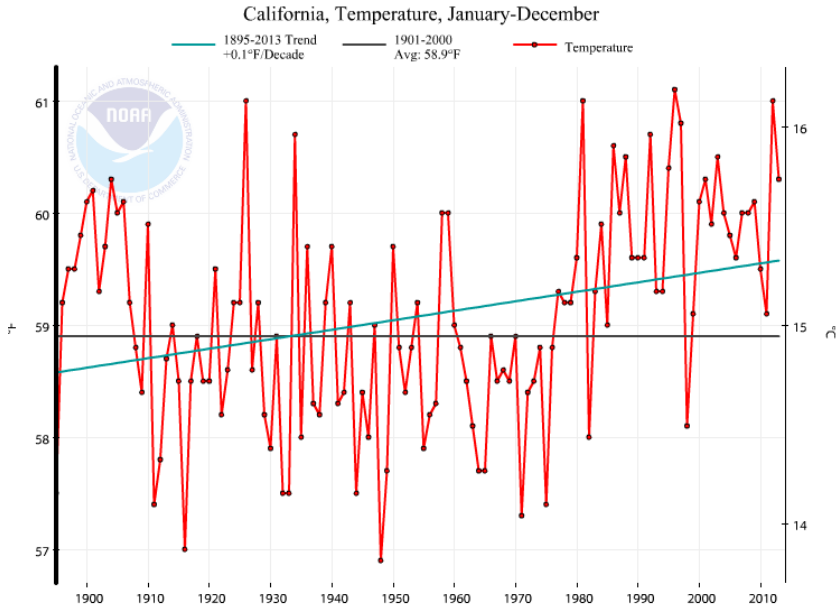
paleoclimatic record. But here is another key point lost to many. Just because the answer is not “yes” does NOT mean the answer is “no.” Get it? This subtle difference is often lost: when a scientist says the current drought doesn’t look much different from some past extreme droughts, this says nothing about causality of the current drought.

2. Is the California drought, no matter the cause, being influenced or affected by climate changes already occurring? This question is much more interesting than the first one about causality. And I think the answer to this is unambiguously “yes,” but we do not know the net/overall influence of current climate changes. We know that climate is changing and that climate changes influence weather. That “influence” can take many forms: changes in storm patterns, precipitation frequency or intensity, the form of precipitation, and so on. And we know that there are many uncertainties about the nature and extent of these changes. But I say the answer to this specific question is “yes” for one simple reason: current average temperatures in California, like average temperatures worldwide, are higher today than they were in the past century because of human-caused climate change (See Figure 1). These higher temperatures worsen the drought through extra soil moisture loss, altered timing of snowmelt, and decreased reservoir levels from extra evaporation. As a new paper in Nature Climate Change states: “Climate change is adding heat to the climate system and on land much of that heat goes into drying.”[1] It is also possible that climate change has influenced other factors, such as the precipitation regime (with more or less precipitation), but the signal of this influence is still buried in the noise. So I’m not saying the net influence of climate change on the current California drought is unambiguously for the worse or better, just that the influence is real, and the most definitive and well-understood effect (higher temperatures) has

decreased current water availability.

Annual temperature trends in California over the past 118 years. (Source: NOAA, 2014)

The increasing trend in annual temperature in California over the past 118 years. (Source: NOAA, 2014). This trend mirrors the global increase.



[https://i0.wp.com/www.circleofblue.org/wp-content/uploads/2014/03/California-Temperature-118-years.png?ssl=1]

The increasing trend in annual temperature in California over the past 118 years. (Source: NOAA, 2014). This trend mirrors the global increase.

3. How will climate changes affect future drought risks in California? There are two answers to this: the first is that climate changes will almost certainly affect California drought risks; but the second is that these effects will be diverse, complicated, and interconnected, and many remain uncertain and exciting topics for current research. Hundreds of research papers have been written about the vulnerability of California water – both the natural hydrology and the complex infrastructure built to manage that hydrology – to climate changes. For the purposes of the current debate around the drought, the question seems to focus on whether future California droughts will be more

or less frequent, and more or less severe. There are many factors that go into assessing this question. The scientific literature has an extensive discussion of all of these factors, some of which have been successfully resolved; others remain highly uncertain. For example, we know with a high degree of certainty that temperatures are going to continue to go up and increase evaporative demands for water. We know with a high degree of certainty that the ratio of rain to snow falling in the mountains will rise, snowpack will diminish, and that the timing of runoff will change. We know with a high degree of certainty that sea-level is rising, with effects on coastal aquifers and brackish water ecosystems. As the Trenberth et al. paper mentioned above notes: “Climate change is adding heat to the climate system and on land much of that heat goes into drying. A natural drought should therefore set in quicker, become more intense, and may last longer. Droughts may be more extensive as a result. Indeed, human-induced warming effects accumulate on land during periods of drought because the ‘air conditioning effects’ of water are absent. Climate change may not manufacture droughts, but it could exacerbate them and it will probably expand their domain in the subtropical dry zone.”

But California droughts are also fundamentally linked to the quantities and timing of precipitation, the dynamics of storm formation in the Pacific Ocean, the impacts of climate change on the frequency and intensity of El Niño and La Niña events and the Pacific Decadal Oscillation, and the behavior of the jet stream as conditions in the Arctic change. These changes are less certain, vitally important to the frequency and intensity of future California drought impacts, and remain serious topics for research and analysis. And as some observers have noted, the risk that future droughts will worsen is more certain for other regions of the world.

The research and the debate over climate change and California droughts will continue. But before commenting, let's make sure we understand what question is actually being asked, what question should be asked, and what question is actually being answered.

Background

Cayan et al., 2010. "Future dryness in the southwest US and the hydrology of the early 21st century drought, PNAS, Vol. 107, December 14, 2010, pp 21271-21276:

Although the recent drought may have significant contributions from natural variability, it is notable that hydrological changes in the region over the last 50 years cannot be fully explained by natural variability, and instead show the signature of anthropogenic climate change.

Dai, A. 2013. "Increasing drought under global warming in observations and models," Nature Climate Change, Vol 3., pp. 52-58. DOI:10.1038/nclimate1633

Trenberth, K. E., A. Dai, G. van der Schrier, P. D. Jones, J. Barichivich, K. R. Briffa, and J. Sheffield, 2014: Global warming and changes in drought. Nature Climate Change, 4, 17-22, doi:10.1038/NCLIMATE2067.

Wehner et al., 2011. "Projections of future drought in the continental United States and Mexico," Journal of Hydrometeorology, Vol. 12, December 2011, pp 1359-1377.

All models, regardless of their ability to simulate the base-period drought statistics, project significant future increases in drought frequency, severity, and extent over the course of the 21st century under the SRES A1B emissions scenario.

Pederson et al., 2011. "The unusual nature of recent snowpack declines in the NorthAmerican Cordillera," Science, Vol. 333, 15 July 2011, pp 332-335.

Over the past millennium, late 20th century snowpack reductions are almost unprecedented in magnitude across the northern Rocky Mountains and in their north-south synchrony across the cordillera. Both the snowpack declines and their synchrony result from unparalleled springtime warming that is due to positive reinforcement of the anthropogenic warming by decadal variability. The increasing role of warming on large-scale snowpack variability and trends foreshadows fundamental impacts on streamflow and water supplies across the western United States.

See also the conclusions from:

The 2013 report of the IPCC's Working Group I, The Science Basis (pdf)

The Technical Summary from IPCC WGI 2013 report (pdf)

[1] Trenberth, K. E., A. Dai, G. van der Schrier, P. D. Jones, J. Barichivich, K. R. Briffa, and J. Sheffield, 2014: Global warming and changes in drought. Nature Climate Change, 4, 17-22, doi:10.1038/NCLIMATE2067.

Tags: California, climate change, Drought, In the Circle: Peter Gleick, sr-cadrought, United States, Water Scarcity

Texas capital may turn to tap Texas capital may turn to tap

Water Resources Highlighted in Climate Adaptation Plans Water Resources Highlighted in Climate Adaptation Plans

El Nino Droughts Take Toll in Africa, Asia, and the Pacific El Nino Droughts Take Toll in Africa, Asia, and the Pacific

Central Texas Drought Is Worst on Record Central Texas Drought Is Worst on Record

American Geophysical Union 2014 Recap: That Sinking, Drying, Sharing Feeling An Ge Ur Re Si Dr Sh Fe

Irrigation Expert Daniel Hillel Wins World Food Prize Irrigation Expert Daniel Hillel Wins World Food Prize

0

REPLIES

Google Analytics Cookies

These cookies collect information that is used either in aggregate form to help us understand how our website is being used or how effective our marketing campaigns are, or to help us customize our website and application for you in order to enhance your experience.

If you do not want that we track your visit to our site you can disable tracking in your browser here:

Other external services

We also use different external services like Google Webfonts, Google Maps, and external Video providers. Since these providers may collect personal data like your IP address we allow you to block them here. Please be aware that this might heavily reduce the functionality and appearance of our site. Changes will take effect once you reload the page.

Google Webfont Settings:

Google Map Settings:

Google reCaptcha Settings:

Vimeo and Youtube video embeds:

Other cookies

The following cookies are also needed - You can choose if you want to allow them:

AGU PUBLICATIONS

Geophysical Research Letters

RESEARCH LETTER
10.1002/2015GL066948

Key Points:
- Anomalous swarm activity associated with White Wolf fault
- Induced seismicity likely caused by localized pressure increase along a seismically active fault
- Induced seismicity may be masked by natural earthquake activity in California

Supporting Information:
- Supporting Information S1
- Tables S1–S8
- Movie S1
- Movie S2
- Figures S1–S22

Correspondence to:
T. H. W. Goebel,
tgoebel@ucsc.edu

Citation:
Goebel, T. H. W., S. M. Hosseini, F. Cappa, E. Hauksson, J. P. Ampuero, F. Aminzadeh, and J. B. Saleeby (2016), Wastewater disposal and earthquake swarm activity at the southern end of the Central Valley, California, *Geophys. Res. Lett.*, *43*, 1092–1099, doi:10.1002/2015GL066948.

Received 8 NOV 2015
Accepted 16 JAN 2016
Accepted article online 22 JAN 2016
Published online 4 FEB 2016

Wastewater disposal and earthquake swarm activity at the southern end of the Central Valley, California

T. H. W. Goebel[1,2], S. M. Hosseini[3], F. Cappa[4,5], E. Hauksson[1], J. P. Ampuero[1], F. Aminzadeh[3], and J. B. Saleeby[1]

[1]Division of Geological and Planetary Sciences, California Institute of Technology, Pasadena, California, USA, [2]Now at Department of Earth and Planetary Sciences, University of California, Santa Cruz, California, USA, [3]Department of Chemical Engineering and Material Sciences, University of Southern California, Los Angeles, California, USA, [4]Geoazur Laboratory, University of Nice Sophia Antipolis, Nice, France, [5]Institut Universitaire de France, Paris, France

Abstract Fracture and fault zones can channel fluid flow and transmit injection-induced pore pressure changes over large distances (>km), at which seismicity is rarely suspected to be human induced. We use seismicity analysis and hydrogeological models to examine the role of seismically active faults in inducing earthquakes. We analyze a potentially injection-induced earthquake swarm with three events above *M*4 near the White Wolf fault (WWF). The swarm deviates from classic main aftershock behavior, exhibiting uncharacteristically low Gutenberg-Richter *b* of 0.6, and systematic migration patterns. Some smaller events occurred southeast of the WWF in an area of several disposal wells, one of which became active just 5 months before the main swarm activity. Hydrogeological modeling revealed that wastewater disposal likely contributed to seismicity via localized pressure increase along a seismically active fault. Our results suggest that induced seismicity may remain undetected in California without detailed analysis of local geologic setting, seismicity, and fluid diffusion.

1. Introduction

Fluid injection into hydrocarbon and geothermal reservoirs can change the local stress field potentially causing earthquakes at several kilometers distance, both immediately and months to years after peak injection [e.g., *Hsieh and Bredehoeft*, 1981; *Bourouis and Bernard*, 2007; *Keranen et al.*, 2013; *Kim*, 2013; *Martínez-Garzón et al.*, 2014; *Schoenball et al.*, 2014; *Rubinstein et al.*, 2014]. Injection-induced fault slip is, in addition to poroelastic stress changes, commonly attributed to an increase in pore pressure, which reduces the frictional resistance on fault surfaces [e.g., *Healy et al.*, 1968; *Hsieh and Bredehoeft*, 1981; *Ellsworth*, 2013; *Keranen et al.*, 2013]. In recent years, induced earthquakes have been observed in the proximity of many high-volume wastewater disposal (WD) wells in the central U.S. [e.g., *Horton*, 2012; *Frohlich and Brunt*, 2013; *Keranen et al.*, 2013; *Kim*, 2013; *Skoumal et al.*, 2014]. Many of the identified injection wells responsible for inducing seismicity are suspected to inject at depth close to the upper basement surface, where critically stressed faults slip more easily when exposed to changing pressures and earthquake ruptures can grow to larger sizes than in sedimentary basins [e.g., *Das and Scholz*, 1983; *Horton*, 2012; *Keranen et al.*, 2013; *Kim*, 2013; *Ellsworth*, 2013].

While many previous studies focused on regions within the central and eastern U.S., where induced event detection is facilitated by low background seismicity rates, detecting induced events in California hydrocarbon basins received less attention, despite extensive injection operations and seismically active faults. Up to now, few injection-induced earthquakes outside of geothermal reservoirs have been observed in California [*Kanamori and Hauksson*, 1992; *Goebel et al.*, 2015]. Consequently, the most acute anthropogenically controlled seismic hazard stems from fluid-injection activity close to active faults. Such injection activity can lead to earthquakes up to M_w5.7 or larger as observed in OK [*Keranen et al.*, 2013].

In California, annual fluid-injection volumes exceed those in OK where induced seismicity is suggested to be widespread. The average number of active injection wells between 2010 and 2013 of ~9900 exceeds the ~8600 wells in OK [*CA Department of Conservation*, 2012]. The wells in California inject on average at depth of ~1.5 km which is about 0.5 km deeper than in OK [*Goebel*, 2015]. More recently and coincident with continuously increasing oil prices between 2001 and 2014 (except 2009 and 2010), California experienced a systematic increase in injection volumes in connection with more extensive well-stimulation operations. This increase

in injection activity together with the lack of available injection sites away from active faults requires a more detailed assessment of possible seismogenic consequences of fluid injection in California.

This study is structured as follows: we first provide an overview of the geologic setting, as well as injection and seismic activity at the southern end of the Central Valley. This is followed by a detailed assessment of the seismically active faults within the study region. We then investigate a possible correlation between fluid injection and seismicity, including variations in frequency-magnitude distribution with the onset of injection rate increase. Lastly, we create a detailed hydrogeological model that incorporates local geology such as active fault structures and examine injection-induced pressure changes.

2. Swarm Activity, Wastewater Disposal Operations, and Geological Setting

This study concentrates on a potentially injection-induced earthquake swarm in 2005, which is associated with the White Wolf fault (WWF) and occurred at the southern end of the Central Valley, CA. The swarm, which is referred to as White Wolf swarm in the following, deviates from standard main shock-aftershock patterns. It is comprised of a M_L4.5 event on 22 September, followed by two M_L4.7 (M_w4.6) and M_L4.3 events the same day as well as some smaller magnitude "fore shocks." The White Wolf swarm is suspected to be connected to fluid-injection activity based on a statistical assessment of injection and seismicity rate changes [*Goebel et al.*, 2015]. The statistical assessment showed that an abrupt increase in injection rates in 2005 was followed by a large increase in seismicity rates, which exceeded the 95% confidence interval of previous rate variations since 1980. In other words, the area did not experience a comparable rate increase within a 10 km radius of the well prior to the start of injection in 2005. Moreover, the strong correlation between rapid injection rate changes and the subsequent seismicity sequence had a ≈3% probability of coinciding by chance based on tests with randomly determined onsets of injection rate changes [*Goebel et al.*, 2015].

The White Wolf swarm occurred at the southern end of Kern County, the largest oil-producing (>75% of the state's oil production) and fluid-injecting (>80% of all injection wells) county in California [*CA Department of Conservation*, 2012]. The region experienced one major earthquake since 1950, i.e., the 1952, M_w7.3 Kern county event, and hosts many seismically active faults including the White Wolf, Wheeler Ridge, and Pleito faults (Figure 1a).

Fluid injection rates at the southern end of the Central Valley increased rapidly from ~20,000 to more than 100,000 m^3/mo between 2001 and 2010 (Figure 1b). The majority of wastewater, i.e., 80 to 95%, was injected within the Tejon Oil Field involving only three closely spaced wells. These wells started injecting in 2001 (WD01), 2004 (WD04), and 2005 (WD05), and injection fluids were generally contained below 950 m. Effective well depths are reported between ~1200 and ~1500 m [*CA Department of Conservation*, 2012]. The injection wells targeted a 25–30 m thin, highly permeable ($0.2–1.0 \cdot 10^{-12}$ m^2) stratigraphic zone within the Monterey formation [*CA Department of Conservation*, 2012]. This injection zone is composed of turbiditic sand lenses with maximum lateral extents of 1 to 2 km (Figure S2 in the supporting information). Moreover, the wellbores include a significant, horizontally drilled portion with 520 to 580 m long perforation zones that maximized injection rates.

Injection into well WD05 occurred at consistently high rates of ~57,000 m^3/mo, starting 5 months before the White Wolf swarm in September 2005. During these five months, the wellbore accommodated more than 75% of the total injection activity of the entire study area. WD05 is located in an area of closely spaced, northwest striking, Early Miocene, buried, normal faults that show evidence of local Holocene reactivation. Based on geological mapping, seismicity, and well-log data, we identified a seismically active normal fault located between the WWF and injection site WD05 (Figure 1a, see supporting information Text S1 and Figures S1–S3 for details of fault identification). This fault is referred to as "Tejon fault" in the following text. Both seismicity and well-log data suggest that the Tejon fault is shallow close to the injection site and deepens toward the northwest below the Wheeler Ridge fault before intersecting with the WWF (Figure 1c). The horizontally elongated perforation zone of well WD05, which extends directly east from the well head, increased the probability of intersecting the Tejon fault with the borehole.

3. Seismicity Analysis and Hydrogeological Modeling

The White Wolf swarm shows evidence of systematic event migration between injection sites and the M_w4.6 hypocenter (Figures 1c and 2). This systematic migration is best seen in relocated catalogs that include newly

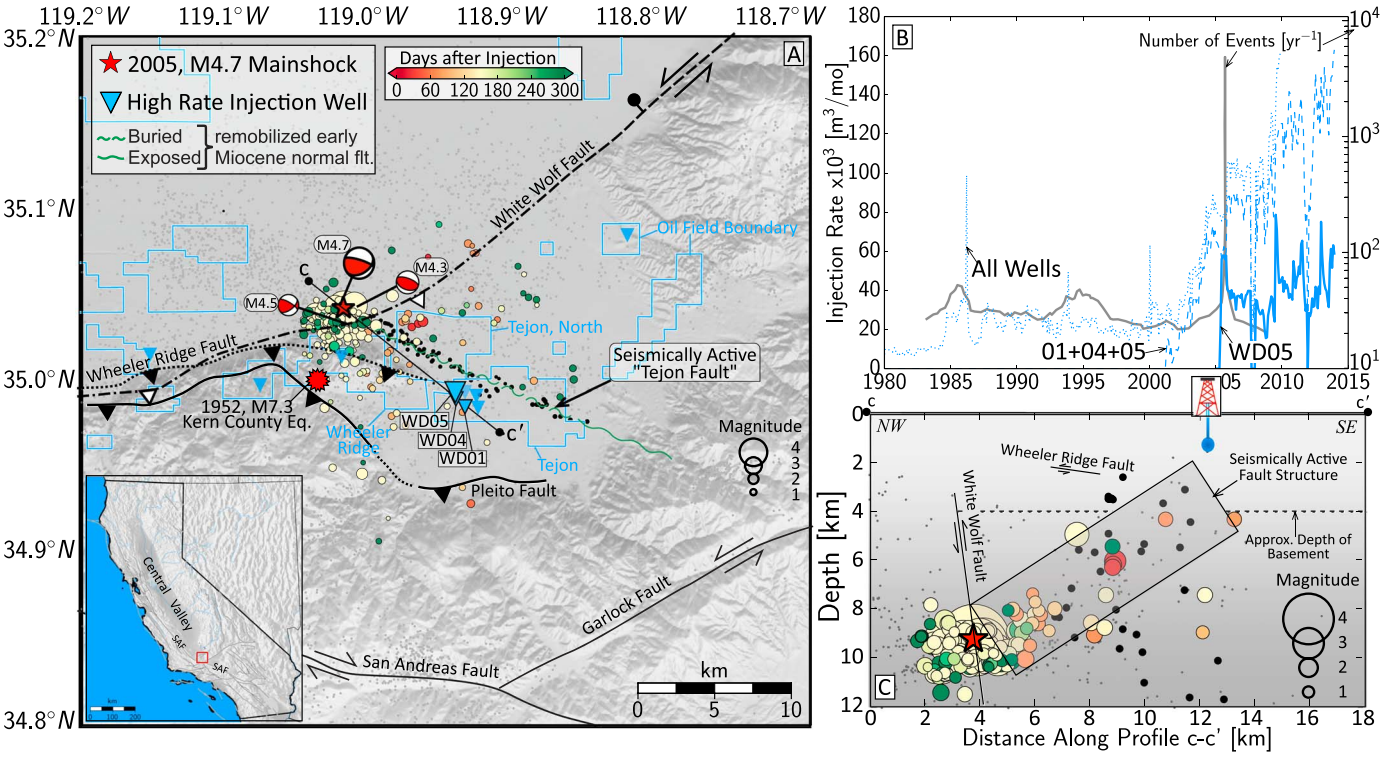


Figure 1. Seismicity and injection activity within the study area centered at the Tejon Oil Field. (a) Fault traces (black and green lines), epicenters (dots), injection wells (blue triangles), and oilfield locations (blue lines). Seismicity is colored according to days after injection into WD05, gray dots show background seismicity. Earthquake locations are from the standard Southern California Seismic Network (SCSN) catalog. (b) Injection (blue) and seismicity rates (gray) between 1980 and 2014. (c) Seismicity cross section within a 5 km zone between c and c′ in Figure 1a. Seismicity up to 300 days after the start of injection is shown in colored dots (see colorbar in Figure 1a), background events by gray dots, and Tejon fault seismicity prior to injection by black dots. The gray rectangle qualitatively highlights a zone of high seismic activity that coincides with the Tejon fault. Earthquake locations are based on a high-quality waveform relocated catalog [*Shearer et al.*, 2005].

detected events using a template-matching method and may easily remain undetected in standard seismicity catalogs (Figure 2). Within the first 2 months of wastewater disposal in WD05, we observed some shallow seismicity at 4 km depth beneath the injection site and on the Tejon fault to the northwest, while little to no seismic activity occurred to the south and southeast. Within the following months, the area northwest of well WD05 in direction of the WWF became progressively active. Much of this seismicity was concentrated just updip of the intersection point between the Tejon and White Wolf fault between ~70 and 150 days after injection (see animations in the supporting information).

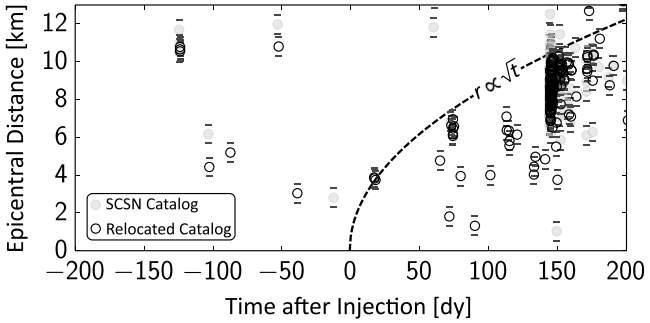


Figure 2. Seismicity migration along the Tejon fault relative to the start of injection in WD05. We show both the SCSN (gray dots) and relocated earthquake catalogs (black circles). The latter includes new event detections using a template-matching approach. Epicentral distances are reported relative to well-head location, error bars show average, absolute location uncertainties. The dashed curve highlights the expected trend for a square-root dependence of distance on time characteristic for a diffusive process.

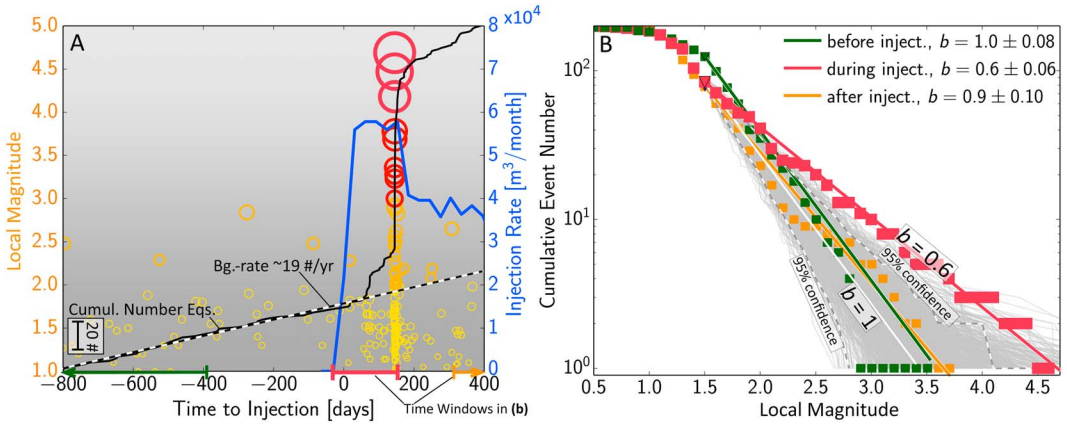


Figure 3. Fluid injection rates in well WD05, seismic activity, and frequency-magnitude distributions (FMDs) of the likely induced White Wolf swarm. (a) Injection rates (blue), cumulative number of earthquakes (black), and event magnitudes (circles) within a 10 km radius of injection in well WD05. (b) FMD before (green), during (red), and after (orange) peak injection rates as well as 95% confidence interval for a FMD with $b = 1$.

In the two years prior to injection into WD05, seismicity rates within a 10 km radius of the well were largely constant (Figure 3a). The start of injection also marked the onset of seismicity rate increase which peaked about 150 days later. The occurrence of the three M_L >4 events was closely followed by a ~30% decrease in injection rates in well WD05 and injection rates kept decreasing over the ensuing 4 years.

In addition to seismicity rate changes, the frequency-magnitude distribution (FMD) strongly deviated from expected behavior of Gutenberg-Richter-type FMDs with *b* values close to unity (Figure 3b). During the period of peak injection in WD05 the *b* value within a 10 km radius was 0.6. This value was significantly lower than before (b=1) and after (b=0.9) peak injection activity. Moreover, we observed a long-term decrease in *b* value coincident with a rate increase in cumulative injection rates in WD01, WD04, and WD05 (Figure S4). Using Monte Carlo simulations of earthquake magnitudes based on the observed *a* value, magnitude of completeness of 1.5, we compute the 95% confidence interval of FMDs with b=1. The observed FMD exceeds the upper confidence bound, indicating a significantly larger proportion of large-magnitude events during the White Wolf swarm which is also confirmed by the corresponding moment magnitudes for events above *M*4 (see supporting information Text S1 for details on moment magnitude computations). Decreasing and low *b* values may be characteristic for gradual fault activation processes, observed in laboratory experiments [*Goebel et al.*, 2013] and during fluid injection [e.g., *Maxwell et al.*, 2009; *Skoumal et al.*, 2014; *Huang and Beroza*, 2015].

To further test if the 2005 White Wolf swarm was connected to wastewater disposal, we model permeability structure and pressure diffusion close to the injection site. Pressure diffusion was likely influenced by high permeability along the seismically active part of the Tejon fault located N-W of well WD05. Slip along this fault may have been pivotal in maintaining elevated permeability down to the depth of the White Wolf swarm. The existence of a zone with higher permeability than the surrounding lithology is supported by several observations such as follows:

1. The asymmetric seismicity distribution concentrated N-W of well WD05 with little to no seismicity to the south and east of the injection sites. Most of these events occurred at depths between the M_w4.6 hypocenter and the injection depth (Figure S5).
2. The presence of background seismic activity on the upper portion of the Tejon fault revealing that part of the fault was seismically active prior to injection.
3. The presence of several mapped faults and fracture zones located N-W of the injection site between WD05 and WWF (Figure S3).

Our 3-D numerical diffusion model includes three principal stratigraphic zones, in addition to the Tejon fault which is implemented as a vertical zone of elevated permeability (Figure 4a). These three zones are (1) the injection zone, i.e., a 20–30 m thin turbiditic sand lens in the Monterey formation with a lateral extent of up to ~1.5 km, labeled as "Transition zone" in the industry data [*CA Department of Conservation*, 2012]; (2) the crystalline (gneissic) basement complex, and (3) the Monterey formation. Permeability is high within the sand

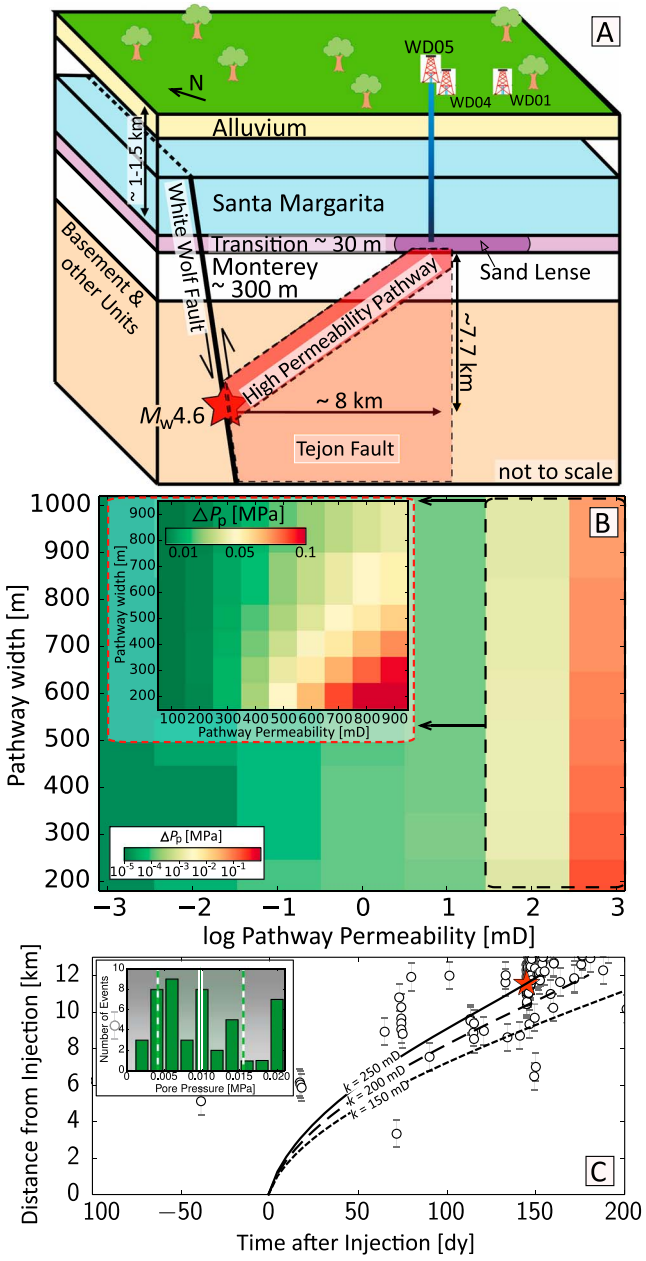


Figure 4. Hydrogeological modeling and predicted pore pressure increase caused by injection in well WD05. (a) Schematic representation of model setup including the high-permeability zone between injection site and M_w4.6 event. The hydrogeological model is based on geologic mapping and industry data described in Text S1 in the supporting information. (b) Model-dependent pore pressure change (ΔP_p) at distance of the M_w4.6 event (~11 km) for different values of fault zone width and permeability. (c) Observed hypocenter migration along the Tejon fault zone (black circles). The theoretical position of a 0.01 MPa pressure front is shown for a 500 m wide fault zone and three different permeability values (150, 200, and 250 mD). The M_w4.6 event is shown by a red star. The inset shows distribution of pore pressures at each hypocenter for $k = 200$ mD and $w = 500$ m, vertical lines are mean and standard deviation.

lenses (i.e. ~1 D) and very low (~10^{-4} mD) above injection depth which is one of the requirements for the selection of an injection site. Similarly, permeability is low (~10^{-4} mD) within the basement and Monterey formations outside of the injection zone (permeabilities are reported in millidarcy, 1 mD ≈10^{-15} m^2).

Our hydrogeological model is based on the most complete available data sets within the upper ~2–3 km of sedimentary basins and includes seismicity records, geologic mapping results, and industry data (i.e., well logs, stratigraphic columns, and interpreted reservoir structure, see supporting information Text S1).

Below these depths, few geophysical data are available except for the seismicity record. To account for larger uncertainty at these depths, we chose a coarse modeling scale which primarily resolves the influence of large-scale structural heterogeneity such as large fault zones. More detailed information about the model setup can be found in the supporting information Text S1.

Our modeling results show that thin fault zones with higher permeability lead to fluid-pressure increase sufficient to trigger earthquakes at distances similar to the M_w4.6 hypocenter. We performed a detailed sensitivity analysis of pressure changes resulting from varying fault zone width and permeability. Permeability was varied over 7 orders of magnitude between 10^{-3} to 10^3 mD to account for generally large uncertainties in permeability measurements [*Manga et al.*, 2012], and fault width was varied between 100 and 1000 m (Figure 4b and supporting information Table S1). The modeled pressure changes experienced by a fault at ~11 km distance span 4 orders of magnitude depending on the initial conditions, with significantly stronger effects of permeability changes compared to variations in fault zone width (Figure 4b). For fault zone permeability above ~300 mD and fault width below ~800 m, we observe a pressure increase at the M_w4.6 hypocenter of at least 0.01 MPa (Figure 4b), which is sufficient to induce seismicity on faults favorably oriented to slip [*Keranen et al.*, 2014; *Hornbach et al.*, 2015]. Fault zones with lower permeability may result in similar magnitude pressure changes if they are sufficiently narrow. The here described seismogenic consequences of thin, high-permeability pressure channels are in agreement with previous studies of induced seismicity in Colorado and Arkansas [*Hsieh and Bredehoeft*, 1981; *Zhang et al.*, 2013].

The migration pattern of seismic events relative to injection into WD05 can be used to constrain a plausible range of permeability values by correlating seismicity and modeled pressure front location along the Tejon fault (Figure 4c). The shape of the pore pressure front in a distance-time plot depends on the particular reservoir geometry and permeability values with a commonly observed square-root dependence of distance on time for simple radial symmetric models (Figure 2) [*Talwani and Acree*, 1984; *Shapiro et al.*, 1997]. Using the more complex 3-D reservoir and fault geometries in our model, we find that fault zone permeability between 150 and 250 mD and a width smaller than 500 m best agrees with the observed seismicity for a change in fluid pressure of 0.01 MPa. This is within the general range of fault zone permeability values inferred from seismicity migration [e.g., *Ingebritsen and Manning*, 2010; *Manga et al.*, 2012]. Much of the seismicity occurred close to the arrival of the initial pressure pulse of ~0.01 MPa, indicating that faults within the area may have been critically stressed, i.e., stress levels were within a narrow range below the shear strength (Figure 4c inset).

4. Discussion

The identification of human-induced earthquakes in tectonically active regions such as California is generally complicated by the many naturally occurring seismicity sequences. To address these challenges, we used detailed statistical analysis methods [*Goebel et al.*, 2015] as well as geological and diffusion models to evaluate a potentially induced origin of an earthquake sequence close to the WWF in 2005. This sequence deviates from commonly observed tectonic sequences in the area by showing significantly elevated seismicity rates above the background associated with a rapid increase in injection rates. Moreover, the seismicity sequence showed evidence for deep migration within the crystalline basement between injection wells and the nearby White Wolf fault suggesting that wastewater disposal likely contributed to triggering the earthquake swarm. The recorded largest magnitude event (M_{max}=4.6) of the sequence and the cumulative injection volume (V_{tot} ~$1.8 \cdot 10^6$ m^3) fall well within the trend of V_{tot} and M_{max} reported by *McGarr* [2014] and are similar to observations of V_{tot} and M_{max} in Timpson, Texas, and Painesville, Ohio [*McGarr*, 2014]. This analysis assumes that injection in all three wells (WD01, WD04, and WD05) contributed to the induced seismicity sequence.

Our results highlight that injection related earthquake triggering processes may involve multiple mechanisms. A plausible mechanism for the triggering of the White Wolf swarm is the diffusion of pressures from the ~1.5 km deep injection site along the northwest striking Tejon fault into the intersection zone with the WWF. This pressure channeling effect may have been further intensified if the WWF acted as flow barrier, thereby trapping the pressure front within the damage zone of the Tejon fault resulting in more rapid pressure increase at the intersection between the two faults. Other triggering mechanisms may have included stress transfer at the front of the pressurized zone as well as injection-induced aseismic slip, which progressively became more seismogenic at larger depth within the basement complex. Shallow aseismic slip is well documented in high-resolution, controlled injection experiments and may hide early fault activation processes [*Cornet et al.*, 1997; *Guglielmi et al.*, 2015]. A potentially injection-induced origin of the White Wolf swarm is intrinsically

connected to the specific geologic setting that accommodated pressure diffusion to seismogenic depth at the southern end of the Central Valley. More detailed assessments of the geologic setting close to injection wells are required to explain the lack of large-scale injection-induced earthquake activity in California hydrocarbon basins [*Goebel*, 2015].

Cases of relatively deep induced seismicity far from injection sites have been reported in several other regions such as Oklahoma, Colorado, and Arkansas, where induced earthquakes occurred at 8 km depth and 7 to 35 km distance from the injection well [*Hsieh and Bredehoeft*, 1981; *Horton*, 2012; *Keranen et al.*, 2014]. In addition to these large distances, there are several other factors that can complicate induced seismicity detection in California hydrocarbon basins: (1) Faults may channel induced pressure changes and lead to localized pore pressure increase; (2) background seismicity rates are generally high so that small-rate variations cannot be detected; (3) small-magnitude earthquakes close to injection sites can easily be missed because of sparse station coverage within hydrocarbon basins; and (4) transient aseismic and seismic slip processes lead to a dynamic increase in permeability and progressive fault activation [*Cornet et al.*, 1997; *Bourouis and Bernard*, 2007; *Guglielmi et al.*, 2015; *Wei et al.*, 2015]. All of these factors can potentially complicate mitigation strategies such as traffic light systems which rely on a systematic seismicity rate increase and event migration from the well.

Based on our empirical results, injection-induced earthquakes are expected to contribute marginally to the overall seismicity in California [see also *Goebel*, 2015; *Goebel et al.*, 2015]. This is in line with physical models of crustal strength distributions which suggest a limit to the amount of strain energy that is available for shallow earthquake ruptures [e.g., *Sibson*, 1974]. Moreover, the frictional properties of shallow, sedimentary faults inhibit rupture growth and diminish the seismogenic impact of surficial pressure perturbation [e.g., *Das and Scholz*, 1983]. However, considering the numerous active faults in California, the seismogenic consequences of even a few induced cases can be devastating.

5. Conclusion

Wastewater injection-induced earthquakes are rare in California compared to widespread tectonic seismicity. Nevertheless, the proximity of high-rate injectors and large active faults can cause noticeable earthquakes under certain geologic conditions. Our results suggest a connection between wastewater disposal and seismicity with events up to M_w4.6 at the southern end of the Central Valley. Wastewater injection within this region should be monitored carefully because of the presence of high-permeability fault structures that connect the injection site with the nearby WWF. The relatively shallow crystalline basement south of the WWF may increase the probability of inducing earthquakes, if fluids migrate beyond the intended geologic formations.

The present example shows that injection-induced earthquakes may remain unidentified in tectonically active regions if only standard seismicity catalogs with comparably high magnitudes of completeness are analyzed. We present a pathway to more reliable identification of possibly induced earthquakes by extending the seismicity records to lower magnitudes and by analyzing waveform relocated catalogs as well as hydrogeological models. Such a detailed analysis of the available data may help recognize regions with increased induced seismicity potential and prevent injection-induced seismicity in California in the future.

Acknowledgments
The authors thank Semechah Lui, Preston Jordan, Bill Foxall, Emily Brodsky, and members of the Induced Seismicity Consortium at USC for helpful comments and discussions. The manuscript benefitted from comments by Art McGarr and Michael Wysession. The well and operational data were supplied by the California Department of Conservation (www.doggr.com), and the seismicity data by the Southern California Earthquake Center (doi: 10.7909/C3WD3xH1) and Advanced National Seismic Systems. This study was funded by NEHRP/USGS grants G14AP00075 and G15AP00095 to Caltech, by the Southern California Earthquake Center (SCEC) under contribution numbers 12017 and 15168 and the Induced Seismicity Consortium (ISC) at USC.

References

Bourouis, S., and P. Bernard (2007), Evidence for coupled seismic and aseismic fault slip during water injection in the geothermal site of Soultz (France), and implications for seismogenic transients, *Geophys. J. Int.*, *169*(2), 723–732.

CA Department of Conservation (2012), Division of oil, gas and geothermal resources: Production history in California. [Available at ftp://ftp.consrv.ca.gov/pub/oil/annual_reports, accessed Sept. 26, 2014.]

Cornet, F., J. Helm, H. Poitrenaud, and A. Etchecopar (1997), Seismic and aseismic slips induced by large-scale fluid injections, *Pure Appl. Geophys.*, *150*, 563–583.

Das, S., and C. Scholz (1983), Why large earthquakes do not nucleate at shallow depths, *Nature*, *305*, 621–623.

Ellsworth, W. L. (2013), Injection-induced earthquakes, *Science*, *341*(6142), 1225942.

Frohlich, C., and M. Brunt (2013), Two-year survey of earthquakes and injection/production wells in the Eagle Ford Shale, Texas, prior to the M_W 4.8 20 October 2011 earthquake, *Earth Planet. Sci. Lett.*, *379*, 56–63.

Goebel, T. H. W. (2015), A comparison of seismicity rates and fluid injection operations in Oklahoma and California: Implications for crustal stresses, *Leading Edge*, *34*(6), 640–648, doi:10.1190/tle34060640.1.

Goebel, T. H. W., D. Schorlemmer, T. W. Becker, G. Dresen, and C. G. Sammis (2013), Acoustic emissions document stress changes over many seismic cycles in stick-slip experiments, *Geophys. Res. Lett.*, *40*, 2049–2054, doi:10.1002/grl.50507.

Goebel, T. H. W., E. Hauksson, F. Aminzadeh, and J.-P. Ampuero (2015), An objective method for the assessment of possibly fluid-injection induced seismicity in tectonically active regions in central California, *J. Geophys. Res. Solid Earth*, *120*, 7013–7032, doi:10.1002/2015JB011895.

Guglielmi, Y., F. Cappa, J.-P. Avouac, P. Henry, and D. Elsworth (2015), Seismicity triggered by fluid injection-induced aseismic slip, *Science*, *348*(6240), 1224–1226.
Healy, J., W. Rubey, D. Griggs, and C. Raleigh (1968), The Denver earthquakes, *Science*, *161*(3848), 1301–1310.
Hornbach, M. J., et al. (2015), Causal factors for seismicity near Azle, Texas, *Nat. Commun.*, *6*, 6728.
Horton, S. (2012), Disposal of hydrofracking waste fluid by injection into subsurface aquifers triggers earthquake swarm in central Arkansas with potential for damaging earthquake, *Seismol. Res. Lett.*, *83*(2), 250–260.
Hsieh, P. A., and J. D. Bredehoeft (1981), A reservoir analysis of the Denver earthquakes: A case of induced seismicity, *J. Geophys. Res.*, *86*(B2), 903–920.
Huang, Y., and G. C. Beroza (2015), Temporal variation in the magnitude-frequency distribution during the Guy-Greenbrier earthquake sequence, *Geophys Res. Lett.*, *42*, 6639–6646, doi:10.1002/2015GL065170.
Ingebritsen, S., and C. Manning (2010), Permeability of the continental crust: Dynamic variations inferred from seismicity and metamorphism, *Geofluids*, *10*(1–2), 193–205.
Kanamori, H., and E. Hauksson (1992), A slow earthquake in the Santa Maria Basin, California, *Bull. Seismol. Soc. Am.*, *82*(5), 2087–2096.
Keranen, K., M. Weingarten, G. Abers, B. Bekins, and S. Ge (2014), Sharp increase in central Oklahoma seismicity since 2008 induced by massive wastewater injection, *Science*, *345*(6195), 448–451.
Keranen, K. M., H. M. Savage, G. A. Abers, and E. S. Cochran (2013), Potentially induced earthquakes in Oklahoma, USA: Links between wastewater injection and the 2011 M_w 5.7 earthquake sequence, *Geology*, *41*(6), 699–702.
Kim, W.-Y. (2013), Induced seismicity associated with fluid injection into a deep well in Youngstown, Ohio, *J. Geophys. Res. Solid Earth*, *118*, 3506–3518, doi:10.1002/jgrb.50247.
Manga, M., I. Beresnev, E. E. Brodsky, J. E. Elkhoury, D. Elsworth, S. Ingebritsen, D. C. Mays, and C.-Y. Wang (2012), Changes in permeability caused by transient stresses: Field observations, experiments, and mechanisms, *Rev. Geophys.*, *50*, RG2004, doi:10.1029/2011RG000382.
Martínez-Garzón, P., G. Kwiatek, H. Sone, M. Bohnhoff, G. Dresen, and C. Hartline (2014), Spatiotemporal changes, faulting regimes, and source parameters of induced seismicity: A case study from the Geysers Geothermal Field, *J. Geophys. Res. Solid Earth*, *119*, 8378–8396, doi:10.1002/2014JB011385.
Maxwell, S. C., et al. (2009), Fault activation during hydraulic fracturing, paper presented at 2009 SEG International Exposition and Annual Meeting, Houston, Tex., 25–30 Oct.
McGarr, A. (2014), Maximum magnitude earthquakes induced by fluid injection, *J. Geophys. Res. Solid Earth*, *119*, 1008–1019, doi:10.1002/2013JB010597.
Rubinstein, J. L., W. L. Ellsworth, A. McGarr, and H. M. Benz (2014), The 2001-present induced earthquake sequence in the Raton Basin of northern New Mexico and southern Colorado, *Bull. Seismol. Soc. Am.*, *104*, 2162–2181.
Schoenball, M., L. Dorbath, E. Gaucher, J. F. Wellmann, and T. Kohl (2014), Change of stress regime during geothermal reservoir stimulation, *Geophys. Res. Lett.*, *41*, 1163–1170, doi:10.1002/2013GL058514.
Shapiro, S. A., E. Huenges, and G. Borm (1997), Estimating the crust permeability from fluid-injection-induced seismic emission at the KTB site, *Geophys. J. Int.*, *131*, F15–F18.
Shearer, P., E. Hauksson, and G. Lin (2005), Southern California hypocenter relocation with waveform cross-correlation, Part 2: Results using source-specific station terms and cluster analysis, *Bull. Seismol. Soc. Am.*, *95*(3), 904–915.
Sibson, R. H. (1974), Frictional constraints on thrust, wrench and normal faults, *Nature*, *249*, 542–544.
Skoumal, R. J., M. R. Brudzinski, B. S. Currie, and J. Levy (2014), Optimizing multi-station earthquake template matching through re-examination of the Youngstown, Ohio, sequence, *Earth Plant. Sci. Lett.*, *405*, 274–280, doi:10.1016/j.epsl.2014.08.033.
Talwani, P., and S. Acree (1984), Pore pressure diffusion and the mechanism of reservoir-induced seismicity, *Pure Appl. Geophys.*, *122*(6), 947–965.
Wei, S., et al. (2015), The 2012 Brawley swarm triggered by injection-induced aseismic slip, *Earth Planet. Sci. Lett.*, *422*, 115–125.
Zhang, Y., et al. (2013), Hydrogeologic controls on induced seismicity in crystalline basement rocks due to fluid injection into basal reservoirs, *Groundwater*, *51*(4), 525–538.

Science of the Total Environment 806 (2022) 150298

Contents lists available at ScienceDirect

Science of the Total Environment

journal homepage: www.elsevier.com/locate/scitotenv

Upstream oil and gas production and ambient air pollution in California

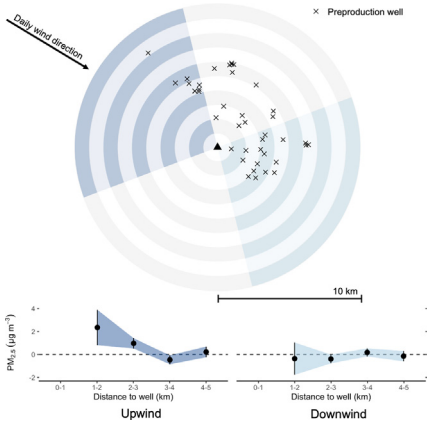
David J.X. Gonzalez [a,b,*], Christina K. Francis [c], Gary M. Shaw [d], Mark R. Cullen [e,1], Michael Baiocchi [f], Marshall Burke [g]

[a] Department of Environmental Science, Policy and Management and School of Public Health, University of California, Berkeley, Berkeley, CA 94720, USA
[b] Emmett Interdisciplinary Program in Environment and Resources, Stanford University, Stanford, CA, USA
[c] Program in Environmental Science and Studies, Johns Hopkins University, Baltimore, MD, USA
[d] Department of Pediatrics, Stanford University, Stanford, CA, USA
[e] Founding Director of the Stanford Center for Population Health Sciences, USA
[f] Department of Epidemiology and Population Health, Stanford University, Stanford, CA, USA
[g] Department of Earth System Science, School of Earth, Energy and Environmental Sciences, Stanford University, Stanford, CA, USA

HIGHLIGHTS

- Oil and gas wells have been linked to adverse health, but mechanisms not well understood.
- Applied a quasi-experimental design with daily air pollution and oil production data
- We leveraged wind direction as source of exogenous variation for exposure to wells.
- Upstream oil and gas production emitted air pollutants at concentrations that may be harmful.
- Evaluated proximity as an appropriate indicator of air pollution exposure from wells

GRAPHICAL ABSTRACT



ARTICLE INFO

Article history:
Received 12 April 2021
Received in revised form 31 August 2021
Accepted 7 September 2021
Available online 11 September 2021

Editor: Lidia Morawska

Keywords:
Oil and gas
Exposure assessment
Ambient air pollution
Particulate matter

ABSTRACT

Background: Prior studies have found that residential proximity to upstream oil and gas production is associated with increased risk of adverse health outcomes. Emissions of ambient air pollutants from oil and gas wells in the preproduction and production stages have been proposed as conferring risk of adverse health effects, but the extent of air pollutant emissions and resulting nearby pollution concentrations from wells is not clear.
Objectives: We examined the effects of upstream oil and gas preproduction (count of drilling sites) and production (total volume of oil and gas) activities on concentrations of five ambient air pollutants in California.
Methods: We obtained data on approximately 1 million daily observations from 314 monitors in the EPA Air Quality System, 2006-2019, including daily concentrations of five routinely monitored ambient air pollutants: $PM_{2.5}$, CO, NO_2, O_3, and VOCs. We obtained data on preproduction and production operations from Enverus and the California Geographic Energy Management Division (CalGEM) for all wells in the state. For each monitor and each day, we assessed exposure to upwind preproduction wells and total oil and gas production volume within 10 km. We used a panel regression approach in the analysis and fit adjusted fixed effects linear regression models for each pollutant, controlling for geographic, seasonal, temporal, and meteorological factors.

* Corresponding author at: 108 Hilgard Hall, University of California, Berkeley, Berkeley, CA 94720, USA.
E-mail address: djxgonz@berkeley.edu (D.J.X. Gonzalez).
[1] Retired.

https://doi.org/10.1016/j.scitotenv.2021.150298

Results: We observed higher concentrations of $PM_{2.5}$ and CO at monitors within 3 km of preproduction wells, NO_2 at monitors at 1-2 km, and O_3 at 2-4 km from the wells. Monitors with proximity to increased production volume observed higher concentrations of $PM_{2.5}$, NO_2, and VOCs within 1 km and higher O_3 concentrations at 1-2 km. Results were robust to sensitivity analyses.
Conclusion: Adjusting for geographic, meteorological, seasonal, and time-trending factors, we observed higher concentrations of ambient air pollutants at air quality monitors in proximity to preproduction wells within 4 km and producing wells within 2 km.

1. Introduction

Recent studies have found that residing in proximity to oil and gas wells is associated with adverse cardiovascular, psychological, perinatal, and other health outcomes (Casey et al., 2015, 2018; Currie et al., 2017; Denham et al., 2021; McKenzie et al., 2014, 2018, 2019; Tang et al., 2020; Whitworth et al., 2017; Willis et al., 2021). Studies in California have found higher risk of preterm birth and low birthweight with exposure to upstream oil production, as well as impaired lung function and higher asthma prevalence (Gonzalez et al., 2020; Johnston et al., 2021; Shamasunder et al., 2018; Tran et al., 2020). Several possible mechanisms have been hypothesized for the observed associations between proximity to wells and adverse health outcomes, including emissions of ambient air contaminants during various stages of upstream oil and gas production (Adgate et al., 2014; Allshouse et al., 2019; Gonzalez et al., 2020; Johnston et al., 2019; McKenzie et al., 2012). There is a potential for widespread risk of exposure to air pollutant emissions from upstream oil and gas development, with an estimated 17.6 million U.S. residents, including 2.1 million Californians, living within 1.6 km (1 mile) of at least one active well (Czolowski et al., 2017).

Despite widespread potential exposure to wells and reported health risks, the effects of upstream oil and gas production on ambient air quality are still not well understood (Johnston et al., 2019). Under the Clean Air Act and its amendments, local regulatory agencies are responsible for maintaining networks of in situ air pollution monitors (Grainger et al., 2017). Agencies routinely monitor criteria air pollutants, which are statutorily regulated under the Clean Air Act and which include fine particulate matter with an aerodynamic diameter less than 2.5 μm ($PM_{2.5}$), carbon monoxide (CO), nitrogen dioxide (NO_2), and ozone (O_3). Other hazardous pollutants are also routinely monitored, including non-methane volatile organic compounds (VOCs) such as acetaldehyde, benzene, ethylbenzene formaldehyde, n-hexane, toluene and xylene. In prior studies, such as in situ monitoring campaigns conducted in California, Colorado, and Texas, investigators have reported elevated concentrations of $PM_{2.5}$, CO, NO_2, O_3, and VOCs near wells (Allshouse et al., 2019; Arbelaez and Baizel, 2015; Garcia-Gonzales et al., 2019a; Schade and Roest, 2016, 2018). Sources of $PM_{2.5}$ emissions associated with upstream oil and gas production may include combustion of diesel fuel from on-site equipment and heavy trucks, dust from construction sites and unpaved roads, and secondary formation in the atmosphere (Adgate et al., 2014); emissions of CO and NO_2 may also be associated with fossil fuel combustion in vehicles and off-road equipment (Holloway et al., 2000; Jackson et al., 2014); O_3 may be formed as a secondary pollutant in photochemical reactions involving nitrous oxides (such as NO_2) and VOCs in the presence of sunlight (Mauzerall et al., 2005; Rodriguez et al., 2009).

Studies have found elevated concentrations of harmful pollutants near oil and gas wells (Garcia-Gonzales et al., 2019b). However, prior studies have been geographically and temporally constrained and often do not mirror methods applied by population health researchers. In particular, exposure characterization is often spatial in nature, whereas population health researchers often seek to exploit temporal variation to isolate the role of exposure to oil and gas wells from exposure to other spatially correlated activities that may affect pollution and health (Currie et al., 2017; Willis et al., 2021). Additionally, the unique geological conditions of California may constrain external validity of air quality studies that investigate oil and gas production-related emissions in other settings (Garcia-Gonzales et al., 2019a). Population health studies investigating exposure to upstream oil and gas production typically use proximity to wells as the indicator of exposure without directly measuring concentrations of air pollutant emissions or other potential hazards, such as noise and water pollution (Casey et al., 2015; Currie et al., 2017; Gonzalez et al., 2020; McKenzie et al., 2014; Rasmussen et al., 2016; Tang et al., 2020; Tran et al., 2020; Willis et al., 2021). Improved understanding of pollutants emitted during upstream oil and gas production, including the classes of pollutants emitted (or secondarily produced) and the distances to which they are transported could help population health scientists more accurately parameterize exposure assessments and determine which aspects of exposure to production activities may adversely affect human health.

In our prior study (Gonzalez et al., 2020), we found that proximity to wells was associated with higher preterm birth risk, but we were not able to measure specific chemical pollutants parents were potentially exposed to during their pregnancy, or to separate proximity to wells from other activities that may also affect preterm birth risk. Our objectives in the current study were to examine how upstream oil preproduction and production activities affected ambient air quality in California from 2006 to 2019, with the aim of informing population health studies of exposure to upstream oil and gas production. We investigated whether marginal changes in preproduction and production activities resulted in increased concentrations of $PM_{2.5}$, CO, NO_2, O_3, and VOCs. Where we observed marginal increases in pollutant concentrations with proximity to wells, we also aimed to determine the distance at which elevated concentrations decay to background levels. To address these objectives, we applied a quasi-experimental design using a panel of publicly available air quality monitoring data.

2. Methods

2.1. Study design

We constructed a panel dataset with repeated daily measures of ambient air pollutant concentrations as well as upstream oil and gas production across California from January 1, 2006, to December 31, 2019. We made use of geospatial and temporal variation in oil and gas extraction activities, including well preproduction (defined as the interval between spudding, or initiation of drilling, and completion) and production (total monthly volume of oil and gas produced), and leveraged daily variation in wind direction as a source of exogenous variation. The type and magnitude of emissions may vary by stage due to differences in activities related to preproduction and production, and the intensity of well pad activity varies within each stage (Allshouse et al., 2017). For each monitor, we assessed daily exposure to upwind wells in preproduction and production during the study period. In the current study, we did not assess exposure of any human populations; rather, we assessed exposure of air quality monitors as a surrogate receptor. Then we used a fixed effects regression approach to assess the effect of exposure to preproduction and producing wells on the concentrations of each pollutant, accounting for geographic, seasonal, and time-trending, and meteorological factors.

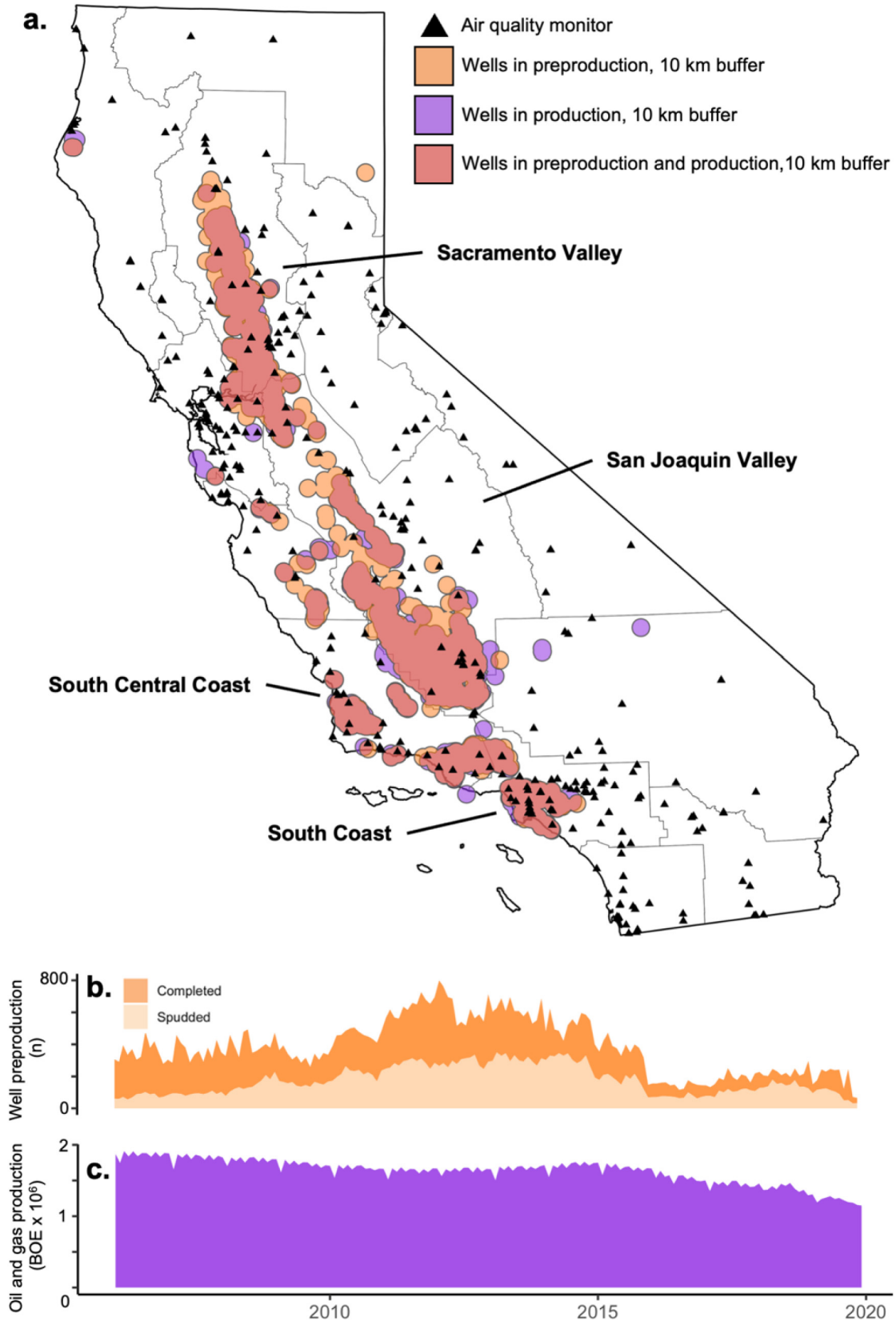


Fig. 1. (a) A map of the study region, showing air basins, air quality monitor locations, and 10 km buffers around wells in preproduction (orange) and production (purple), as well as the overlap (red). (b) Count of wells spudded and completed by month across California, including recompletions of previously drilled wells. (c) Total oil and gas production by month for all wells in California, reported as million barrels of oil equivalent (BOE).

2.2. Data

We obtained air quality data from the U.S. Environmental Protection Agency (EPA) Air Quality System (AQS). This dataset comprised daily measurements of seven air pollutants, with daily mean concentrations of $PM_{2.5}$ (μg m^{-3}) as well as daily max concentrations of CO (ppm), NO_2 (ppb), O_3 (ppb), and non-methane VOCs (ppb C). In all analyses, the unit of observation was the pollutant concentration at each monitor for each day, or the monitor-day. We included data for all 314 AQS monitors in California that were operating during the study period and that monitored for the five pollutants of interest (Fig. 1). Missing air pollution data were omitted from the analyses; we did not impute missing air pollution data. Due to the sparse monitoring of VOCs compared to other pollutants, we included data on VOC measurements for 1999-2005; we excluded pre-2006 measurements for other pollutants because data for wildfire smoke plumes, described below, were not available before 2006. Air qualtity monitors detected and measured non-methane VOC concentrations via the EPA Method TO-3 for ethylbenzene, n-hexane, toluene, benzene, and ethylene using cryogenic preconcentration techniques, gas chromatography, and flame ionization detection. Xylene concentrations were estimated using preconcentration techniques, gas chromatography, and Saturn 2000 ion mass spectrometry. Acetaldehyde and formaldehyde concentrations were measured using 2,4-dinitrophenylhydrazine (DNPH) silica gel cartridges, an O_3 scrubber, and ultraviolet absorption spectroscopy.

Data on the oil and gas wells, including development dates and monthly production volume, was obtained from the California Geologic Energy Management Division (CalGEM) and Enverus, a private data aggregation service. The analytic dataset included 38,157 wells that were in the preproduction and 90,697 wells in production in California during the study period (Table S1). We defined the preproduction stage of the well as starting with the reported spud date (when drilling begins) and ending with the completion date. We assessed monitors as exposed to proximate preproduction wells on days when the well was between the dates of spudding and completion. Preproduction wells were included in the study if the preproduction interval (spudding to completion) occurred during the study period. For wells with missing data for spud date, we assumed that the preproduction interval began 30 days before completion; for wells missing completion date, we assumed the preproduction stage ended 30 days after spudding. Wells missing both spud and completion dates were assumed to have been drilled outside the study period; since the record dates to the late 19th century, we expected there to be missingness in these variables for wells drilled prior to 1999. Wells in the production stage were included for all sites with any reported oil or gas production during the study period. Because oil and gas are frequently produced from the same wells, we used a combined metric of oil and gas production reported as barrels of oil equivalent (BOE). The dataset comprised 8,064,549 well-month observations of a total of approximately 3.8 billion BOE.

We obtained meteorological data from the North American Regional Reanalysis (NARR), a product developed by the National Centers for Environmental Prediction. This dataset included modeled daily mean wind direction and speed, reported as vectors (*u* and *v*), as well as observations of mean daily surface temperature (°C) and total daily precipitation (mm). There were no missing estimates for these meteorological variables. We also obtained administrative shapefiles for air basins across the state from the California Air Resources Board (CARB). We used data from the 2010 decennial census to determine whether monitors were located in urban areas (with 50,00 or more residents) or urban clusters (with 2500-50,000 residents) compared with rural areas, which comprise all other areas. To control for potential effects of wildfire smoke on daily concentrations ambient air pollutants, we used data on the daily location of wildfire smoke plumes from the Hazard Mapping System of the National Oceanic and Atmospheric Administration (NOAA), which assessed the number of overhead smoke plumes at the zip code level (Schroeder et al., 2008).

2.3. Exposure assessment

We constructed a panel dataset where, for each monitor and each day with a pollutant observation, we summed (a) the number of upwind wells in preproduction and (b) the total volume of upwind oil and gas production (BOE) in 1 km increments out to 10 km (Fig. 2). We determined the wind direction for each monitor and day from the *u* and *v* vector components from the NARR wind product. The resultant of the *u* and *v* vector components conveys wind direction and speed (magnitude). Preproduction and production wells that intersected the upwind quadrant on each day for each monitor comprised the primary exposure variables; wells outside the quadrant were excluded in the primary analyses.

As sensitivity analyses, we also assessed exposure to wells in the downwind quadrant as a placebo exposure. Additionally, we assessed exposure to all preproduction wells and production volume in 1 km annuli (or rings) radiating out from the monitor, i.e., without taking wind into account.

The receptor in our exposure assessment was the air quality monitor; this study did not consider any human receptors or health outcomes. Our aim was to use air monitors as a proxy for the residential receptors typically targeted in population health studies that assess exposure to oil and gas wells.

2.4. Identification strategy

We leveraged daily variation in wind direction as a plausibly exogenous source of variation, uncorrelated with well preproduction and production activities as well as other sources of pollution. This strategy allowed us to, by design, isolate the marginal contributions of additional preproduction wells and production volume to ambient air pollutant concentrations.

2.5. Statistical analyses

We used adjusted fixed effects linear regression models to assess how marginal changes in (a) the count of wells in preproduction or (b) the volume of oil and gas production affects concentrations of each observed pollutant ($PM_{2.5}$, CO, NO_2, O_3, and VOCs). For each combination of pollutants and well stage (preproduction or production), we fit the following model:

$$Y_{md} = U_{mda} + D_{mda} + O_{mda} + C_{md} + \gamma_{md} + \delta_{by} + e_{md},$$

where Y is the observed daily concentration of the pollutant at monitor *m* on day *d*; U is a vector of either the (a) upwind count preproduction wells or (b) upwind sum oil and gas production on day *d* in annulus *a* (0-1 km, 1-2 km, … 9-10 km) radiating from monitor *m*; D is similar to U but for downwind wells; O is also similar to U, but were wells in the two quadrants orthogonal to the upwind quadrant (i.e., lateral wells); C is a vector of covariates (day of week, precipitation in mm, temperature in °C, wind speed in ms^{-1}, and the count of overhead smoke plumes) at monitor *m* on day *d*; γ is a fixed effect for monitor by month, *n*; δ is a fixed effect for air basin, *b*, by year, *y*; *and e* is an error term representing unmodeled sources of variation in pollution at monitor *m* on day *d*. We fit additional models with polynomial terms for each exposure bin to examine whether the response was nonlinear.

We compared the point estimates for upwind wells with downwind placebos. As sensitivity analyses, we also modified the fixed effects in the model, using monitor-by-year and air basin-by-month-by-year fixed effects in the model. Additionally, we fit models as described above in the primary analysis but using exposure assessment data that did not take wind into account (i.e., the sum of all preproduction wells or production volume within each annulus). Finally, as an additional sensitivity analysis for co-exposure to wildfire smoke, we fit models

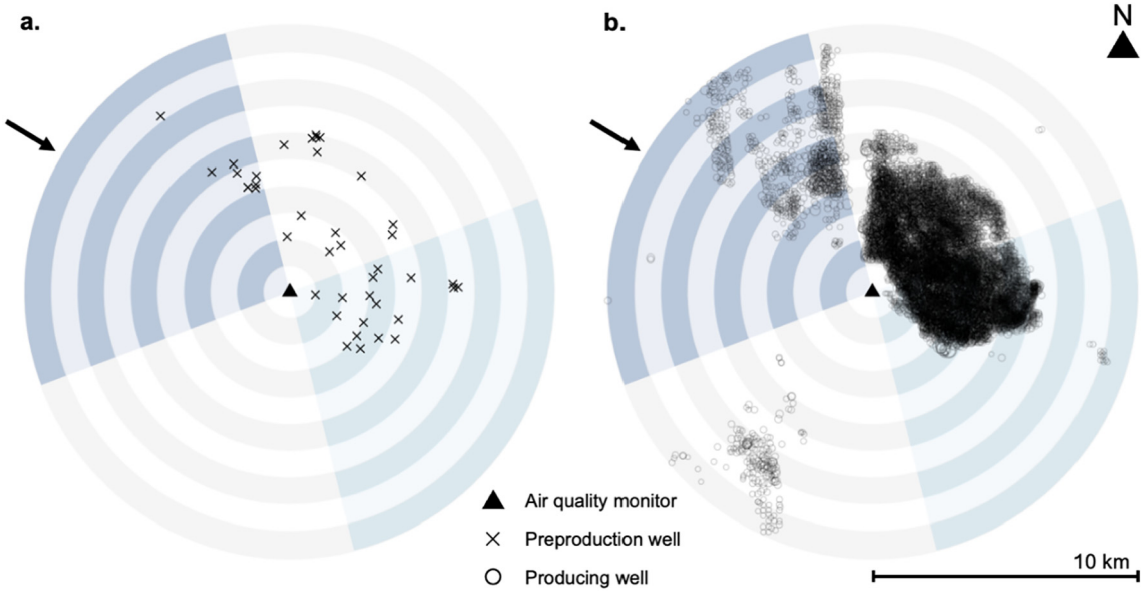


Fig. 2. A visualization of the exposure assessment method at a monitor located in Bakersfield, California, using sample data from July 1, 2009, when the wind was blowing from the northwest (arrow). For each monitor-day, we assessed exposure to (a) the count of wells in preproduction and (b) the total volume of oil and gas produced upwind (darker shaded area) of the monitor. As a placebo test, we assessed exposure to wells downwind (lighter shade) of the monitor.

for $PM_{2.5}$ where monitor-day observations that had >0 smoke plumes overhead were omitted.

In total we fit 27 models, and, as the primary analysis, we focused on the adjusted fixed effects regression models for exposure to preproduction wells and production volume. In particular, we were interested in the point estimates for exposure to upwind wells and production within 5 km of the monitor.

All data preparation and analyses were conducted using R v. 4.0 (R Core Team, 2020).

3. Results

3.1. Descriptive statistics

The analytic dataset comprised 1,058,230 daily observations of the five pollutants from 314 monitors across California collected from 2006 to 2019, with additional observations for VOCs from 1999 to 2005 (Table 1). Most (208) monitors were located in urban areas and approximately half (158) were in the four air basins with the majority of oil and gas wells (96.4%) and production (87.2%): Sacramento Valley, San Joaquin Valley, South Central Coast, and South Coast (Table S1). Not all monitors collected data for all pollutants. The majority (79.5%) of monitor-days included observations for O_3, with 43% of monitor-days including data for NO_2 and $PM_{2.5}$. Some 31% of monitor-days included CO observations and 8.9% included observations of VOCs. Among the 94,349 monitor-days with an observation for VOCs, 39.3% were in the San Joaquin Valley and 12.8% were in the South Coast basin, both basins where most oil and gas wells were concentrated. For each pollutant, there were more observations at monitors more than 10 km from wells than monitors near wells. More observations were collected in the later years of the study period compared to earlier in the study period. The number of monitors in operation throughout the study period was relatively consistent from year to year; the minimum number of monitors in operation was 223 in 2006 and the maximum was 245 in both 2012 and 2014, with a median of 239 (Fig. S4). The number of monitors that assessed $PM_{2.5}$ concentrations increased throughout the study period. Concentrations of pollutants at monitors within 10 km of wells were similar to the concentrations at monitors further away (Table 1).

Wells in all production stages were concentrated in the San Joaquin Valley, which includes Kern County, with substantial production in the South Coast air basin, which includes Los Angeles County (Table S1). Among the 314 monitors included in the analytic dataset, 79 (25.2%) were within 10 km of at least one oil or gas well, 33 (10.5%) were within 3 km, and 11 (3.5%) were within 1 km. Of the monitor-days included in the analysis, 46,477 (4.4%) were exposed to at least one preproduction or production well within 1 km, 115,648 (10.9%) were within 3 km, and 239,764 (22.7%) were within 10 km. For monitor-days with data for $PM_{2.5}$ and VOCs, there were no preproduction wells within 1 km.

Among exposed monitor-days, the median number of preproduction wells within each upwind 1-km bin was between 1 and 4, with a maximum of 41 (Table S2). For producing wells, median upwind exposure spanned 7.2 to 166.9 BOE, with a right-skew and a maximum of 24,166.1 BOE. There was both seasonal and geographic variation in wind direction: in the San Joaquin Valley, the wind predominantly originated in the northwest; in the South Coast basin, wind predominantly came from the southwest (Fig. S1). Exposure to preproduction wells was correlated with exposure to production volume for all annuli beyond 1 km. Across producing wells, daily production volume was right-skewed, with a median of 7.3 BOE per day and mean (± SD) of 17.1 (± 50.6) BOE per day. Exposure to preproduction wells was highly correlated for adjacent annuli and moderately correlated with further annuli; we observed a similar trend for production volume (Table S3). Exposure to preproduction wells was moderately correlated with exposure to production volume at distances greater than 1 km from wells.

3.2. Primary analyses

In the primary analysis, we observed increased concentrations of $PM_{2.5}$, CO, NO_2, and O_3 with proximity to preproduction wells (Fig. 3). For $PM_{2.5}$, we observed an increase of 2.35 μg m^{-3} (95% CI: 0.81, 3.89) for each additional upwind preproduction well site within 2 km of the monitor, and 0.97 μg m^{-3} (0.52, 1.41) for an additional well between 2 and 3 km from the monitor. For CO, we observed an increase of 0.09 ppm (-0.0004, 0.18) with an additional upwind well within 2 km and 0.02 (0.004, 0.032) for a well at 2-3 km. Concentrations of NO_2 increased 2.27 with well at 0-1 km, 2.91 (0.99, 4.84) for a well at 1-2 km, and 0.65 (0.31, 0.99) for a well at 2-3 km upwind. For O_3, there

Table 1
Descriptive statistics of the air monitors, pollutant concentrations, and meteorological factors during the study period, 2006-2019. The unit of observation is the monitor-day; some monitors observe multiple pollutants. VOCs in the dataset comprise non-methane volatile organic compounds.

	≤ 10 km to wells	> 10 km to wells	All
Monitors, *n* (column %)	79 (25.2)	235 (74.8)	314 (100)
Urban	57 (72.2)	151 (64.3)	208 (66.2)
Rural	22 (27.8)	84 (35.7)	106 (33.8)
Sacramento Valley	16 (20.2)	26 (11.1)	42 (26.6)
San Joaquin Valley	18 (22.8)	24 (10.2)	42 (26.6)
South Central Coast	15 (19.0)	14 (6.0)	29 (18.4)
South Coast	15 (19.0)	30 (12.8)	45 (28.5)
$PM_{2.5}$	43 (54.4)	155 (66.0)	198 (63.1)
CO	34 (43.0)	76 (32.3)	110 (35.0)
NO_2	45 (57.0)	94 (40.0)	139 (44.3)
O_3	65 (82.3)	172 (73.2)	237 (75.5)
VOCs	24 (30.4)	24 (10.2)	48 (15.3)
Observations, *n* (column %)	307,095 (29.0)	751,135 (71.0)	1,058,230 (100)
Urban	214,011 (69.7)	507,287 (67.5)	721,298 (68.2)
Rural	93,084 (30.3)	243,848 (32.5)	336,932 (31.8)
$PM_{2.5}$	137,657 (44.8)	317,065 (42.2)	454,722 (43.0)
CO	98,165 (32.0)	229,646 (30.6)	327,811 (31.0)
NO_2	157,567 (51.3)	297,197 (39.6)	454,764 (43.0)
O_3	252,572 (82.2)	588,448 (78.3)	841,020 (79.5)
VOCs[a]	44,992 (14.7)	49,357 (6.6)	94,349 (8.9)
2006–2009	77,013 (25.1)	200,404 (26.7)	277,417 (26.2)
2010–2014	104,839 (34.1)	264,066 (35.2)	368,905 (34.9)
2015–2019	107,248 (34.9)	268,876 (35.8)	376,124 (35.5)
Smoke plume overhead	21,780 (7.1)	54,299 (7.2)	76,079 (7.2)
Pollutant concentrations, daily mean ± SD			
$PM_{2.5}$ ($\mu g/m^3$)	10.6 ± 9.5	9.9 ± 9.0	10.1 ± 9.1
CO (ppm)	0.5 ± 0.4	0.5 ± 0.4	0.5 ± 0.4
NO_2 (ppb)	21.4 ± 14.6	22.1 ± 14.5	21.9 ± 14.5
O_3 (ppm)	0.04 ± 0.01	0.04 ± 0.02	0.04 ± 0.02
VOCs (ppb C)	120 ± 166	104 ± 142	112 ± 155
Meteorological factors, daily mean ± SD			
Precipitation (mm)	0.9 ± 4.0	1.2 ± 5.1	1.1 ± 4.8
Temperature (°C)	18.6 ± 7.8	17.2 ± 9.1	17.6 ± 8.8
Wind speed (m/s)	3.0 ± 2.1	3.2 ± 2.0	3.1 ± 2.0

[a] The data for VOCs includes observations for 1999-2019.

were no significant changes for an additional well within 2 km, an increase of 0.31. (0.20, 0.42) with an additional well at 2-3 km, and an increase of 0.14 (0.05, 0.23) with a well at 3-4 km. There were no increases in concentration with upwind exposure to VOCs, though notably there was no exposure to preproduction wells within 1 km. Across all pollutants, we did not observe any substantial increased concentrations beyond 4 km. In the placebo test, with exposure assessed to downwind wells, we did not observe any substantial increases in pollutant concentrations.

We observed increased concentrations of $PM_{2.5}$, NO_2, O_3, and VOCs with higher exposure to upwind production (Fig. 4). We estimated the marginal effect of exposure to an additional 100 BOE of daily total oil and gas volume within each 1-km annulus. This degree of exposure roughly corresponds with median upwind production volume within each annulus among exposed monitor-days (Table S2) and is comparable to cutoffs used in recent population health work (Tran et al., 2020). For each additional 100 BOE of total oil and gas production within 1 km, we observed an increase of 1.93 $\mu g\ m^{-3}$ (95% CI: 1.08, 2.78) in the concentration of $PM_{2.5}$. For NO_2, we observed an increase of 0.62 ppb (0.37, 0.86) with an additional 100 BOE within 1 km. The concentration of O_3, increased by 0.11 ppb (0.08, 0.14) with for each 100 additional BOE at 1-2 km. There was an increase in VOC concentrations of 0.04 (0.01, 0.07) ppb C for an additional 100 BOE of production within 1 km. We did not observe any substantial changes in CO concentrations with upwind exposure to production volume. In the downwind placebo tests, we observed an increase in $PM_{2.5}$ concentrations for exposure to increased production within 1 km, a small increase in NO_2 concentrations at 1-2 km, and an increase in O_3 at 3-4 km.

3.3. Sensitivity analyses

We performed several sensitivity analyses. Fitting models that included exposure variables for both preproduction and production did not substantially change the results; point estimates and confidence intervals were similar in models with exposure variables for both preproduction and production compared to models examining each exposure separately (Fig. S4). In models with polynomial term for exposure we did not see evidence of non-linear responses to upwind exposure. Changing model specification in the primary analysis for preproduction wells (Table S4) or for production volume did not qualitatively change findings (Table S5). In a sensitivity analysis, we fit the model as described above but omitted the 35,422 monitor-days with smoke plumes overhead, comprising 7.8% of the $PM_{2.5}$ analytic dataset. The results were similar to the smoke-adjusted results for exposure to wells in both the preproduction and production stages (Fig. S3).

4. Discussion

We observed higher concentrations of ambient air pollutants at air monitors exposed to wells in both the preproduction and production stages. Concentrations of $PM_{2.5}$ were substantially higher on days when a well was in preproduction within 3 km of the monitor, and also when production volume increased within 1 km of the monitor. Notably, we observed increases in $PM_{2.5}$ within 1 km of producing wells with and without considering wind direction. There are several possible explanations for this result: it may be attributable to high volume of producing wells near monitors in San Joaquin Valley orthogonal to the upwind direction, imperfect data on wind direction,

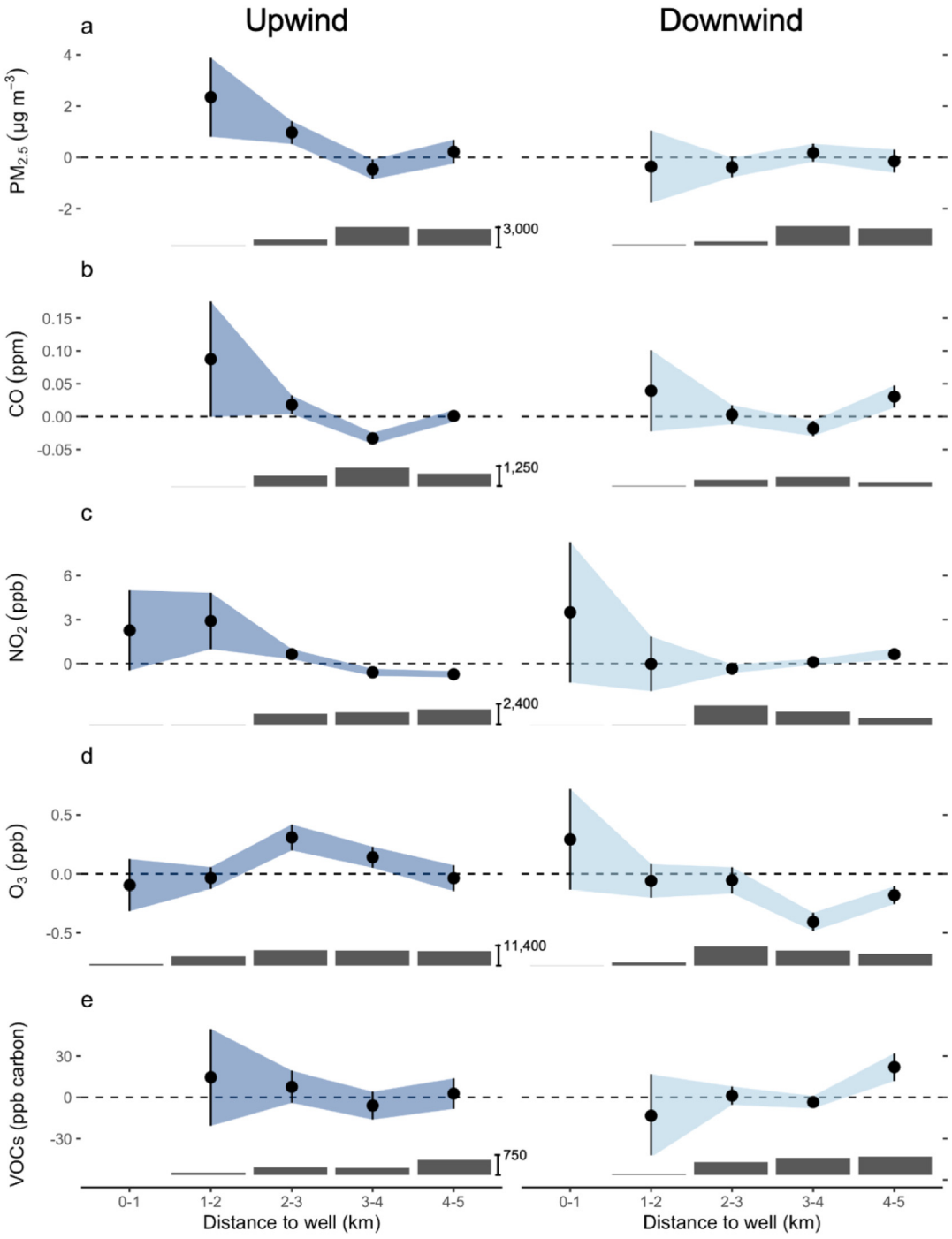


Fig. 3. Point estimates (95% CIs) for the marginal effect of one additional preproduction well upwind (left column) and downwind (right column) of the monitor. The bar plots show the number of monitor-days with exposure at least one preproduction well within each distance bin.

or shifts in wind direction during the day that were not adequately captured when we integrated wind direction over the course of a 24 h period. In addition to elevated $PM_{2.5}$ levels, concentrations of O_3 increased when production activity increased between 1 and 4 km upwind of the monitor, but not for activity within 1 km of the monitor. This result may be attributable to secondary formation from primary pollutants emitted from during preproduction and production. Ground-level O_3 may be secondarily formed from photochemical reactions involving CO, NO_x, and VOCs, all of which we also observed were emitted from wells (Real et al., 2007; Rodriguez et al., 2009). We observed increased CO concentrations on days when preproduction wells were drilled within 3 km of the monitor. Concentrations of NO_2 were higher on days when there was a preproduction well within 2 km or increased production volume within 1 km. For VOCs, we found higher concentrations when production volume increased within 1 km of the monitor. In the current study, VOCs comprised non-methane organic compounds including acetaldehyde, benzene, ethylene, and formaldehyde.

In models that considered both preproduction wells and production volume, we observed similar estimates to the models where we considered preproduction and production separately, as shown in Fig. S4. Preproduction activity near monitors was correlated with production volume, though this may not be apparent based on the correlation matrix in Table S3, which shows low correlation between preproduction

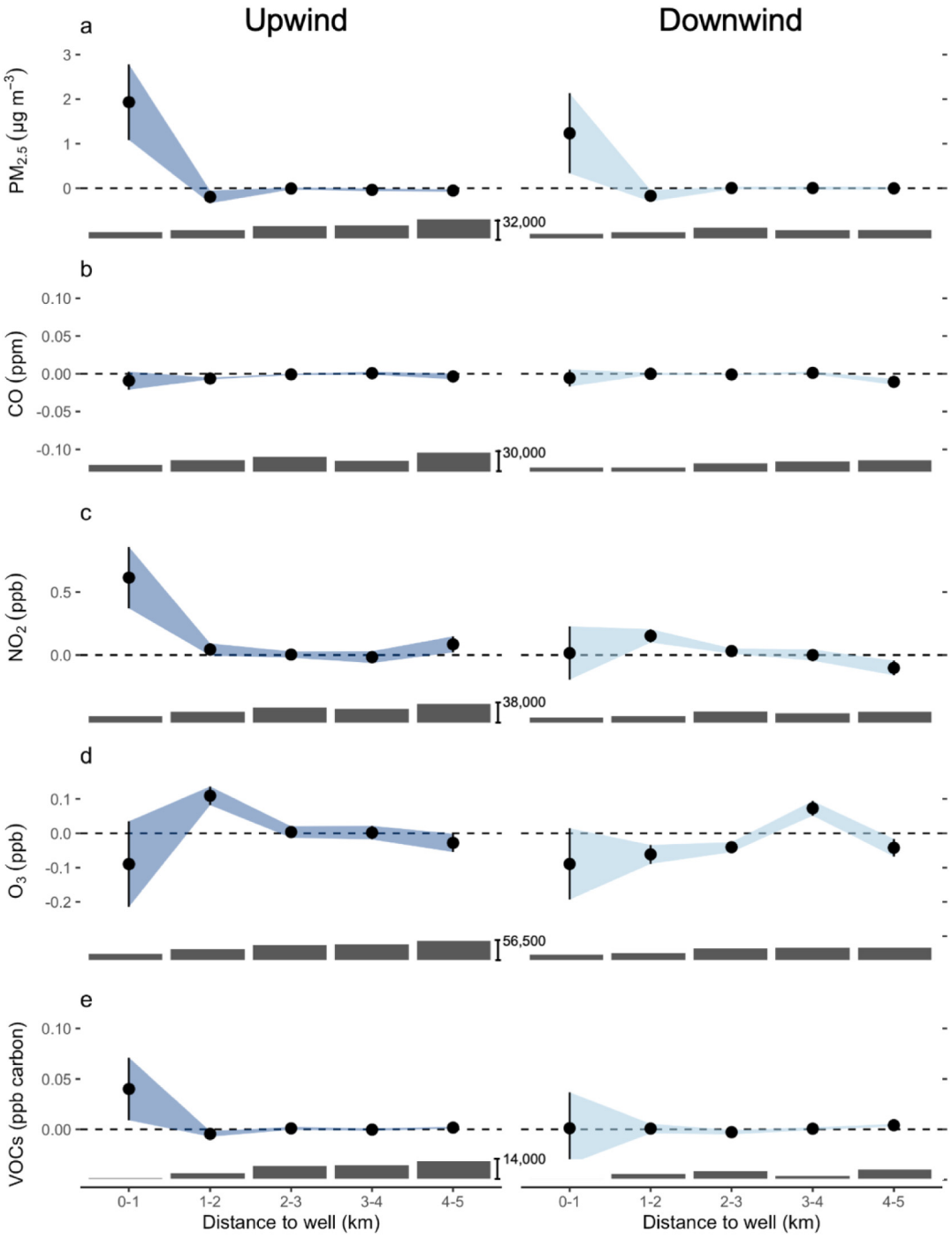


Fig. 4. Point estimates (95% CIs) for the marginal effect of 100 additional barrels of oil equivalent (BOE) of daily production volume, for wells upwind (left column) and downwind (right column) of the monitor. The bar plots show the number of monitor-days with exposure at least 1 BOE of daily production volume within each distance bin. Note that more monitor-days had exposure to production volume than preproduction wells.

wells and production volume. However, among all monitor-days with a preproduction well within 1 km of the monitor, there was also >0 BOE of production volume.

In this study, we conducted a quasi-experimental analysis that relied on the existing network of air quality monitors. The siting of air quality monitors is delegated to local authorities and prior studies have found evidence of bias in where monitors are sited, which should be considered when interpreting the results from the current study (Grainger et al., 2017; Grainger and Schreiber, 2019). For example, in counties just marginally in attainment for National Ambient Air Quality Standards (NAAQS), regulators had an incentive to place new monitors far from pollution sources, whereas in areas already in non-attainment, the regulators were incentivized to place monitors close to polluting sources (Grainger et al., 2017). This could lead to biased estimates of emissions from oil and gas wells, as monitors may be sited away from the most intensively producing oil fields. There is also evidence that monitors are less likely to be located in communities with racially and socioeconomically marginalized populations, which could lead to underestimation of oil and gas-related emissions if oil production in excluded areas was more intensive and polluting (Grainger and Schreiber, 2019). In the current study, the majority of oil and gas production was concentrated in Kern and Los Angeles Counties, both of which were in

non-attainment for $PM_{2.5}$ throughout the study period (Environmental Protection Agency, 2021).

Findings from the current study indicate both primary emission and secondary formation of pollutants from upstream oil and gas production activities. However, identifying specific processes that resulted in observed pollutant emissions was outside the scope of the study.

4.1. Comparison to prior studies

Using proximity as a metric of exposure to upstream oil and gas production appears to adequately capture exposures to chemical contaminants. Proximity-based methods, such as inverse distance weighting or estimating production activity within 1 km of receptors, have been used in prior population health studies to estimate acute or chronic exposure to wells. The five pollutants we examined in this study represent a subset of potential hazards associated with exposure to oil and gas wells, which may include other air pollutants as well as water and noise pollution (Adgate et al., 2014; Jackson et al., 2014). Recent studies from California have reported fugitive methane from idle and unplugged wells, as well as urban oil and gas infrastructure, which may correlate with emissions of benzene, toluene, ethylene, xylene, and other air toxics (Lebel et al., 2020; Okorn et al., 2021). To differentiate risks conferred by air pollutants, population health researchers could utilize variations in wind direction.

Prior field studies have also found emissions of pollutants from upstream oil and gas facilities. A 2018 study in Texas found high concentrations of nitrous oxides and saturated hydrocarbons associated with oil and gas production in the Eagle Ford Shale (Schade and Roest, 2018). Another recent study in Colorado, which combined in situ monitoring and cancer risk assessment, found higher exposure to benzene and other non-methane hydrocarbons (toluene, ethylbenzene, and xylene) and elevated risk of cancer and other adverse health outcomes with close proximity to oil and gas facilities (McKenzie et al., 2018). Notably, the dataset in the current study did not include toluene, ethylbenzene, and xylene. Garcia-Gonzales et al. (2019a) found higher concentrations of VOCs downwind of a well site in Los Angeles. A study in Pennsylvania found that exposure metrics used in prior epidemiological studies were poorly correlated with observed pollutant concentrations (Wendt Hess et al., 2019). However, this study assessed exposure to wells at distances greater than 10 km, where we would not expect to detect increases in pollution, and the authors did not account for meteorological factors that may affect pollutant concentrations (Buonocore et al., 2020).

In prior studies, Tran et al. (2020) and Gonzalez et al. (2020) used differing proximity metrics to assess exposure to upstream oil and gas production and adverse birth outcomes in California. For their analysis of production volume and adverse birth outcomes, Tran et al. used a similar exposure assessment method to the one we employed in the current study, assessing "high" exposure to births with >100 BOE within 1 km of the residence. In the current analysis, we modeled exposure to production volume continuously rather than categorically. We found substantial increases in concentrations of $PM_{2.5}$, NO_2, and O_3 with exposure to an additional 100 BOE within 1 km, indicating that the metrics employed by Tran et al. likely were effective in capturing aspects of air pollution near active wells. Gonzalez et al. used inverse distance-squared weighting (IDW), a different approach that relies on the assumption that both density and proximity of wells confers risk of air pollution exposures. Notably, Gonzalez et al. (2020) conducted an exploratory analysis of the association between proximity to oil and gas wells, assessed using an IDW index, and concentrations of four pollutants (NO_2, O_3, PM_{10}, and $PM_{2.5}$). For that supplemental analysis, Gonzalez et al. also used data from EPA Air Quality System for mean monthly concentrations of air pollutants and fit fixed effects linear regression models estimating the effect of "high" exposure to wells (the highest tertile of the IDW index). These authors observed substantially higher concentrations of PM_{10} and $PM_{2.5}$, lower concentrations of NO_2, and no substantial changes for O_3; for all pollutants, effects. This indicates that the IDW method may be less effective as an exposure metric for the air pollutants investigated in this study than the methods employed in the current study. Additionally, the approaches in both Tran et al. (2020) and Gonzalez et al. (2020) may not adequately capture exposure to secondary pollutants such as O_3, which in the current study had higher concentrations several km downwind of wells.

4.2. Limitations and strengths

The current study had several limitations. We relied on daily changes in wind direction as a source of exogenous variation. On days with variable wind direction, estimating mean wind direction integrated over the course of the day could lead to exposure misclassification if, for example, wind blew from multiple directions during the course of a 24-h period. Data for many pollutants that may be emitted during upstream oil and gas production operations are not routinely monitored and reported in the EPA Air Quality System. Consequently, the results of the current study likely reflect only a subset of pollutants potentially emitted from upstream oil and gas production. Population health studies referring to our estimates of chemical contaminant exposure should consider the possibility of co-exposures to additional pollutants emitted during oil and gas production. We also did not have sufficient data to investigate specific VOC constituents, which may be associated with particular health endpoints of interest. Additionally, there were relatively few monitor-days with exposure to preproduction wells within 1 km. None of the monitors that measure concentrations of $PM_{2.5}$ and VOCs were within 1 km of a preproduction well. We found evidence that drilling sites up within 1 to 3 km of air monitors increased $PM_{2.5}$ concentrations, and concentrations of $PM_{2.5}$ within 1 km of preproduction wells may be similar to or higher than our estimates for wells at 1-3 km. We did not expect to observe changes in VOC concentrations further than 1 km, as prior work has reported decay of VOCs within 100-200 m from well sites (Garcia-Gonzales et al., 2019a; Zielinska et al., 2014). Because of this, we were unable to make any inferences about the effect of preproduction activities on concentrations of VOCs.

In the primary analyses, we adjusted for exposure to wildfire smoke plumes to account for potential contributions of smoke to the pollutants of interest. Exposure was assessed as the number of overhead plumes for each monitor-day, but this method may not accurately indicate smoke conditions at ground level. A sensitivity analysis for $PM_{2.5}$ omitting smoke days from the analysis yielded similar results to the smoke-adjusted models, suggesting that our statistical adjustment for smoke plumes was sufficient.

For the analyses of wells in the production stage, data on total oil and gas production volume were available at the monthly level. Because of this constraint, in the exposure assessment we assumed that production occurred evenly throughout the month. This could lead to exposure misclassification if production was concentrated in certain days of the month. Future researchers building on these findings should consider obtaining daily production volume data, if possible. Finally, we were not able to differentiate between drilling or production methods (i.e., conventional vs. unconventional methods, such as hydraulic fracturing), so we were not able to determine whether certain unconventional methods resulted in higher emissions.

Strengths of this study include the large panel dataset, comprising over 1 million daily observations from high quality air monitors with broad geographic and temporal variation. We were able to control for unobserved potential confounders through the study design, using wind as a plausibly exogenous source of variation uncorrelated to both upstream oil production and other sources of pollution. The monitor fixed effect accounts for average differences between monitoring locations, such as from pollution sources unrelated to oil and gas. Leveraging temporal variation from oil production activities and daily changes in wind direction accounts for other nearby pollution sources that are not both spatially collocated and temporally correlated with

oil and gas production. Based on this analytic approach, we think there is unlikely to be residual confounding. Additionally, we conducted several tests to validate the robustness of the results.

5. Conclusion

We conducted a quasi-experimental study to examine whether upstream oil and gas production results in emissions of ambient air pollutants. Adjusting for geographic, meteorological, seasonal, and time-trending factors, and leveraging daily changes in wind direction as an exogenous source of variation, we observed that proximity to oil and gas wells in both preproduction and production increased concentrations of $PM_{2.5}$, CO, NO_2, O_3, and VOCs at distances up to 4 km downwind of wells. These findings indicate that proximity to wells is an appropriate metric for air pollution-related exposures in population health studies. Notably, increases in $PM_{2.5}$ concentrations near wells could be a mediating factor for previously reported increases in risk of adverse birth outcomes with proximity to wells in California (Bekkar et al., 2020; Gonzalez et al., 2020; Tran et al., 2020). Further research on hazards associated with upstream oil and gas production would improve understanding of potential health and environmental risks. Acute emissions of particular pollutants may be associated with specific steps of oil and gas preproduction or production, and more work is needed to determine if this is the case and, if so, which processes produce high emissions. Mitigating exposure to oil and gas wells would likely reduce exposure to ambient air pollutants.

Funding

This work was supported by the Ford Foundation Predoctoral Fellowship, the Vice Provost for Graduate Education at Stanford University, and the Stanford School of Earth, Energy, and Environmental Sciences. The funding sources did not have any involvement in the design, conduct, or writing of this study.

CRediT authorship contribution statement

David J.X. Gonzalez: Conceptualization, Data curation, Methodology, Formal analysis, Visualization, Writing – original draft, Writing – review & editing. **Christina K. Francis:** Data curation, Writing – original draft. **Gary M. Shaw:** Writing – review & editing. **Mark R. Cullen:** Methodology, Writing – review & editing. **Michael Baiocchi:** Methodology, Writing – review & editing. **Marshall Burke:** Conceptualization, Methodology, Supervision, Writing – review & editing.

Data availability

Data and code used in this analysis are available at https://github.com/djxgonzalez/cal-drilling-air-quality.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

We would like to thank Sam Heft-Neal and Anne Driscoll for assistance with the smoke plume data.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scitotenv.2021.150298.

References

Adgate, J.L., Goldstein, B.D., McKenzie, L.M., 2014. Potential public health hazards, exposures and health effects from unconventional natural gas development. Environ. Sci. Technol. 48, 8307–8320.
Allshouse, W.B., Adgate, J.L., Blair, B.D., McKenzie, L.M., 2017. Spatiotemporal industrial activity model for estimating the intensity of oil and gas operations in Colorado. Environ. Sci. Technol. 51, 10243–10250.
Allshouse, W.B., McKenzie, L.M., Barton, K., Brindley, S., Adgate, J.L., 2019. Community noise and air pollution exposure during the development of a multi-well oil and gas pad. Environ. Sci. Technol. 53, 7126–7135.
Arbelaez, J., Baizel, B., 2015. Californians at Risk: An Analysis of Health Threats From Oil and Gas Pollution in Two Communities.
Bekkar, B., Pacheco, S., Basu, R., DeNicola, N., 2020. Association of air Pollution and Heat Exposure with Preterm Birth, low birth weight, and stillbirth in the US: a systematic review. JAMA Netw. Open 3, e208243.
Buonocore, J.J., Casey, J.A., Croy, R., Spengler, J.D., McKenzie, L., 2020. Air monitoring stations far removed from drilling activities do not represent residential exposures to Marcellus shale air pollutants. Response to the paper by Hess et al. on proximity-based unconventional natural gas exposure metrics. Int. J. Environ. Res. Public Health, 17 https://doi.org/10.3390/ijerph17020504.
Casey, J.A., Savitz, D.A., Rasmussen, S.G., Ogburn, E.L., Pollak, J., Mercer, D.G., 2015. Unconventional natural gas development and birth outcomes in Pennsylvania, USA. Epidemiology 1.
Casey, J.A., Wilcox, H.C., Hirsch, A.G., Pollak, J., Schwartz, B.S., 2018. Associations of unconventional natural gas development with depression symptoms and disordered sleep in Pennsylvania. Sci. Rep. 8. https://doi.org/10.1038/s41598-018-29747-2.
Currie, J., Greenstone, M., Meckel, K., 2017. Hydraulic fracturing and infant health: new evidence from Pennsylvania. Sci. Adv. 3, e1603021.
Czolowski, E.D., Santoro, R.L., Srebotnjak, T., Shonkoff, S.B.C., 2017. Toward consistent methodology to quantify populations in proximity to oil and gas development: a National Spatial Analysis and review. Environ. Health Perspect. 125, 086004.
Denham, A., Willis, M.D., Croft, D.P., Liu, L., Hill, E.L., 2021. Acute myocardial infarction associated with unconventional natural gas development: a natural experiment. Environ. Res. 195, 110872.
Environmental Protection Agency, 2021. Green book.Available: https://www3.epa.gov/airquality/greenbook/anayo_ca.html [accessed 25 March 2021].
Garcia-Gonzales, D.A., Shamasunder, B., Jerrett, M., 2019a. Distance decay gradients in hazardous air pollution concentrations around oil and natural gas facilities in the city of Los Angeles: a pilot study. Environ. Res. 173, 232–236.
Garcia-Gonzales, D.A., Shonkoff, S.B.C., Hays, J., Jerrett, M., 2019b. Hazardous air pollutants associated with upstream oil and Natural gas development: a critical synthesis of current peer-reviewed literature. Annu. Rev. Public Health 40, 283–304.
Gonzalez, D.J.X., Sherris, A.R., Yang, W., Stevenson, D.K., Padula, A.M., Baiocchi, M., et al., 2020. Oil and gas production and spontaneous preterm birth in the San Joaquin Valley, CA: a case-control study. Environ. Epidemiol. 4, e099.
Grainger, C., Schreiber, A., 2019. Discrimination in ambient air pollution monitoring? AEA Pap. Proc. 109, 277–282.
Grainger, C., Schreiber, A., Chang, W., 2017. How States Comply With Federal Regulations: Strategic Ambient Pollution Monitoring.
Holloway, T., Levy II, H., Kasibhatla, P., 2000. Global distribution of carbon monoxide. J. Geophys. Res. 105, 12123–12147.
Jackson, R.B., Vengosh, A., Carey, J.W., Davies, R.J., Darrah, T.H., O'Sullivan, F., et al., 2014. The environmental costs and benefits of fracking. Annu. Rev. Environ. Resour. 39, 327–362.
Johnston, J.E., Lim, E., Roh, H., 2019. Impact of upstream oil extraction and environmental public health: a review of the evidence. Sci. Total Environ. 657, 187–199.
Johnston, J.E., Enebish, T., Eckel, S.P., Navarro, S., Shamasunder, B., 2021. Respiratory health, pulmonary function and local engagement in urban communities near oil development. Environ. Res. 197, 1–10 111088.
Lebel, E.D., Lu, H.S., Vielstädte, L., Kang, M., Banner, P., Fischer, M.L., et al., 2020. Methane emissions from abandoned oil and gas Wells in California. Environ. Sci. Technol. 54, 14617–14626.
Mauzerall, D.L., Sultan, B., Kim, N., Bradford, D.F., 2005. NOx emissions from large point sources: variability in ozone production, resulting health damages and economic costs. Atmos. Environ. 39, 2851–2866.
McKenzie, L.M., Witter, R.Z., Newman, L.S., Adgate, J.L., 2012. Human health risk assessment of air emissions from development of unconventional natural gas resources. Sci. Total Environ. 424, 79–87.
McKenzie, L.M., Guo, R., Witter, R.Z., Savitz, D.A., Newman, L.S., Adgate, J.L., 2014. Birth outcomes and maternal residential proximity to natural gas development in rural Colorado. Environ. Health Perspect. 122, 412–417.
McKenzie, L.M., Blair, B., Hughes, J., Allshouse, W.B., Blake, N.J., Helmig, D., et al., 2018. Ambient nonmethane hydrocarbon levels along Colorado's northern front range: acute and chronic health risks. Environ. Sci. Technol. 52, 4514–4525.
McKenzie, L.M., Crooks, J., Peel, J.L., Blair, B.D., Brindley, S., Allshouse, W.B., et al., 2019. Relationships between indicators of cardiovascular disease and intensity of oil and natural gas activity in northeastern Colorado. Environ. Res. 170, 56–64.
Okorn, K., Jimenez, A., Collier-Oxandale, A., Johnston, J., Hannigan, M., 2021. Characterizing methane and total non-methane hydrocarbon levels in Los Angeles communities with oil and gas facilities using air quality monitors. Sci. Total Environ. 777, 146194.
R Core Team, 2020. R: A Language and Environment for Statistical Computing.
Rasmussen, S.G., Ogburn, E.L., McCormack, M., Casey, J.A., Bandeen-Roche, K., Mercer, D.G., et al., 2016. Association between unconventional natural gas development in the Marcellus shale and asthma exacerbations. JAMA Intern. Med. 176, 1334–1343.

Real, E., Law, K.S., Weinzierl, B., Fiebig, M., Petzold, A., Wild, O., et al., 2007. Processes influencing ozone levels in alaskan forest fire plumes during long-range transport over the North Atlantic. J. Geophys. Res. 112. https://doi.org/10.1029/2006jd007576.
Rodriguez, M.A., Barna, M.G., Moore, T., 2009. Regional impacts of oil and gas development on ozone formation in the western United States. J. Air Waste Manage. Assoc. 59, 1111–1118.
Schade, G.W., Roest, G., 2016. Analysis of Non-methane Hydrocarbon Data From a Monitoring Station Affected by Oil and Gas Development in the Eagle Ford Shale, Texas. https://doi.org/10.12952/journal.elementa.000096.
Schade, G.W., Roest, G., 2018. Source Apportionment of Non-methane Hydrocarbons, NOx and H2S Data From a Central Monitoring Station in the Eagle Ford Shale, Texas. https://doi.org/10.1525/elementa.289.
Schroeder, W., Ruminski, M., Csiszar, I., Giglio, L., Prins, E., Schmidt, C., et al., 2008. Validation analyses of an operational fire monitoring product: the Hazard mapping system. Int. J. Remote Sens. 29, 6059–6066.
Shamasunder, B., Collier-Oxandale, A., Blickley, J., Sadd, J., Chan, M., Navarro, S., et al., 2018. Community-based health and exposure study around urban oil developments in South Los Angeles. Int. J. Environ. Res. Public Health 15 (138). https://doi.org/10.3390/ijerph15010138.
Tang, I.W., Langlois, P.H., Vieira, V.M., 2020. Birth defects and unconventional natural gas developments in Texas, 1999–2011. Environ. Res. 194, 1–10 110511.
Tran, K.V., Casey, J.A., Cushing, L.J., Morello-Frosch, R., 2020. Residential proximity to oil and gas development and birth outcomes in California: a retrospective cohort study of 2006–2015 births. Environ. Health Perspect. 128, 067001.
Wendt Hess, J., Bachler, G., Momin, F., Sexton, K., 2019. Assessing agreement in exposure classification between proximity-based metrics and air monitoring data in epidemiology studies of unconventional resource development. Int. J. Environ. Res. Public Health 16, 3055.
Whitworth, K.W., Marshall, A.K., Symanski, E., 2017. Maternal residential proximity to unconventional gas development and perinatal outcomes among a diverse urban population in Texas. PLoS One 12, e0180966.
Willis, M.D., Hill, E.L., Boslett, A., Kile, M.L., Carozza, S.E., Hystad, P., 2021. Associations between residential proximity to oil and gas drilling and term birth weight and small-for-gestational-age infants in Texas: a difference-in-differences analysis. Environ. Health Perspect. 129, 077002. https://doi.org/10.1289/ehp7678.
Zielinska, B., Campbell, D., Samburova, V., 2014. Impact of emissions from natural gas production facilities on ambient air quality in the Barnett shale area: a pilot study. J. Air Waste Manage. Assoc. 64, 1369–1383.

AN ABSTRACT OF THE DISSERTATION OF

Britton C. Goodale for the degree of Doctor of Philosophy in Toxicology presented on August 12, 2013.

Title: Developmental Toxicity of Polycyclic Aromatic Hydrocarbons: Defining Mechanisms with Systems-based Transcriptional Profiling

Abstract approved:_____
Robert L. Tanguay

Polycyclic aromatic hydrocarbons (PAHs) are ubiquitous in the environment as components of fossil fuels and by-products of combustion. Defining toxicity mechanisms for this large family of multi-ring structures and substituted derivatives is a substantial challenge. Several PAHs, such as benzo(a)pyrene (BaP), are mutagenic, toxic to wildlife, and classified as probable carcinogens to humans. PAHs are present in the environment both in the gaseous phase as well as associated with particulates, and exposures occur via complex mixtures; combustion emissions contain PAHs along with many other contaminants. Cardiac dysfunction and adverse birth outcomes associated with exposure to airborne PAHs suggest that this family of compounds may have non-mutagenic biological activities that affect human health. Some PAHs exert toxic effects via binding the aryl hydrocarbon receptor (AHR), a ligand-activated transcription factor that mediates transcription of many downstream target genes, including cytochrome P450 metabolizing enzymes. Unlike planar halogenated hydrocarbons, such as 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD), PAHs are readily metabolized by CYP1A, CYP1B1 and other enzymes, which create reactive intermediates and/or facilitate excretion. Mechanisms of PAH toxicity therefore include canonical AHR signaling, induction of oxidative stress, and other lesser-understood activities that do not require the AHR. We employed zebrafish as a model to rapidly assess developmental toxicity, global transcriptional responses and AHR activation in embryos exposed to parent and oxygenated PAHs (OPAHs). Using comparative analysis of mRNA expression profiles from microarrays with embryos exposed to benz(a)anthracene (BAA), dibenzothiophene (DBT) and pyrene (PYR), we identified expression biomarkers and

disrupted biological processes that precede developmental abnormalities. These transcriptional responses were associated with PAH body burdens in the embryos detected by GC-MS. We found that uptake data were essential for discerning molecular pathways from dose-related differences, and identified two primary toxicity profiles. While BAA disrupted transcripts involved in vasculogenesis, DBT and PYR misregulated ion homeostasis and muscle-related genes. NfKB signaling was predicted to be involved in both responses, but canonical AHR signaling was only activated by BAA. In order to study the role of the AHR in mediating toxicity of PAHs, we developed an AHR2 mutant zebrafish line, which has a mutation in the transactivation domain of AHR2. We used AHR agonists TCDD and leflunomide as toxicological probes to characterize AHR activity in the mutant line, and determined that the mutants were functionally null. Finally, we used AHR2 deficient zebrafish embryos to investigate mechanisms by which two four-ring OPAHs induced developmental effects. 1,9 benz-10-anthrone (BEZO) and benz(a)anthracene-7,12-dione (7,12-B[a]AQ) both caused malformations in developing embryos, but they differentially induced CYP1A expression. Despite this difference, the toxicity produced from both compounds was AHR2-dependent. We used mRNA-seq to compare the transcriptional profiles of BEZO and 7,12-B[a]AQ, and identified transcriptional networks that will be investigated further to determine how ligands differentially modulate AHR activity. We also discovered novel transcripts that are potentially important mediators of AHR toxic effects. Comparison across all five parent and OPAHs highlighted clusters of genes that, surprisingly, were similarly expressed in response to the OPAHs, DBT and PYR. These commonly-regulated transcripts may be important to consider when investigating toxicity of PAH mixtures. Together, these studies show that PAHs act via different transcriptional mechanisms, but can be categorized based on transcriptional profiles and differential AHR activation. The clusters of transcripts identified may be involved in common pathways; further investigation of transcription factors and coactivators that interact with mixexpressed genes is a promising area of research for elucidating diverse functions of the AHR.

Developmental Toxicity of Polycyclic Aromatic Hydrocarbons: Defining Mechanisms with Systems-based Transcriptional Profiling

by

Britton C. Goodale

A DISSERTATION

submitted to

Oregon State University

in partial fulfillment of

the requirements for the

degree of

Doctor of Philosophy

Presented August 12, 2013

Commencement June 2014

Doctor of Philosophy dissertation of Britton C. Goodale presented on August 12, 2013.

APPROVED:

_____

Major Professor, representing Toxicology

_____

Head of the Department of Environmental and Molecular Toxicology

_____

Dean of the Graduate School

I understand that my dissertation will become part of the permanent collection of Oregon State University libraries. My signature below authorizes release of my dissertation to any reader upon request.

_____

Britton C. Goodale, Author

ACKNOWLEDGEMENTS

The completion of this dissertation has by no means been a solitary effort, and I am grateful to the many people who made it possible. I would like to express my sincere gratitude to my graduate mentor, Robert Tanguay, for giving me the opportunity to join the lab and pursue what were, five years ago, fairly specific interests in toxicology. Thank you for sharing your endless excitement about new ideas, and for broadening my perspective and enthusiasm for this field of research more than I could have imagined. None of the work presented here would have been possible without the support I had during my time at OSU, and I am appreciative of the excellent resources that the Tanguay lab has provided. I would like to thank the many members of the Tanguay lab who have helped me over the years: Jane La Du, for her humor and for always being there to help in a pinch; Kate Saili, Tamara Tal, Lisa Truong, Galen Miller and Sumitra Sengupta for providing training in the lab, and for their mentorship and friendship; Andrea Knecht, Mike Simonich, and Annika Swanson for their help and patience with the OPAH studies; Leah Wehmas, Derik Haggard, Siba Das and Cory Gerlach for editing, experiment assistance, and helping me through this final year with excellent company in the lab; Sean Bugel for sharing the office with the window and the challenge assay, providing thoughtful answers to my many questions, and for the excellent discussions; Jill Franzosa for encouragement and advice, both in the lab and on the climbing wall; and Margaret Corvi for helping me keep perspective, making the OPAH body burden studies possible, and being an inspiration to work with. All of the studies in this dissertation were truly made possible by the SARL staff, and I am thankful to Carrie Barton and Cari Buchner for their dedication to the zebrafish colony, particularly the AHR2 mutants (they will some day express their gratitude, too, I'm sure). Thank you to Greg Gonnerman, Chapell Miller for screening help, and to all of the other staff who have kept SARL running.

I would like to thank others at OSU for their support; I am grateful to my committee members Daniel Sudakin, Michael Freitag, Kim Anderson, Barbara Taylor and Jeffrey Greenwood for their wisdom and guidance of this project. The Anderson lab welcomed me to OSU, and I can't imagine my time here without them. Thank you to the many folks who helped me with chemistry questions, compound requests, advised me on environmental

relevance for my studies, and gave me good excuses to go to campus. I thank the EMT department for making my graduate work possible, especially department head Craig Marcus for helping me accomplish many goals, and all of the EMT office staff for making things run smoothly.

I am especially grateful to my friends who, from near and far, supported me through every step of graduate school. Thank you for being my family when I arrived, exploring the west coast with me, and making Oregon home. Since graduate work feels, much of the time, like a process of many failures, successes in other arenas were an essential part of life the past five years. Thank you to everyone who buoyed my sanity with triumphant climbs, bikes, and foraging adventures. I am so thankful to Erik Endrulat, for his support through this endeavor, and for gently reminding me to think about balance. I was unable to conduct a controlled study on the topic, but nevertheless attribute my perplexing decrease in flu susceptibility the past two years to the good food and abundance of laughter I've enjoyed with him. Most of all, I am forever grateful to my family, for their love and support through this entire journey, especially when I chose to move across the country. To my sister, Jane: thank you for your incredible insight, and for your gift of finding the humor in whatever situation I presented you with. Thank you to my Dad, Steve, for teaching me to be a problem solver, and continually giving me new things to think about. To my Mom, Anne: thank you for your words of advice, even when I didn't want to hear it, cheering for me always, and the steadfast assurance that I wasn't doing this on my own.

CONTRIBUTION OF AUTHORS

Many people at Oregon State University and Pacific Northwest National Lab contributed to the studies presented in this thesis. In Chapter 2, Susan Tilton statistically analyzed the microarray data, conducted transcription factor enrichment/pathway analysis, provided guidance and training for other bioinformatics techniques employed, and contributed intellectually to the manuscript. Margaret Corvi contributed experiment planning guidance and assisted with all aspects of sample preparation for the PAH body burden studies. Glenn Wilson conducted GC-MS analysis of PAH body burden samples. Derek Janszen statistically analyzed the PAH developmental toxicity data. Kim Anderson and Katrina Waters both contributed training and intellectual guidance for the manuscript.

In Chapter 3, Jane La Du conducted outcrossing and screening for *ahr2*^{hu3335} carriers, and assisted with characterization and AHR knockdown studies. William Bisson conducted the in silico AHR docking studies. Derek Janszen carried out statistical analysis of developmental toxicity data. Katrina Waters provided statistical expertise and contributed intellectually to the studies.

In Chapter 4, Susan Tilton provided bioinformatic support and conducted transcription factor network prediction and enrichment analysis for biological functions associated with RNA-seq transcripts. Christopher Sullivan processed RNA-seq data, and provided bioinformatic core support for mapping, assembly, and statistical analysis of RNA-seq data.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

DEDICATION

This dissertation is dedicated to my grandparents, Edith and John Artz, Walter Goodale, and Garrett and Pearl Morrell, whose love and support of all educational and creative endeavors have been incredible gifts to me.

Developmental toxicity of polycyclic aromatic hydrocarbons: defining mechanisms with systems-based transcriptional profiling

Chapter 1 - Introduction

Combustion of fuels for transportation, heating and industrial activities produces volatile and fine particulate matter emissions that decrease air quality and contribute to environmental contamination, particularly in urban areas. Polycyclic aromatic hydrocarbons (PAHs), a group of chemicals comprised of multiple fused benzene rings, are formed from these combustion (pyrogenic) processes and are components of fossil fuels (petrogenic sources). Environmental samples contain a diversity of parent PAHs, which differ in the number and arrangement of rings, as well as substituted (alkyl-, nitro-, amino-, and oxy-) structures (Ciganek et al. 2004). PAHs are contaminants of extant concern because of their carcinogenic properties and ubiquity both in high-population areas and hazardous waste sites (Schoeny 1993; Collins et al. 1998). Recently, PAH exposure has been associated with non-cancer health effects such as immune system deficiency, cardiovascular disease, low birth weight, neural tube defects and learning deficits in children (Burstyn et al. 2005; Choi et al. 2006; Hertz-Picciotto et al. 2008; Perera et al. 2009; Lee et al. 2011; Ren et al. 2011). Both pyrogenic and petrogenic PAHs are ubiquitous in the natural world, but anthropogenic activities such as automobile combustion, fossil fuel burning, oil refining and coal tar seal coating have contributed to increasing concentrations in local environments (Mahler et al. 2005; Polidori et al. 2010; Van Metre and Mahler 2010). Humans and wildlife are inveterately exposed to this family of compounds; however, certain environmental conditions, occupational settings, residential combustion practices and dietary habits are associated with increased disease risk that compelled the inclusion of 16 PAHs in the EPA list of priority pollutants (EPA 2012).

PAH-containing mixtures such as soot, coal tar mixtures and tobacco smoke have long been known carcinogens in humans (Bostrom et al. 2002). Animal studies have provided data on the ability of individual PAH structures to induce tumors, as well as shed light on the mechanisms by which PAHs induce toxicity. Carcinogenic mechanisms of Benzo(a)pyrene (BaP), a commonly detected PAH classified as a probable human carcinogen, have been well-studied in a variety of animal and cell models. In order to estimate cancer risk for environmental mixtures, which contain multiple PAH structures, the US EPA employed a Toxic Equivalency Factor (TEF) approach with available data to rank seven PAHs for

potency as carcinogens in comparison to BaP (Schoeny 1993). Since then, potency equivalency factors and risk assessments have been published for a wider range of PAHs, and the EPA has provided a draft document on the development of a relative potency factor (RPF) approach for PAH mixtures (Collins et al. 1998; Bostrom et al. 2002; EPA 2010). Assessment of health risk from PAH exposure is driven primarily by carcinogenicity data for parent PAHs, as data is limited and uncertainty surrounds the toxicity endpoints of non-carcinogenic PAHs (Jennings 2012)

Determining the contribution of individual PAHs to the increased risk of morbidity such as heart attack, asthma and low birth weight remains a daunting challenge, as the mechanisms by which PAHs may cause these effects are not defined. Though PAHs are not new to the human exposure paradigm, the recent epidemiological associations of PAH exposure with multiple diseases, as well as increased ability to detect a broad spectrum of substituted PAHs in environmental samples, is cause to consider them emerging contaminants of concern to human health. The goal of this work is to investigate biological activity and group PAHs based on proposed molecular mechanisms, providing an important step towards understanding the mechanisms by which this diverse family of chemicals affects the health of humans and wildlife.

Human exposure to PAHs

Exposure to PAHs in the general population occurs primarily via inhalation of aerosols or fine particulate matter from smoke (combustion emissions as well as cigarette), and through ingestion of smoked or grilled meats, fish and charred foods. Dermal exposures can also occur from exposure to petroleum products, and accidental ingestion of PAHs through house dust is a concern for small children (Ramesh et al. 2004). Cigarette smoke is a primary source of PAHs for individuals who smoke or are exposed regularly to second-hand smoke. Several studies have shown that for non-smoking individuals, the largest contributor to PAH exposures is the diet (Menzie et al. 1992). This is true particularly for the higher molecular weight (and more carcinogenic) PAHs, which are less volatile and associate with particulate matter. Deposition of PAH particles on crops also contributes PAHs to the diet and may be of concern for agricultural sites located near major industrial areas or roadways.

In urban environments, airborne carcinogenic PAHs are predicted to increase lung cancer risk. The low molecular weight PAHs partition into the volatile fraction of air, while higher molecular weight PAHs associated with small particulates can travel deep into the lungs (Ramirez et al. 2011). PAHs are lipophilic so upon exposure, they are readily absorbed by organisms. However, they can also be metabolized, which complicates measurement of exposure, as well as their toxic effects. PAH metabolites are detected in urine, where 1-hydroxypyrene (1-OHP) is a commonly measured biomarker of PAH exposure (Hansen et al. 2008). High 1-OHP levels are detected in coke oven and aluminum smelter workers, as well as residents living near industries with high PAH emissions, such as coal fired power plants (Hu et al. 2011). Increased levels of PAH metabolites are observed in children in polluted areas. For instance, 6-7 year old children who attended an elementary school near a heavily trafficked road in Guangzhou, China had higher levels of PAH metabolites in their urine than children who attended a school farther from large roadways (Fan et al. 2012).

Reactive PAH metabolites form adducts with DNA, RNA and protein. Adduct formation is part of the toxic mechanism of many PAHs, leading to DNA damage and mutations, but can also be used as a measure of exposure (Baird et al. 2005). Human PAH exposures can be monitored by detection of PAH-DNA adducts in white blood cells and other tissues. These biomarkers of PAH exposure have been associated with various cancers and other health endpoints such as reduced fetal growth (Kriek et al. 1998; Tang et al. 2006). Many studies estimate PAH exposure by monitoring PAHs in air, either from point sources or personal air monitors. While these studies do not determine internal PAH dose, they provide exposure information for a more complete spectrum of PAHs. Background ranges of PAHs are reported at 0.02-1.2 ng/m^3 in rural areas and 0.15-19.3 ng/m^3 in urban air; average total exposure in the U.S. has been estimated at 3 mg/day (Mumtaz and George 1995). Much higher PAH concentrations occur in major cities, occupational settings such as petroleum and dye industries, and in homes where low-efficiency fuels are used indoors. A number of studies associate increased cancer risk with these exposures. PAHs associated with small particulate matter measured at a school in Delhi, India were predicted to cause an Incremental Lifetime Cancer Risk of 3.18×10^{-6}, which is higher than the acceptable risk level of 10^{-6} (Jyethi et al. 2013). A study of air control measures implemented during the Beijing Olympics found that controlling emissions could substantially decrease risk of

excess cancer cases (Jia et al. 2011). Few studies have directly predicted effects of reduced emissions on other health endpoints. Research suggests, however, that reducing PAH exposure may have many other positive implications, such as reduced inflammatory and vascular disease.

Emerging concerns: cardiac function and effects during development

Multiple studies have shown increased risk of cardiac dysfunction with exposure to fine (pm 2.5) particulate matter, which contains PAHs along with many other contaminants. A smaller set of studies have specifically investigated relationships between PAHs and cardiac function. Occupational exposure to PAHs in asphalt workers is associated with an increased risk of fatal ischemic heart disease (Burstyn et al. 2005). Higher 1-OHP levels were associated with decreased heart rate variability in boilermakers and coke oven workers, suggesting an acute effect of PAH exposure on cardiac autonomic function (Lee et al. 2011; Li et al. 2012). In myocardial infarction survivors, an association was observed between exposure to particulate matter and symptoms of cardiovascular disease (Kraus et al. 2011). Analysis of National Health and Nutrition Examination Survey data also identified a higher prevalence of peripheral arterial disease in subjects with greater than average fluorene and phenanthrene metabolites (Xu et al. 2013). These smaller PAHs are generally present in the gas phase of emissions, and can be more prevalent than the larger PAHs (Bostrom et al. 2002). Reducing exposure to particulate air pollution has been shown to improve cardiovascular health of patients with heart disease (Langrish et al. 2011).

While it is difficult to discern effects of PAHs from other co-occurring contaminants in combustion-related exposures, epidemiological studies collectively suggest that PAHs affect cardiac function and increase risk of cardiac-related injury. A number of studies in animals have supported these associations. BaP exposure increased atherosclerosis and disrupted gene expression in the aortas of mice, as well as altered blood pressure patterns in rats (Jules et al. 2012; Kerley-Hamilton et al. 2012). In utero exposure to BaP also caused cardiac dysfunction later in life in rats (Jules et al. 2012). PAH exposure similarly affects cardiac function in fish, and developmental exposure to PAHs causes cardiac defects in developing zebrafish (Incardona et al. 2004). While epidemiological studies suggest that smaller PAHs (2-3 rings) are associated with cardiac toxicity, few studies in mammalian models have investigated the effects of these individual compounds on heart development. Studies in

zebrafish, however, have identified structure-related differences in the mechanisms by which these compounds affect the heart (Incardona et al. 2011).

The effects of PAH exposure on vascular function and inflammation may have increased impact during embryonic development. Adverse pregnancy outcomes, such as low birth weight, are associated with living near major roadways and in other areas with high vehicle emissions (Wilhelm et al. 2012). Oxidative stress is believed to play a role in this toxicity, and was supported by a study that showed dietary vitamin C reduced risk of reduced fetal growth associated with BaP exposure (Duarte-Salles et al. 2012) PAH exposure in rodents decreases vascularization in the placenta, and studies in fish embryos demonstrate PAHs disrupt molecular pathways important for proper heart formation (Rennie et al. 2011). While neural tube defects, asthma, and learning deficits are also associated with PAH exposure in epidemiological studies, the mechanisms by which PAHs may interfere with developmental processes, and the individual PAHs responsible, remain to be elucidated (Perera et al. 2009; Ren et al. 2011).

Mechanisms of PAH toxicity: activation of the aryl hydrocarbon receptor

PAHs exhibit varied non-genotoxic activity, which can not only contribute to their carcinogenicity but also mediate multiple other toxic effects. Some PAHs, including BaP, can cause toxicity by binding the aryl hydrocarbon receptor (AHR), a ligand-activated member of the basic helix-loop-helix Per-ARNT-Sim (bHLH-PAS) family of transcription factors. Ligand binding induces dimerization with the Ah receptor nuclear translocator (ARNT), translocation to the nucleus, and alteration of gene transcription, including cytochrome P450 phase 1 (*CYP1A, CYP1B1*) and phase 2 (*UGT1A6, ALDH3A1*) metabolizing enzymes (Figure 1)(Nebert et al. 2000; Sartor et al. 2009). While parent PAHs are generally unreactive, metabolism by CYP1A and other metabolizing enzymes forms more reactive metabolites such as PAH epoxides and radical cation intermediates (Cavalieri and Rogan 1995). These reactive compounds can cause cellular damage by forming DNA and protein adducts. Additionally, they can activate redox-responsive genes containing antioxidant response elements (AREs), including phase II metabolizing enzymes (Bock 2012). Further metabolism by hydroxylases forms hydroxy-PAHs, and glucoronidases and sulfotransferases conjugate these oxygenated PAHs, facilitating their excretion. Because of this complex process involving multiple intermediates, metabolism induced by AHR

activation can increase toxicity or allow for excretion, depending on the PAH structure. In the case of BaP, carcinogenicity in mice is dependent on a functional AHR (Shimizu et al. 2000). The AHR pathway and cytochrome P450 enzymes are conserved among vertebrates. Differences exist, however, in affinity of compounds for AHRs between species, as well as within populations, which affects their carcinogenicity and potency as toxicants (Hahn 2002; Wirgin et al. 2011). The work presented in this thesis focuses on non-carcinogenic mechanisms. However, the large body of research on mechanisms by which BaP interacts with the AHR to cause cancer has contributed greatly to our understanding of AHR function. While the molecular signaling pathways by which BaP induces its diverse array of toxicological effects are not fully elucidated, BaP toxicity is known to be mediated by AHR activation in many species. Beyond this, the ability of multiple CYP enzymes, which vary between tissue types, individuals and species, to metabolize PAHs creates a complex array of toxicological profiles.

In addition to toxicological effects caused via AHR induction and the formation of reactive PAH intermediates, sustained activation of the AHR leads to a number of other adverse effects. These have been well-characterized in studies with halogenated hydrocarbons, such as 2,3,7,8-tetrachlorodibenzo–*p*-dioxin (TCDD) and polychlorinated biphenyls (PCBs), which have high affinity for the AHR but are not readily metabolized. AHR activation during development causes teratogenic effects in rodents and fish, which include malformations in the heart and jaw. AHR activation additionally causes a wide range of neurologic, immune and reproductive effects (reviewed in (White and Birnbaum 2009)). A TEF approach has been employed by the World Health Organization (WHO) to rank the potency of halogenated hydrocarbons for assessment of health risk to humans and wildlife (Van den Berg et al. 2006). In contrast to the TEFs for PAHs, which are based on carcinogenic potency, TEFs for dioxin-like compounds are determined from a variety of AHR-mediated endpoints, including chronic toxicity, enzyme induction, tumor promotion and lethality, and generally correlate with ligand affinity for the receptor.

Because AHR activation by dioxin-like compounds leads to transcriptional activation of CYP1A, the enzyme has been widely used as a biomarker of AHR activation. This tool has been particularly useful for investigating exposure to dioxin-like compounds in wild

populations (Hahn 2002; Sarkar et al. 2006; Jensen et al. 2010). As interactions of the AHR such as ligand and DNA binding have been characterized, many other assays have also been developed to identify AHR-activating compounds. Several assays screen for AHR activators using dioxin responsive elements (DREs) in promoters driving reporter genes such as luciferase or GFP. The chemical activated luciferase gene expression (CALUX) assay is widely used to screen compounds for AHR activation activity (Murk et al. 1996). Several *in silico* models of the AHR ligand binding pocket have also been created to predict binding and screen for alternative AHR ligands. For example, a chemical library screen and an *in silico* AHR molecular docking study identified leflunomide, a rheumatoid arthritis drug, as an AHR agonist, which was confirmed in human, mouse and zebrafish (O'Donnell et al. 2010). PAHs that have high affinity for the AHR induce AHR-mediated toxicological effects similar to those caused by exposure to dioxin-like compounds. Because of their structural diversity and aforementioned metabolism, however, PAHs have a wide range of additional biological activities that complicate the interpretation of CYP1A activity as a biomarker for exposure and toxic effects. Some PAHs, such as fluoranthene, inhibit CYP1A, and can lead to synergistic toxicity in fish embryos when combined with other PAHs (Billiard et al. 2006; Timme-Laragy et al. 2007). Other conditions, such as hypoxia, can also inhibit CYP1A induction (Fleming and Di Giulio 2011). Finally, CYP1A induction can be elevated by PAHs (such as chrysene) in the absence of other signs of toxicity (Incardona et al. 2006). Because of these complex interactions, AHR affinity on its own is not a sufficient predictor of PAH toxicity.

Effects of PAH exposure on fish development

PAH exposure causes developmental abnormalities in fish embryos, including pericardial and yolk sac edema, disrupted cardiac function, craniofacial and spinal malformations, anemia and reduced growth, which have been described in many studies addressing toxicity of PAH mixtures to wild fish populations (Barron et al. 2004). Many of these effects are similar to those described for planar halogenated compounds (PCBs). Fish are exposed to high levels of PAHs from events such as oil spills, from runoff, and from sites contaminated by industrial activity. Research on fish populations exposed to PAHs has contributed to our understanding of the AHR, its crosstalk with other signaling pathways and the complex mechanisms of PAH toxicity. While dioxin-like compounds are toxic to marine life, there are

several examples of fish populations adapted to live in heavily contaminated sites (Nacci et al. 2009; Bugel et al. 2010). These phenotypes provide fascinating information about interactions between pathways that facilitate adaptation to the external environment; many of the adaptive mechanisms have yet to be fully determined. A study of Atlantic tomcod in the Hudson River found that populations resistant to contaminants had a deletion in AHR2 that rendered it non-functional (Wirgin et al. 2011). Atlantic killifish from a heavily contaminated wood treatment facility site on the Elizabeth River, VA are exposed to high concentrations of PAHs and exhibit higher levels of DNA damage compared to fish from a reference site with low PAH levels (Wills et al. 2010; Jung et al. 2011). Their embryos are resistant to developmental defects induced by PAHs and PCBs, and their adaptive phenotype highlights the complexity of AHR regulation. PCB 126, a dioxin-like compound that is not readily metabolized, does not induce Cyp1a expression in these embryos, while PAHs BaP and benzo(k)fluoranthene (BkF) induce Cyp1a (though at lower levels than reference site embryos). This suggests that ligands can interact differently with the AHR and/or other transcription factors to induce Cyp1a expression both in the presence and absence of overt toxicity.

Research on the effects of crude and weathered oil in various fish species has also contributed to our understanding of biological effects of PAHs. In adult fish, narcosis has been described as a toxicological endpoint of exposure to low molecular weight PAHs, and is characterized by loss of balance, lethargy and decreased respiration which is reversible but can result in death during prolonged exposure to high concentrations (Vanwezel and Opperhuizen 1995). In fish embryos, however, three ring PAHs disrupt heart function, causing arrhythmia and eventual heart failure (Incardona et al. 2004). Studies from a number of labs have demonstrated that PAHs induce malformations in embryos via different mechanisms, depending on their structures. 3-ring PAHs induce cardiac toxicity with a suggested mechanism of ion channel disruption (Incardona et al. 2004), Many 4 and 5-ring PAHs bind the aryl hydrocarbon receptor, causing cardiac toxicity along with other effects (Incardona et al. 2006). Because of the different mechanisms, predicting toxicity of PAH mixtures remains a challenge. A need for studies that clarify the biological effects of low molecular weight PAHs has been recognized in order to better understand the effect of PAH contamination in aquatic ecosystems (Hylland 2006). Characterizing the different

mechanisms of PAH developmental toxicity is an important first step in predicting toxicity of mixtures, both in aquatic systems and human populations.

Discovering endogenous functions of the AHR

As previously discussed, the AHR pathway has been well-studied and plays a central role in the toxicological mechanisms of many PAHs. Despite over 30 years since its discovery as the receptor responsible for mediating TCDD toxicity, characterization of endogenous functions of the receptor remains a rapidly developing area of research. PAS (Per-ARNT-Sim) family transcription factors, of which the AHR is a member, are involved in a variety of physiological processes including circadian rhythms and oxygen sensing (McIntosh et al. 2010). The AHR dimerization partner required for transcriptional activation, ARNT (also known as Hif1b), is a member of the Hypoxia Inducible Factor (HIF) family of proteins. AHR binding partners such hsp90 and p300 also interact with other transcription factors (Beischlag et al. 2008). Many studies have demonstrated AHR crosstalk with other transcriptional regulators such as NFkB, ER and GCR via direct or indirect mechanisms (Puga et al. 2009). A handful of studies have also shown AHR dimerization with other proteins, such as klf6, and activation of other downstream genes via an alternative recognition sequence (Wilson et al. 2013). This propensity to interact with other transcription factors supports the notion that AHR-mediated developmental toxicity may be, at least in part, caused by disruption of endogenous functions mediated in concert with other interacting proteins. This is supported by studies that have demonstrated that *Cyp1a* and other metabolic genes highly induced by AHR are not responsible for the toxic effects of TCDD (Antkiewicz et al. 2006). The means by which AHR activation leads to downregulation of genes, in particular, is not well-defined. A study of transcriptional binding of the AHR with and without exogenous ligand identified a large number of genes involved in developmental and vascular processes that were bound by the AHR in the absence of TCDD and BaP. Upon ligand binding, the targets shifted to genes involved in xenobiotic metabolism (Sartor et al. 2009). Along the same lines, recent studies have identified roles for the AHR in a myriad of processes, including progenitor cell expansion and differentiation (Smith et al. 2013). It would appear that many functions of the AHR in normal development have yet to be discovered.

AHR knockout mouse strains developed by three different groups illustrate the importance of the AHR in normal liver development and immune function, and continue to expand understanding of the receptor's role in both toxicological responses and normal physiology (Fernandez-Salguero et al. 1995; Schmidt et al. 1996; Lahvis et al. 2005). Because their development can be observed non-invasively, fish have provided much insight into mechanisms of AHR-mediated toxicity during development. Three AHR isoforms have been identified in zebrafish: AHR1A, AHR1B, and AHR2 (Tanguay et al. 1999; Andreasen et al. 2002; Hahn 2002; Karchner et al. 2005). Numerous studies with known AHR ligands, however, have identified AHR2 as the primary mediator of early life stage toxicological effects in zebrafish (Prasch et al. 2003; Teraoka et al. 2003; Antkiewicz et al. 2006). Other genes, including *foxq1a* and *sox9b* have been uncovered as mediators of TCDD-induced effects on jaw and heart development (Xiong et al. 2008; Planchart and Mattingly 2010; Hofsteen et al. 2013). The ability to transiently knock down genes in zebrafish has enabled study of the roles of these genes during development. Knockdown of AHR2 has also highlighted crosstalk between the AHR pathway and NRF2, which provides protection against oxidative stress induced by PAHs (Timme-Laragy et al. 2009). While AHR2 knockdown is able to rescue TCDD-induced toxicity, it does not completely prevent Ahr2 activity, and is a transient effect. An AHR2 knockout zebrafish would therefore greatly expand capability to investigate biological functions of the receptor during development and throughout the lifespan.

The zebrafish model

The zebrafish is an excellent system in which to pursue mechanisms of toxicity during development, which is rapid. By 5 days post-fertilization all organ systems are functional (Figure 2)(Kimmel et al. 1995; Saili 2012). Development external to the mother allows for non-invasive observation and imaging over the course of development. Additionally, environmental factors such as chemical exposure can be meticulously controlled. A fully sequenced genome with ever-increasing annotation allows for investigation of genetic targets of interest. Genes can be specifically targeted with antisense oligos (morpholinos) to transiently knock down expression during development. Many transgenic zebrafish lines are available for studies. The small size of zebrafish makes them adaptable to development in 96 well plates and amenable for large scale genetic screens. Forward and reverse genetic

screens have been employed to identify thousands of mutants with specific mutations and phenotypes. More recently, zinc finger and TALEN technologies have enabled the creation of targeted knockouts in zebrafish. Chemical screens can be conducted in zebrafish with amounts of chemical comparable to cell-culture studies, allowing for rapid screening of large numbers of chemicals *in vivo*. Recent development of high-throughput technology has enabled much-needed investigation of compounds that are detected in environmental samples but lack toxicity data.

Substituted PAHs: adding complexity to toxicity evaluation

PAHs in the environment exist not only as parent PAH structures, but as substituted derivatives such as oxygenated PAHs (OPAHs). Because substitutions occur via both biotic and abiotic processes, it is expected that concentrations of substituted PAHs may be higher than parent PAHs under certain conditions. As advances in detection methods allow for quantification of a wider range of compounds that constitute exposure paradigms, there is an accompanying need for toxicity data in order to assess health risks. The large number of substituted PAH structures poses a significant challenge; identifying groups of structures that behave similarly would help the prioritization of studies to determine toxicological mechanisms of these compounds, and eventually improve predictive capability for modeling toxicity of PAHs and mixtures. Our laboratory conducted a toxicity screen for developmental toxicity and Ahr activation of 38 oxygenated PAHs (OPAHs) in zebrafish embryos (Knecht et al. 2013). Embryos were statically exposed from 6 to 120 hours post fertilization (hpf) to 0.8 – 500 uM concentrations of OPAH in embryo media, then screened for malformations. For each compound, embryos exposed to a concentration that caused ~80% malformations but not mortality were examined for Cyp1a expression with immunohistochemistry. We found that OPAHs induced toxicity at a wide range of concentrations. Some observations could be made based on structure; several quinones (1,4-naphthoquione, phenanthrene-quinone, 1,2-napthoquinone) had steep dose-response curves and caused mortality at concentrations < 4 μM. Cyp1a expression was not observed at these concentrations (Knecht et al. 2013). A substantial group of compounds caused malformations such as pericardial edema and yolk sac edema at concentrations < 20 μM. Some of these, such as benzo(a)fluorenone, phenanthrene-1,4-dione, and benz(a)anthracene-7,12-dione, induced Cyp1a expression in the vasculature. Many,

including 1,9-benz-10-anthrone and 9-hydroxybenzo(a)pyrene, induced malformations but no Cyp1a expression. A large number of PAHs did not induce malformations below 20 μM. Of these, some induced Cyp1a expression that was specifically expressed in the liver at higher concentrations. We also observed interesting patterns of Cyp1a expression in the lateral line of zebrafish exposed to oxygenated napthalenes. The differential Cyp1a expression patterns observed with OPAHs that were similarly toxic suggested differential involvement of the aryl hydrocarbon receptor.

Categorizing compounds, predicting PAH toxicity

In the studies presented here, I used whole genome microarrays to identify transcriptional profiles of developmental toxicity induced by three parent PAHs and two OPAHs in zebrafish. Differential proposed mechanisms of dibenzothiophene (DBT), pyrene (PYR) and benz(a)anthracene (BAA) – induced developmental toxicity in zebrafish embryos are discussed in Chapter 2. I examined PAH body burdens following exposures and found large differences in the amount of PAH in embryos. Measuring uptake was important for discerning dose-dependent differences from biological mechanisms. Expanding the investigation of different PAH structures, I chose two 4-ring OPAHs, benz(a)anthracene-7,12-dione (7,12-B[a]AQ) and 1,9-benz-10-anthrone (BEZO) from the OPAH toxicity screen conducted in our laboratory to further investigate transcriptional profiles. 7,12-B[a]AQ is an oxygenated derivative of parent PAH BAA, while BEZO is a mono-oxygenated PAH with a different ring arrangement. In Chapter 4, I investigated the role of AHR2 in mediating the toxicity of these two compounds.

A handful of other studies have compared transcriptional profiles across several PAH structures in other model systems, with a similar goal of identifying biomarkers of exposure and increasing toxicity prediction capability for this class of compounds. A study of gene expression changes induced by 3-4 ring PAHs in leukemia (THP-1) cells identified groups of PAHs that induced similar gene expression profiles. Predictor genes were identified, which included calcium binding proteins, transcription factors, immune response and genes with oxidoreductase activity (Wan et al. 2008) In a similar study in liver hepatoma cells (HepG2) gene expression profiles were determined from cells exposed to 2-5 ring PAHs (Song et al. 2012). Transcriptional signatures were compared between known carcinogens and non-carcinogens. Interestingly, clustering did not predict known carcinogenicity; however,

known carcinogens induced genes involved in oxidative stress, while non-carcinogenic PAHs such as fluoranthene did not. An interesting study of human lymphocytes identified a small number of genes that were significantly differentially expressed in PAH-exposed coke oven workers (Wu et al. 2011). These genes were involved in metal ion binding and transport, and included myosin XVB and solute carrier family 25 member 34. These studies have begun to identify biomarkers and propose mechanisms for diverse PAH structures, including the non-genotoxic PAHs. However, the majority of these studies have been carried out in cell lines, and none have explored PAH toxicity in developing embryos. Our *in vivo* approach provides a unique set of data that can be used to group PAHs based on a large set of genes that are important during early development. The availability of an annotated genome and high homology of genes between vertebrates allows us to compare biomarkers and eventually validate across species. Global transcriptional analysis of PAH exposure in multiple systems will create a powerful dataset from which to identify biological mechanisms associated with structural differences. The overarching objective of the studies presented here is to employ transcriptional profiling techniques to identify potential differential mechanisms by which PAHs induce developmental toxicity, and to further characterize the role of the aryl hydrocarbon receptor in biological pathways that are disrupted by PAH exposure.

Summary and study objectives

We hypothesized that PAHs induce developmental effects in embryos via distinct mechanisms that could be identified by comparative investigation of global changes in transcription that occur following chemical exposure. We compared the transcriptional profiles of 5 PAH structures that induce different malformation profiles in developing zebrafish (Figure 3). In Chapter 2, we used a whole genome mRNA microarray to compare parent PAHs which differentially activate the aryl hydrocarbon receptor, DBT, PYR and BAA. Using concentrations that induced malformations but not mortality, we identified genes that were differentially regulated over time and in response to the three PAH structures. PAH body burdens were analyzed at both time points, which was important for discerning dose-related differences from those that represented unique molecular mechanisms. By analyzing functional roles of misregulated genes and their predicted regulatory transcription factors, we showed that the BAA response (AHR activated) could be

distinguished from regulatory networks disrupted by DBT and PYR exposure (AHR not activated).

In chapter 3, we developed a zebrafish line with a mutation in *ahr2* to enable deeper mechanistic investigation of the role of the AHR in PAH-induced toxicity. We characterized AHR activity in the mutant line using TCDD and leflunomide as toxicological probes to investigate function, ligand binding and Cyp1a induction patterns of paralogues AHR2, AHR1A and AHR1B. In these studies, I determined that *ahr2*^{hu3335} zebrafish are functionally null. We then further explored function of the other zebrafish AHR paralogues, and demonstrated differential ligand binding and Cyp1a expression patterns mediated by the three receptors.

Because the *ahr2*^{hu3335} line was developed from a founder identified in a screen of a mutant library generated by random mutagenesis (TILLING, Targeting Induced Local Lesions In Genomes), the line required multiple generations of outcrosses to reduce background mutations. Embryo production and quality was variable over the first three outcrosses, and limited our ability to conduct reliable studies of PAH-induced toxicity in the *ahr2*^{hu3335} line. We investigated BAA, DBT and PYR-induced toxicity in *ahr2*^{hu3335} and *ahr2*$^{+}$ zebrafish, and confirmed the differential involvement of the AHR in mediating toxicological effects (Appendix 1). Background malformations in these experiments were higher than normal, however, so we utilized an AHR2 morpholino to knock down expression in the remaining studies in this dissertation.

While BAA induced toxicity dependent on the AHR, DBT and PYR caused toxicity via other mechanisms. We conducted preliminary characterization of other phenotypes associated with these PAHs, with the goal of identifying more sensitive endpoints to assess for toxic effects. We observed a unique hyperactive phenotype in PYR-exposed embryos, and investigated this response over a wider concentration range. We also investigated whether localized inflammation could be visualized in the transgenic mpx:gfp zebrafish line, which expresses green fluorescent protein (driven by myeloid-specific peroxidase promoter) in neutrophils. Preliminary data from these studies are presented in Appendix 2.

In Chapter 4, We explored the role of the AHR in mediating toxicity induced by 4-ring OPAHs BEZO and 7,12-B[a]AQ. We showed that despite very different Cyp1a expression profiles, both BEZO and 7,12-B[a]AQ induced toxicity via AHR2. We used RNA-seq to compare transcriptional profiles induced by the OPAHs at 48 hpf, and identified potential novel targets of the AHR as well as intriguing mechanistic differences by which the AHR may interact with other transcription factors to differentially regulated target genes. We additionally compared sets of transcripts across both platforms (microarray and RNA-seq), and identified patterns of expression across all 5 PAH structures.

Together these studies show that PAHs act through multiple mechanisms that differentially involve the AHR to induce developmental toxicity. We identified clusters of transcripts involved in mechanisms, which can be further pursued to unravel molecular targets of PAHs, as well as be utilized as biomarkers to begin to predict effects of additional PAH structures. These studies demonstrate the power of transcriptomics approaches for comparing toxicity pathways of structurally-related compounds, identifying biomarkers of toxic effects, and generating hypotheses to further mechanistic understanding of a large family of compounds

References

Andreasen, E. A., M. E. Hahn, et al. (2002). "The zebrafish (Danio rerio) aryl hydrocarbon receptor type 1 is a novel vertebrate receptor." Mol Pharmacol **62**(2): 234-249.

Antkiewicz, D. S., R. E. Peterson, et al. (2006). "Blocking expression of AHR2 and ARNT1 in zebrafish larvae protects against cardiac toxicity of 2,3,7,8-tetrachlorodibenzo-p-dioxin." Toxicol Sci **94**(1): 175-182.

Baird, W. M., L. A. Hooven, et al. (2005). "Carcinogenic polycyclic aromatic hydrocarbon-DNA adducts and mechanism of action." Environ Mol Mutagen **45**(2-3): 106-114.

Barron, M. G., M. G. Carls, et al. (2004). "Evaluation of fish early life-stage toxicity models of chronic embryonic exposures to complex polycyclic aromatic hydrocarbon mixtures." Toxicol Sci **78**(1): 60-67.

Beischlag, T. V., J. Luis Morales, et al. (2008). "The aryl hydrocarbon receptor complex and the control of gene expression." Crit Rev Eukaryot Gene Expr **18**(3): 207-250.

Billiard, S. M., A. R. Timme-Laragy, et al. (2006). "The role of the aryl hydrocarbon receptor pathway in mediating synergistic developmental toxicity of polycyclic aromatic hydrocarbons to zebrafish." Toxicol Sci **92**(2): 526-536.

Bock, K. W. (2012). "Ah receptor- and Nrf2-gene battery members: Modulators of quinone-mediated oxidative and endoplasmic reticulum stress." Biochem Pharmacol **83**(7): 833-838.

Bostrom, C. E., P. Gerde, et al. (2002). "Cancer risk assessment, indicators, and guidelines for polycyclic aromatic hydrocarbons in the ambient air." Environ Health Perspect **110 Suppl 3**: 451-488.

Bugel, S. M., L. A. White, et al. (2010). "Impaired reproductive health of killifish (Fundulus heteroclitus) inhabiting Newark Bay, NJ, a chronically contaminated estuary." Aquat Toxicol **96**(3): 182-193.

Burstyn, I., H. Kromhout, et al. (2005). "Polycyclic aromatic hydrocarbons and fatal ischemic heart disease." Epidemiology **16**(6): 744-750.

Cavalieri, E. L. and E. G. Rogan (1995). "Central role of radical cations in metabolic activation of polycyclic aromatic hydrocarbons." Xenobiotica **25**(7): 677-688.

Choi, H., W. Jedrychowski, et al. (2006). "International studies of prenatal exposure to polycyclic aromatic hydrocarbons and fetal growth." Environ Health Perspect **114**(11): 1744-1750.

Ciganek, M., J. Neca, et al. (2004). "A combined chemical and bioassay analysis of traffic-emitted polycyclic aromatic hydrocarbons." Sci Total Environ **334-335**: 141-148.

Collins, J. F., J. P. Brown, et al. (1998). "Potency equivalency factors for some polycyclic aromatic hydrocarbons and polycyclic aromatic hydrocarbon derivatives." Regul Toxicol Pharmacol **28**(1): 45-54.

Duarte-Salles, T., M. A. Mendez, et al. (2012). "Dietary benzo(a)pyrene and fetal growth: effect modification by vitamin C intake and glutathione S-transferase P1 polymorphism." Environ Int **45**: 1-8.

EPA, U. (2010). Development of a relative potency factor (RPF) approach for polycyclic aromatic hydrocarbon (PAH) mixtures (External review draft). U. S. E. P. Agency. Washington, DC.

EPA, U. (2012). "Integrated Risk Information System." from http://www.epa.gov/iris/.

Fan, R., D. Wang, et al. (2012). "Preliminary study of children's exposure to PAHs and its association with 8-hydroxy-2'-deoxyguanosine in Guangzhou, China." Environ Int **42**: 53-58.

Fernandez-Salguero, P., T. Pineau, et al. (1995). "Immune system impairment and hepatic fibrosis in mice lacking the dioxin-binding Ah receptor." Science **268**(5211): 722-726.

Fleming, C. R. and R. T. Di Giulio (2011). "The role of CYP1A inhibition in the embryotoxic interactions between hypoxia and polycyclic aromatic hydrocarbons (PAHs) and PAH mixtures in zebrafish (Danio rerio)." Ecotoxicology **20**(6): 1300-1314.

Hahn, M. E. (2002). "Aryl hydrocarbon receptors: diversity and evolution." Chem Biol Interact **141**(1-2): 131-160.

Hahn, M. E. (2002). "Biomarkers and bioassays for detecting dioxin-like compounds in the marine environment." Sci Total Environ **289**(1-3): 49-69.

Hansen, A. M., L. Mathiesen, et al. (2008). "Urinary 1-hydroxypyrene (1-HP) in environmental and occupational studies--a review." Int J Hyg Environ Health **211**(5-6): 471-503.

Hertz-Picciotto, I., H. Y. Park, et al. (2008). "Prenatal exposures to persistent and non-persistent organic compounds and effects on immune system development." Basic Clin Pharmacol Toxicol **102**(2): 146-154.

Hofsteen, P., J. Plavicki, et al. (2013). "Sox9b is Required for Epicardium Formation and Plays a Role in TCDD-induced Heart Malformation in Zebrafish." Mol Pharmacol.

Hu, S. W., Y. J. Chan, et al. (2011). "Urinary levels of 1-hydroxypyrene in children residing near a coal-fired power plant." Environ Res **111**(8): 1185-1191.
Hylland, K. (2006). "Polycyclic aromatic hydrocarbon (PAH) ecotoxicology in marine ecosystems." J Toxicol Environ Health A **69**(1-2): 109-123.
Incardona, J. P., T. K. Collier, et al. (2004). "Defects in cardiac function precede morphological abnormalities in fish embryos exposed to polycyclic aromatic hydrocarbons." Toxicol Appl Pharmacol **196**(2): 191-205.
Incardona, J. P., H. L. Day, et al. (2006). "Developmental toxicity of 4-ring polycyclic aromatic hydrocarbons in zebrafish is differentially dependent on AH receptor isoforms and hepatic cytochrome P4501A metabolism." Toxicol Appl Pharmacol **217**(3): 308-321.
Incardona, J. P., T. L. Linbo, et al. (2011). "Cardiac toxicity of 5-ring polycyclic aromatic hydrocarbons is differentially dependent on the aryl hydrocarbon receptor 2 isoform during zebrafish development." Toxicol Appl Pharmacol **257**(2): 242-249.
Jennings, A. A. (2012). "Worldwide regulatory guidance values for surface soil exposure to noncarcinogenic polycyclic aromatic hydrocarbons." J Environ Manage **101**: 173-190.
Jensen, B. A., C. M. Reddy, et al. (2010). "Developing tools for risk assessment in protected species: Relative potencies inferred from competitive binding of halogenated aromatic hydrocarbons to aryl hydrocarbon receptors from beluga (Delphinapterus leucas) and mouse." Aquat Toxicol **100**(3): 238-245.
Jia, Y., D. Stone, et al. (2011). "Estimated reduction in cancer risk due to PAH exposures if source control measures during the 2008 Beijing Olympics were sustained." Environ Health Perspect **119**(6): 815-820.
Jules, G. E., S. Pratap, et al. (2012). "In utero exposure to benzo(a)pyrene predisposes offspring to cardiovascular dysfunction in later-life." Toxicology.
Jung, D., C. W. Matson, et al. (2011). "Genotoxicity in Atlantic killifish (Fundulus heteroclitus) from a PAH-contaminated Superfund site on the Elizabeth River, Virginia." Ecotoxicology **20**(8): 1890-1899.
Jyethi, D. S., P. S. Khillare, et al. (2013). "Risk assessment of inhalation exposure to polycyclic aromatic hydrocarbons in school children." Environ Sci Pollut Res Int.
Karchner, S. I., D. G. Franks, et al. (2005). "AHR1B, a new functional aryl hydrocarbon receptor in zebrafish: tandem arrangement of ahr1b and ahr2 genes." Biochem J **392**(Pt 1): 153-161.
Kerley-Hamilton, J. S., H. W. Trask, et al. (2012). "Inherent and Benzo[a]pyrene-Induced Differential Aryl Hydrocarbon Receptor Signaling Greatly Affects Life Span, Atherosclerosis, Cardiac Gene Expression, and Body and Heart Growth in Mice." Toxicol Sci **126**(2): 391-404.
Kimmel, C. B., W. W. Ballard, et al. (1995). "Stages of embryonic development of the zebrafish." Dev Dyn **203**(3): 253-310.
Knecht, A. L., B. C. Goodale, et al. (2013). "Comparative developmental toxicity of environmentally relevant oxygenated PAHs." Toxicol Appl Pharmacol.
Kraus, U., S. Breitner, et al. (2011). "Particle-associated organic compounds and symptoms in myocardial infarction survivors." Inhalation Toxicology **23**(7): 431-447.
Kriek, E., M. Rojas, et al. (1998). "Polycyclic aromatic hydrocarbon-DNA adducts in humans: relevance as biomarkers for exposure and cancer risk." Mutat Res **400**(1-2): 215-231.

Lahvis, G. P., R. W. Pyzalski, et al. (2005). "The aryl hydrocarbon receptor is required for developmental closure of the ductus venosus in the neonatal mouse." Mol Pharmacol **67**(3): 714-720.

Langrish, J. P., X. Li, et al. (2011). "Reducing personal exposure to particulate air pollution improves cardiovascular health in patients with coronary heart disease." Environ Health Perspect **120**(3): 367-372.

Lee, M. S., S. Magari, et al. (2011). "Cardiac autonomic dysfunction from occupational exposure to polycyclic aromatic hydrocarbons." Occup Environ Med **68**(7): 474-478.

Li, X., Y. Feng, et al. (2012). "The dose-response decrease in heart rate variability: any association with the metabolites of polycyclic aromatic hydrocarbons in coke oven workers?" PLoS One **7**(9): e44562.

Mahler, B. J., P. C. Van Metre, et al. (2005). "Parking lot sealcoat: an unrecognized source of urban polycyclic aromatic hydrocarbons." Environ Sci Technol **39**(15): 5560-5566.

McIntosh, B. E., J. B. Hogenesch, et al. (2010). "Mammalian Per-Arnt-Sim proteins in environmental adaptation." Annu Rev Physiol **72**: 625-645.

Menzie, C. A., B. B. Potocki, et al. (1992). "Exposure to Carcinogenic Pahs in the Environment." Environmental Science & Technology **26**(7): 1278-1284.

Mumtaz, M. and J. George (1995). Toxicological profile for polycyclic aromatic hydrocarbons (PAHs). Atlanta, GA, Agency for Toxic Substances and Disease Registry, U.S. Department of Health and Human Services.

Murk, A. J., J. Legler, et al. (1996). "Chemical-activated luciferase gene expression (CALUX): a novel in vitro bioassay for Ah receptor active compounds in sediments and pore water." Fundam Appl Toxicol **33**(1): 149-160.

Nacci, D., M. Huber, et al. (2009). "Evolution of tolerance to PCBs and susceptibility to a bacterial pathogen (Vibrio harveyi) in Atlantic killifish (Fundulus heteroclitus) from New Bedford (MA, USA) harbor." Environ Pollut **157**(3): 857-864.

Nebert, D. W., A. L. Roe, et al. (2000). "Role of the aromatic hydrocarbon receptor and [Ah] gene battery in the oxidative stress response, cell cycle control, and apoptosis." Biochem Pharmacol **59**(1): 65-85.

O'Donnell, E. F., K. S. Saili, et al. (2010). "The anti-inflammatory drug leflunomide is an agonist of the aryl hydrocarbon receptor." PLoS One **5**(10).

Perera, F., W. Y. Tang, et al. (2009). "Relation of DNA methylation of 5'-CpG island of ACSL3 to transplacental exposure to airborne polycyclic aromatic hydrocarbons and childhood asthma." PLoS One **4**(2): e4488.

Perera, F. P., Z. Li, et al. (2009). "Prenatal Airborne Polycyclic Aromatic Hydrocarbon Exposure and Child IQ at Age 5 Years." Pediatrics.

Planchart, A. and C. J. Mattingly (2010). "2,3,7,8-Tetrachlorodibenzo-p-dioxin upregulates FoxQ1b in zebrafish jaw primordium." Chem Res Toxicol **23**(3): 480-487.

Polidori, A., J. Kwon, et al. (2010). "Source proximity and residential outdoor concentrations of PM(2.5), OC, EC, and PAHs." J Expo Sci Environ Epidemiol **20**(5): 457-468.

Prasch, A. L., H. Teraoka, et al. (2003). "Aryl hydrocarbon receptor 2 mediates 2,3,7,8-tetrachlorodibenzo-p-dioxin developmental toxicity in zebrafish." Toxicol Sci **76**(1): 138-150.

Puga, A., C. Ma, et al. (2009). "The aryl hydrocarbon receptor cross-talks with multiple signal transduction pathways." Biochem Pharmacol **77**(4): 713-722.

Ramesh, A., S. A. Walker, et al. (2004). "Bioavailability and risk assessment of orally ingested polycyclic aromatic hydrocarbons." Int J Toxicol **23**(5): 301-333.

Ramirez, N., A. Cuadras, et al. (2011). "Risk assessment related to atmospheric polycyclic aromatic hydrocarbons in gas and particle phases near industrial sites." Environ Health Perspect **119**(8): 1110-1116.

Ren, A., X. Qiu, et al. (2011). "Association of selected persistent organic pollutants in the placenta with the risk of neural tube defects." Proc Natl Acad Sci U S A **108**(31): 12770-12775.

Rennie, M. Y., J. Detmar, et al. (2011). "Vessel tortuousity and reduced vascularization in the fetoplacental arterial tree after maternal exposure to polycyclic aromatic hydrocarbons." Am J Physiol Heart Circ Physiol **300**(2): H675-684.

Saili, K. S. (2012). Developmental neurobehavioral toxicity of bisphenol A in zebrafish (Danio rerio) [electronic resource] / by Katerine Schletz Saili. Corvallis, Or. :, Oregon State University.

Sarkar, A., D. Ray, et al. (2006). "Molecular Biomarkers: their significance and application in marine pollution monitoring." Ecotoxicology **15**(4): 333-340.

Sartor, M. A., M. Schnekenburger, et al. (2009). "Genomewide analysis of aryl hydrocarbon receptor binding targets reveals an extensive array of gene clusters that control morphogenetic and developmental programs." Environ Health Perspect **117**(7): 1139-1146.

Schmidt, J. V., G. H. Su, et al. (1996). "Characterization of a murine Ahr null allele: involvement of the Ah receptor in hepatic growth and development." Proc Natl Acad Sci U S A **93**(13): 6731-6736.

Schoeny, R. a. K. P. (1993). Provisional guidance for quantitative risk assessment of polycyclic aromatic hydrocarbons. U. S. E. P. Agency. Washington, DC.

Shimizu, Y., Y. Nakatsuru, et al. (2000). "Benzo[a]pyrene carcinogenicity is lost in mice lacking the aryl hydrocarbon receptor." Proc Natl Acad Sci U S A **97**(2): 779-782.

Smith, B. W., S. S. Rozelle, et al. (2013). "The aryl hydrocarbon receptor directs hematopoietic progenitor cell expansion and differentiation." Blood.

Song, M. K., M. Song, et al. (2012). "Identification of molecular signatures predicting the carcinogenicity of polycyclic aromatic hydrocarbons (PAHs)." Toxicol Lett **212**(1): 18-28.

Tang, D., T. Y. Li, et al. (2006). "PAH-DNA adducts in cord blood and fetal and child development in a Chinese cohort." Environ Health Perspect **114**(8): 1297-1300.

Tanguay, R. L., C. C. Abnet, et al. (1999). "Cloning and characterization of the zebrafish (Danio rerio) aryl hydrocarbon receptor." Biochim Biophys Acta **1444**(1): 35-48.

Teraoka, H., W. Dong, et al. (2003). "Induction of cytochrome P450 1A is required for circulation failure and edema by 2,3,7,8-tetrachlorodibenzo-p-dioxin in zebrafish." Biochem Biophys Res Commun **304**(2): 223-228.

Timme-Laragy, A. R., C. J. Cockman, et al. (2007). "Synergistic induction of AHR regulated genes in developmental toxicity from co-exposure to two model PAHs in zebrafish." Aquat Toxicol **85**(4): 241-250.

Timme-Laragy, A. R., L. A. Van Tiem, et al. (2009). "Antioxidant responses and NRF2 in synergistic developmental toxicity of PAHs in zebrafish." Toxicol Sci.

Van den Berg, M., L. S. Birnbaum, et al. (2006). "The 2005 World Health Organization reevaluation of human and Mammalian toxic equivalency factors for dioxins and dioxin-like compounds." Toxicol Sci **93**(2): 223-241.

Van Metre, P. C. and B. J. Mahler (2010). "Contribution of PAHs from coal-tar pavement sealcoat and other sources to 40 U.S. lakes." Sci Total Environ **409**(2): 334-344.

Vanwezel, A. P. and A. Opperhuizen (1995). "Narcosis Due to Environmental-Pollutants in Aquatic Organisms - Residue-Based Toxicity, Mechanisms, and Membrane Burdens." Critical Reviews in Toxicology **25**(3): 255-279.

Wan, B., J. W. Yarbrough, et al. (2008). "Structure-related clustering of gene expression fingerprints of thp-1 cells exposed to smaller polycyclic aromatic hydrocarbons." SAR QSAR Environ Res **19**(3-4): 351-373.

White, S. S. and L. S. Birnbaum (2009). "An overview of the effects of dioxins and dioxin-like compounds on vertebrates, as documented in human and ecological epidemiology." J Environ Sci Health C Environ Carcinog Ecotoxicol Rev **27**(4): 197-211.

Wilhelm, M., J. K. Ghosh, et al. (2012). "Traffic-related air toxics and term low birth weight in Los Angeles County, California." Environ Health Perspect **120**(1): 132-138.

Wills, L. P., C. W. Matson, et al. (2010). "Characterization of the recalcitrant CYP1 phenotype found in Atlantic killifish (Fundulus heteroclitus) inhabiting a Superfund site on the Elizabeth River, VA." Aquat Toxicol **99**(1): 33-41.

Wilson, S. R., A. D. Joshi, et al. (2013). "The tumor suppressor Kruppel-like factor 6 is a novel aryl hydrocarbon receptor DNA binding partner." J Pharmacol Exp Ther **345**(3): 419-429.

Wirgin, I., N. K. Roy, et al. (2011). "Mechanistic basis of resistance to PCBs in Atlantic tomcod from the Hudson River." Science **331**(6022): 1322-1325.

Wu, M. T., T. C. Lee, et al. (2011). "Whole genome expression in peripheral-blood samples of workers professionally exposed to polycyclic aromatic hydrocarbons." Chem Res Toxicol **24**(10): 1636-1643.

Xiong, K. M., R. E. Peterson, et al. (2008). "Aryl hydrocarbon receptor-mediated down-regulation of sox9b causes jaw malformation in zebrafish embryos." Mol Pharmacol **74**(6): 1544-1553.

Xu, X., H. Hu, et al. (2013). "Studying the effects of polycyclic aromatic hydrocarbons on peripheral arterial disease in the United States." Sci Total Environ **461-462C**: 341-347.

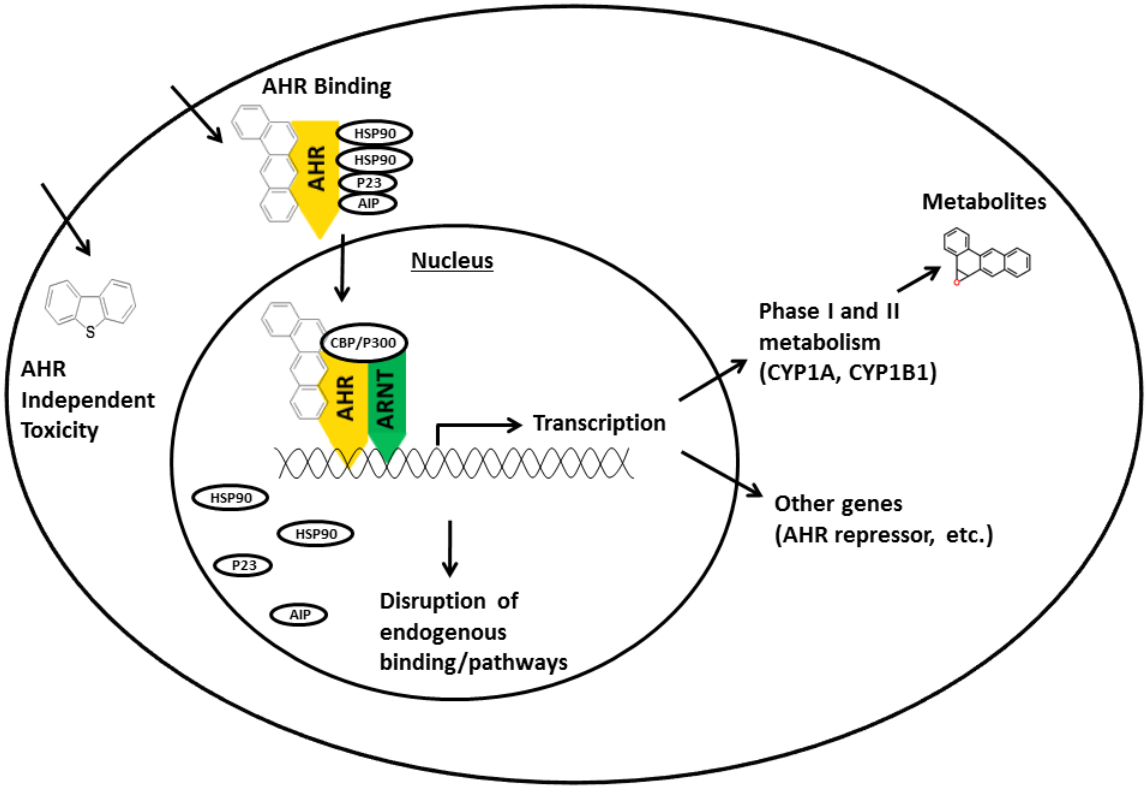


Figure 1-1 Schematic diagram of the aryl hydrocarbon receptor pathway

Upon entering the cell, some PAHs are bound by the aryl hydrocarbon receptor (AHR), which resides in the cytosol, and in its unliganded state is bound by 90 kDa heat-shock protein (HSP90), co-chaperone p23, and aryl hydrocarbon interacting protein (AIP). Upon ligand binding, the AHR translocates to the nucleus and dimerizes with the aryl hydrocarbon receptor nuclear translocator (ARNT). Together with other interacting proteins, such as CREB binding protein (CBP/P300), the AHR/ARNT heterodimer binds to aryl hydrocarbon receptor response elements (AHREs) in the genome and activates transcription of many genes. Genes directly activated by the AHR include phase I and II metabolizing enzymes such as cytochrome p4501A and 1B1 (CYP1A and CYP1B1). These enzymes metabolize PAHs to more reactive metabolites, which can be further metabolized and excreted, but can also cause toxicity by interacting with DNA and proteins. The battery of genes induced by the AHR includes the AHR repressor as well as other targets which may mediate PAH-induced toxicity. Binding of AHR to ARNT and localization to AHRE in response to ligand activation may additionally disrupt endogenous pathways, leading to toxic effects.

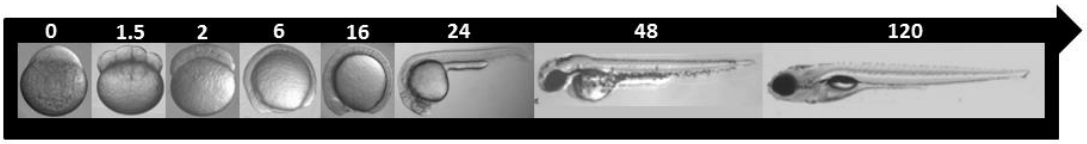


Figure 1-2 Zebrafish early development

Stages of zebrafish development from fertilization to 120 hours post fertilization (hpf) (adapted from Saili 2012).

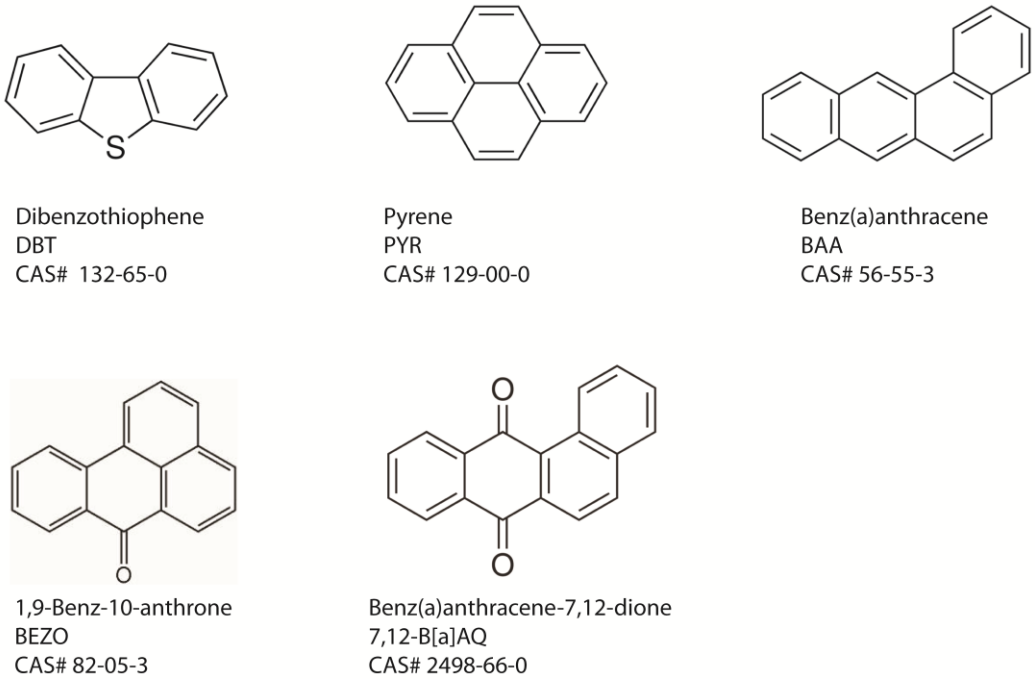


Figure 1-3 Polycyclic aromatic hydrocarbon structures

Compounds investigated in this dissertation include parent PAHs dibenzothiophene, pyrene, and benz(a)anthracene, and OPAHs 1,9-Benz-10-anthrone and benz(a)anthracene-7,12-dione.

Chapter 2 - Structurally distinct polycyclic aromatic hydrocarbons induce differential transcriptional responses in developing zebrafish

Britton C. Goodale[1], Susan C. Tilton[2], Margaret M. Corvi[1], Glenn R. Wilson[1], Derek B. Janszen[2], Kim A. Anderson[1], Katrina M. Waters[2] and Robert L. Tanguay[1*]

1.Department of Environmental and Molecular Toxicology, the Environmental Health Sciences Center, Oregon State University, Corvallis, OR,

2.Computational Biology and Bioinformatics, Pacific Northwest National Laboratory

Toxicology and Applied Pharmacology

Reprinted with permission

Abstract

Polycyclic aromatic hydrocarbons (PAHs) are ubiquitous in the environment as components of fossil fuels and by-products of combustion. These multi-ring chemicals differentially activate the aryl hydrocarbon receptor (AHR) in a structurally dependent manner, and induce toxicity via both AHR-dependent and-independent mechanisms. PAH exposure is known to induce developmental malformations in zebrafish embryos, and recent studies have shown cardiac toxicity induced by compounds with low AHR affinity. Unraveling the potentially diverse molecular mechanisms of PAH toxicity is essential for understanding the hazard posed by complex PAH mixtures present in the environment. We analyzed transcriptional responses to PAH exposure in zebrafish embryos exposed to benz(a)anthracene (BAA), dibenzothiophene (DBT) and pyrene (PYR) at concentrations that induced developmental malformations by 120 h post-fertilization (hpf). Whole genome microarray analysis of mRNA expression at 24 and 48 hpf identified genes that were differentially regulated over time and in response to the three PAH structures. PAH body burdens were analyzed at both time points using GC-MS, and demonstrated differences in PAH uptake into the embryos. This was important for discerning dose-related differences from those that represented unique molecular mechanisms. While BAA misregulated the least number of transcripts, it caused strong induction of *cyp1a* and other genes known to be downstream of the AHR, which were not induced by the other two PAHs. Analysis of functional roles of misregulated genes and their predicted regulatory transcription factors also distinguished the BAA response from regulatory networks disrupted by DBT and PYR exposure. These results indicate that systems approaches can be used to classify the toxicity of PAHs based on the networks perturbed following exposure, and may provide a path for unraveling the toxicity of complex PAH mixtures.

Keywords: AHR; microarray; dibenzothiophene; pyrene; benz(a)anthracene; systems toxicology

Introduction

Polycyclic aromatic hydrocarbons (PAHs) are a diverse class of chemicals composed of multiple fused benzene rings, which originate from both petrogenic and pyrogenic sources and are ubiquitous in the environment. Many PAHs are biologically active, cause toxicity in a variety of organisms, and can adversely affect human health. Increasing PAH concentrations in the environment, particularly in urban areas, has been attributed to anthropogenic activities such as fossil fuel burning, automobile exhaust, oil refining and coal tar seal coating (Van Metre and Mahler 2005; Polidori et al. 2010; Van Metre and Mahler 2010). PAHs are present in the ultrafine particulate fraction as well as the gas phase of ambient air, and are considered carcinogenic components of cigarette smoke, vehicle exhaust, wood smoke and other emissions (Bostrom et al. 2002; Ramirez et al. 2011). The primary routes of human exposure are inhalation and ingestion. PAHs associated with ultrafine particulate matter can accumulate in the bronchial epithelium, while volatile PAHs are readily absorbed through the alveolar epithelium (Ramirez et al. 2011). For non-smoking individuals, ingestion via foods and unintentional consumption of household dust (of particular concern for young children) is a primary contributor to PAH exposure (Menzie et al. 1992; Ramesh et al. 2004). Seven non-substituted PAHs are considered possible carcinogens (group 2B) by the US EPA, and 16 PAHs are listed as priority pollutants because of their prevalence in urban and suburban air (EPA 2012). PAH-containing coal tar mixtures are known to be carcinogenic in humans (International Agency for Research on Cancer) (Collins et al. 1998). Human exposure to PAHs almost always occurs within complex mixtures, which may contain multiple PAHs and often include other chemicals such as halogenated hydrocarbons and metals. Because of complex exposure patterns, it is difficult to associate health effects in human populations with individual PAHs.

While the bulk of research on PAHs has focused on mutagenic and carcinogenic properties, exposure to PAH mixtures and ultrafine particulate matter is associated with an array of other health effects in humans, including immune system deficiency, cardiovascular disease and impaired development (Burstyn et al. 2005; Choi et al. 2006; Hertz-Picciotto et al. 2008; Lee et al. 2011; Ren et al. 2011). Activation of the aryl hydrocarbon receptor (AHR) and generation of reactive oxygen species (ROS) are key modes of action initiated by some PAHs, but the full extent of the molecular responses that result from exposure to this

diverse set of compounds has not been characterized. A number of PAHs, including benzo(a)pyrene (BaP) and dimethylbenz(a)anthracene (DMBA) , bind the AHR and induce expression of phase I and II metabolizing genes, such as *CYP1A1*, *GSTA1*, *NQO1* and *UGT1A6*, along with many other downstream transcripts (Guengerich 2000; Nebert et al. 2000). Activation of the AHR pathway and metabolism of PAHs can result in a protective effect against PAH toxicity. In many cases, however, AHR activation and metabolism by CYP enzymes increase PAH reactivity and toxicity, which is consequential to the PAH, route of exposure, and exposure concentration (Nebert et al. 2004; Shi et al. 2010). The low molecular weight PAHs (2-3 rings) are generally poor AHR ligands and less potent carcinogens, but are often detected at higher levels in environmental samples and human urine than their higher molecular weight counterparts (Durant et al. 1996; Naumova et al. 2002; Ciganek et al. 2004; Hecht et al. 2010).

Several studies have associated PAH exposure during pregnancy with adverse birth outcomes such as reduced fetal growth and neural tube defects (Choi et al. 2006; Ren et al. 2011). In rodents, exposure to BaP and DMBA induces abnormal vasculature in the placenta and interferes with fetal growth (Detmar et al. 2008; Rennie et al. 2011). Developmental exposure to BaP also impairs cardiac function later in life (Jules et al. 2012).

In zebrafish embryos, BaP-induced cardiac toxicity is mediated by the aryl hydrocarbon receptor (AHR2) (Incardona et al. 2011). However, other PAH structures induce cardiac toxicity and developmental effects via distinct mechanisms that are not AHR-dependent (Incardona et al. 2005). Analyses of global mRNA transcriptional responses to individual PAH exposures demonstrate that structurally-distinct PAHs induce unique gene expression patterns in both human macrophage leukemia (THP-1) cells and circulating leukocytes of rats (Wan et al. 2008; Jung et al. 2011). Little is known, however, about the toxicity pathways and molecular signatures of these diverse exposures during embryonic development.

We used whole genome mRNA microarrays to investigate transcriptional responses that lead to developmental toxicity of three distinct PAHs in developing zebrafish. Dibenzothiophene (DBT), pyrene (PYR) and benz(a)anthracene (BAA) all induce developmental abnormalities by 5 days post fertilization, but have different proposed

toxicity mechanisms. DBT (3 rings) induces cardiac toxicity that is independent of the AHR (Incardona et al. 2004). BAA (4 rings) induces Cyp1a expression and developmental toxicity via activation of AHR2, while PYR (4 rings) toxicity was shown to be metabolism-dependent (Incardona et al. 2006). We determined PAH body burden and corresponding transcriptional profiles in PAH-exposed zebrafish embryos at 24 and 48 hours post-fertilization, before toxicity could be visibly identified. We found that DBT, PYR and BAA induce mRNA expression profiles that differentially implicate AHR activity, and highlight multiple pathways that can be disrupted by exposure to PAHs over the course of vertebrate development.

Methods

Zebrafish lines and embryos:

Adult wild type 5D zebrafish were housed at the Sinnhuber Aquatic Research Laboratory on a recirculating system maintained at 28±1°C with a 14 h light/10 h dark schedule. Embryos were collected from group spawns of adult zebrafish as described previously (Reimers et al. 2006) and all experiments were conducted with fertilized embryos according to Oregon State University Institutional Animal Care and Use Protocols.

Chemical Exposures and Developmental Toxicity Assessment:

Dibenzothiophene (>99%), pyrene (99%) and 1,2-benzanthracene (99%) were purchased from Sigma-Aldrich and dissolved in DMSO (J.T. Baker) at 50 mM, 50 mM and 25 mM concentrations, respectively. Embryos were cleaned, developmentally staged, and batch-exposed in glass vials at 6 h post fertilization (hpf) (chorions intact) to PAHs or vehicle control with 1% final DMSO concentration in E2 embryo medium (Kimmel et al. 1995). For all experiments, exposures were conducted on a rocker and embryos were protected from light until the experimental time points. For developmental toxicity experiments, PAH solutions were removed at 48 hpf and embryos were rinsed 4x and incubated in fresh embryo medium until 120 hpf, when they were assessed visually for malformations as previously described (Truong et al. 2011). Preliminary range-finding studies were conducted with each PAH and all further developmental toxicity assessments were conducted at 25 μM with 20 embryos per vial in 2 ml exposure solution. Microarray and body burden exposures were conducted with 40 embryos per vial in 4 ml solution.

Analysis of Developmental Toxicity Endpoints:

Embryos were anesthetized with tricaine methanesulfonate and visually assessed at 120 hpf for yolk sac, axis, trunk, somite, fin, cardiac, eye, snout, jaw, otic vesicle, brain and pigment malformations. Mortality and the percentage of embryos with each malformation were calculated for each treatment group with the vial (20 embryos) as the experimental unit. The experiment was repeated 3 times. A generalized linear model (binomial distribution, logit link) one-way ANOVA was performed for the 8 endpoints which were observed in at least 3 embryos across all treatment groups. If the overall p-value indicated differences among the treatment percentages, individual comparisons were conducted using Tukey's all pairwise post hoc test in R version 2.12.

Detection of PAH body burden in zebrafish embryos:

Embryos were exposed to 0, 1, 5, 10 and 25 μM PAH (1% DMSO) solutions in glass vials as described previously, with 40 embryos per vial in 4 ml exposure solution. As with all exposures in this study, embryos were exposed at 6 hpf with chorions intact and incubated at 28˚C on a rocker. Control embryos hatched just before 48 hpf; exposure to 10 and 25 μM PAH delayed hatching by 3-4 h, but all treatment groups hatched on their own by 72 hpf. Because several exposure concentrations are above solubility for PAHs in embryo medium, PAH precipitate accumulated on the outside of the chorion. In order to measure the amount of PAH internalized by the embryos, chorions were removed immediately following exposure and before analysis as described below. For each biological replicate, 2 vials were combined after exposure.

For analysis at 24 hpf, embryos were rinsed with fish water and transferred to a clean glass petri dish. They were incubated in 82 μg/ml pronase (Sigma-Aldrich) at room temperature, gently agitated for 3 min, then rinsed thoroughly using an automated dechorionating system as previously described (Mandrell et al. 2012). Following rinsing, embryos were placed in a 28˚C incubator for 20 min, after which >95% of chorions were removed by gentle agitation of the dish.

At 48 hpf, the majority of embryos had hatched and did not require batch dechorionation. They were chilled on ice to reduce activity, PAH solution was removed, and embryos were transferred to a clean glass petri dish with cold fish water. Chorions were removed from any

remaining embryos with forceps, and embryos were gently agitated and rinsed 4x with fish water. Immediately following dechorionation, 50 embryos from each treatment group were loaded into microcentrifuge tubes with approximately 80 mg 1 mm glass beads and placed on ice for at least 10 min. Embryos were homogenized in 500 µl ethyl acetate with a bullet blender (Next Advance, Averill Park, NY). Samples were then vortexed and incubated 15 min before centrifuging for 5 min at 16,000 RCF. 400 µl of supernatant was stored in amber vials at 4˚C until analysis.

Percent PAH recovery for this method was calculated from 4 replicates of unexposed 24 and 48 hpf embryo samples loaded into microcentrifuge tubes as above and spiked with 12.5 µl PAH stock in DMSO. Samples were processed identically to experimental samples. Zebrafish extracts were analyzed using an Agilent 5975B Gas Chromatograph-Mass Spectrometer (GC-MS) with a DB-5MS column (30 m x 0.25 mm x 0.25 µm) in electron impact mode (70 eV) using selective ion monitoring (SIM). The GC parameters were as follows: injection port maintained at 300 °C, 1.0 ml min^{-1} helium flow, 70 °C initial temperature, 1 min hold, 10 °C min^{-1} ramp to 300 °C, 4 min hold, and 10 °C min^{-1} ramp to 310 °C, 4 min hold. The MS temperatures were operated at 150, 230 and 280 °C for the quadrupole, source and transfer line respectively. Standards for BAA, DBT and PYR (>97% purity) were purchased from AccuStandard (New Haven, CT). Isotopically labeled chrysene-D12 and acenaphthylene-D8 were purchased from C/D/N incorporated (Quebec, Canada). A nine point calibration curve (10 pg/ µl to 10 ng/µl) was conducted to determine relative response ratios of PAHs to deuterated surrogate standards; chrysene-D12 was used as the deuterated surrogate for PYR (r^2 = 0.9992) and BAA (r^2 = 0.9982), acenaphthylene-D8 was used for DBT (r^2 = 0.9991).

Calibration verification standards for target analytes and surrogates were analyzed at least every 22 samples and reported values within ±20% of the true value were considered to meet our data quality objectives (DQO). Only results from samples run between two calibration verifications that met the DQO were accepted; the majority were within ±10% of the true value. PAHs in all laboratory blanks (solvent-exposed embryos) were below detection except for 5 samples in which DBT and BAA were detected. This possible contamination was <10% of the levels detected in our lowest exposure sample groups, and

deemed negligible. Body burden (µmol/g embryo) was calculated using average embryo weights of 0.4 mg at 24 hpf and 0.3 mg at 48 hpf. Pairwise comparisons were conducted between PAH-exposed samples and time-matched controls, as well between 24 and 48 hpf at each exposure concentration with Mann-Whitney Rank Sum tests using SigmaPlot software.

Microarray analysis of mRNA expression:

Embryos batch-exposed in groups of 40 to 25 µM DBT, PYR, BAA or 1% DMSO control were homogenized in TRI Reagent (Molecular Research Center, Cincinnati, OH) at 24 and 48 hpf for RNA isolation. Four independent biological replicates were prepared for each treatment. Total RNA was isolated with phenol-chloroform extraction, and RNA was quantified and quality confirmed with a NanoDrop ND-1000 UV-Vis spectrophotometer and Agilent Bioanalyzer 2100. Microarray analysis was performed by the University of Wisconsin McArdle Laboratory of Cancer Research Microarray Facility. Briefly, cDNA was synthesized from 1.2 µg of total RNA from each sample and labeled with cy3 (experimental samples) or cy5 (pooled control sample) according to the Agilent protocol with minor modifications. Equal amounts of cy3 and cy5 labeled samples were mixed, fragmented, and hybridized to Agilent Zebrafish V2 array chips. Slides were scanned immediately with an Agilent microarray scanner (Agilent Technologies, Santa Clara, CA). Microarray files were submitted to the NCBI Gene Expression Omnibus, accession number GSE44130 http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE44130.

Microarray analysis:

Raw intensity data were processed by Agilent Feature Extraction software using Lowess normalization. Quality control analysis was performed on preprocessed data in GeneSpring v.11 (Silicon Genetics, Redwood City, CA) software using feature intensity distributions from box-whisker plots to determine interquartile range span and median intensity value across the experiment. The intra-group versus between-group comparisons were made using correlation matrix plots, followed with principle components analysis to determine potential outliers. One biological replicate from the DBT 48 hpf treatment group was removed as an outlier, resulting in an N=3 for that treatment group. Normalized data were transformed to time-specific controls and analyzed by one-way ANOVA for unequal

variances (Welch's ANOVA) with Tukey's post hoc test and 5% false discovery rate calculation (Benjamini and Hochberg 1995). Values are reported as fold change (log2) with associated Benjamini-Hochberg adjusted p value in each treatment group compared to time-matched control. Correlation analysis between treatment groups was performed by linear regression of log2 fold change values, using the union of significant genes from both groups. Based on the significant correlation between DBT and PYR treatments at both time points, these datasets were further filtered to identify the subset of genes that were significantly different between them ($p<0.05$, 1.5-fold change). Genes that did not meet these criteria were considered similar between the DBT and PYR treatments for functional and transcription factor analysis.

Bioinformatic analysis:

Unsupervised hierarchical clustering of microarray data was performed using Euclidean distance metric and centroid linkage clustering to group gene expression patterns by similarity. The clustering algorithms, heat map visualizations and centroid calculations were performed with Multi-Experiment Viewer (Saeed et al. 2003) software based on log2 expression ratio values. For downstream bioinformatic analysis, zebrafish identifiers on the Agilent platform were converted to human orthologs using Bioinformatics Resource Manager v. 2.3 (Tilton et al. 2012). Genes that did not have human orthologs were still included in the bioinformatic analysis using their zebrafish identifier. Both MetaCore (GeneGO) and DAVID software recognize mixed identifiers (Entrez Gene ID) from human and zebrafish. Significant targets from the microarray and genes of interest are referred to by zebrafish gene identifiers, where zebrafish-derived information was available in the literature. Functional annotation and network information, however, were primarily derived from other species, and data for many genes of interest were only available in the mammalian literature; we present this information with human gene identifiers throughout the results and discussion. Functional enrichment was determined using the DAVID functional annotation tool (Huang da et al. 2009), which utilizes the Fisher Exact test to measure gene enrichment in biological process Gene Ontology (GO) category terms for significant genes compared to background, which consisted of all genes on the Agilent platform. GO biological process categories from levels 3, 4, and 5 were included for enrichment calculation. Since the DAVID functional annotation tool clusters GO terms by

similarity to reduce redundancy, the biological processes are presented in the results with a representative process from each significant cluster ($p<0.05$) that represented at least 1% of genes from the exposure group. To identify major transcriptional regulators of gene expression by PAHs, the Statistical Interactome tool was used in MetaCore to measure the interconnectedness of genes in the experimental dataset relative to all known interactions in the background dataset. Statistical significance of over-connected interactions was calculated using a hypergeometric distribution, where the p value represents the probability of a particular mapping arising by chance for experimental data compared to the background (Nikolsky et al. 2009). Networks were constructed in MetaCore for experimental data using an algorithm that identifies the shortest path to directly connect nodes in the dataset to transcription factors. Network visualizations were generated in Cytoscape (Shannon et al. 2003).

Quantitative RT-PCR

Validation of gene expression changes identified in the microarray analysis was conducted for a group of transcripts selected to represent differential regulation patterns by the three PAHs at 24 and 48 hpf. Gene-specific primers (MWG Operon) for qRT-PCR amplification are listed in Table S1. Sub aliquots of 10 µg total RNA from the microarray analysis were reverse transcribed using Superscript III (Invitrogen) according to manufacturer instructions. All qRT-PCR assays were performed in 20 µl reactions consisting of 10 µl Power SYBR Green PCR master mix (Applied Biosystems), 0.4 µl each primer, 9.2 µl H_2O and 50 ng equivalents of cDNA. Amplification (StepOnePlus, Applied Biosystems) was performed with cycling parameters as follows: 95°C for 10 min; 40 cycles of 95°C for 15 s, 60°C for 1 min; 95°C for 15 sec and 60°C for 1 min. A melt curve was performed at 3° increments to assess for multiple products. Relative fold change values in PAH-treated samples compared to vehicle controls were calculated for genes of interest, normalized to β-actin, by the method described by Pfaffl (Pfaffl 2001). Three independent biological replicates were assessed and statistically analyzed by one-way ANOVA with Tukey's post-hoc test using SigmaPlot software.

Results and discussion

Dibenzothiophene, pyrene, and benz(a)anthracene induce developmental toxicity in zebrafish embryos.

Exposure to DBT, PYR or BAA caused a significant increase in the incidence of abnormal embryos compared to the vehicle control exposure at 120 hpf. All three compounds induced pericardial edema, snout and jaw malformations (Fig. 1). PYR and BAA exposures caused significant increases in yolk sac edema, while DBT did not. DBT, however, induced distinct axis malformations (Fig. 1B) which were not present in BAA- or PYR-exposed embryos (Tables 1, 2). Interestingly, the 25 μM concentration of all three PAHs induced malformations in >80% of embryos by 120 hpf, while mortality was < 10%, not significantly different from control (Table 1). The phenotypes induced by these PAHs suggested different underlying pathologies. BAA induced more severe edema, while necrotic tissue was observed in PYR-exposed embryos, particularly in the anterior yolk sac, liver and digestive tract (Fig. 1C); these embryos did not survive more than a couple hours past 120 hpf. This was observed previously by Incardona et al, who demonstrated that DBT, PYR and BAA induced malformations in zebrafish that were differentially dependent on activation of the AHR and metabolism by Cyp1a (Incardona et al. 2004; Incardona et al. 2005). The differential proposed mechanisms of these PAHs presented an ideal opportunity to investigate the diversity of molecular pathways that lead to developmental effects of PAH exposure. The objective of this study was not to mimic environmental exposures, but rather to identify molecular pathways that precede morphological changes induced by different PAH structures. Based on our developmental toxicity data, 25 μM was identified as an appropriate concentration for microarray analysis of early gene expression changes elicited by DBT, PYR and BAA exposure.

Zebrafish exposed to dibenzothiophene, pyrene and benz(a)anthracene in embryo medium accumulate differential PAH body burdens.

The internal body burden of PAH in embryos was measured after exposures as described for the microarray analysis. To allow for structure-toxicity comparisons between the three compounds, as well as to relate gene expression data to other model systems, DBT, PYR and BAA were detected by GC-MS in zebrafish embryos exposed to a range of concentrations (1-25 μM). PAH recovery averaged between 80 and 125% (Table S2). Measured PAH values were therefore reported as detected in experimental samples, unadjusted for recovery. The

amount of PAH detected in embryos revealed stark differences between the three PAH structures. At all concentrations and time points, DBT body burden in embryos was the highest, averaging 3.4 and 5.3 μmol/g at 24 and 48 hpf, respectively, following exposure to 25 μM DBT (Fig. 2A). DBT had the highest solubility in water, and uptake did not plateau in the range of concentrations tested here. PAH body burden of embryos exposed to 25 μM PYR averaged 1.0 and 2.9 μmol/g at 24 hpf and 48 hpf, respectively, and uptake appeared to reach a plateau, likely because of low compound solubility in embryo medium (Fig. 2B). BAA body burden was markedly lower than the other two PAHs at all exposure concentrations. An apparent saturation was reached at 0.10 μmol/g embryo at 24 hpf, an order of magnitude lower than PYR (Fig. 2C). Water solubility of BAA was the lowest of these PAHs, and the high concentrations employed in this study were above solubility with 1% DMSO in embryo medium. At 48 hpf, BAA concentrations averaged 0.19 μmol/g in embryos from the 25 μM exposure group.

Studies of early-life exposure to PAHs showed that the bioconcentration factor (BCF) in fish embryos correlated with the octanol water partition coefficient (K_{ow}) (Mathew et al. 2008). The log K_{ow} values of DBT, PYR and BAA are 4.38, 4.88 and 5.79, respectively (Hansch 1995). BAA would therefore be predicted to have the highest BCF of the PAHs in our study. BCFs have primarily been calculated for larvae (post-hatch), however, and the short duration exposures employed in our study did not allow steady-states to be achieved. Steady-state PAH concentrations were similarly not attained in zebrafish eggs in a study reported by Petersen and colleagues (Petersen and Kristensen 1998). Metabolism could also explain differences in parent PAH concentration, but is expected to be low during the developmental stages chosen for gene expression analysis in this study (Petersen and Kristensen 1998). Metabolism increases upon hatching in Atlantic killifish embryos, and zebrafish exhibit greater inducibility of *cyp1a* starting at 48 hpf, the approximate time of hatching in our laboratory (Binder and Stegeman 1984; Andreasen et al. 2002). While metabolism could potentially explain the small decrease in BAA between 24 and 48 hpf at the lower concentrations, it is unlikely to explain the large difference in body burdens observed between PAH structures. Differences between PAHs in this study appear to be driven by their solubility in embryo medium, rather than their BCFs or metabolism. The 25 μM exposures for the microarray study represent an acute exposure intended to identify

mRNA expression changes that precede appearance of morphological abnormalities. While total dose and maximum exposure calculations were beyond the scope of this study, the measurement of parent PAH in the embryos at the time of gene expression analysis provided important information for mechanistic comparison between the PAHs.

mRNA expression profiles induced by PAH exposure are different at 24 and 48 h post fertilization.

Pairwise analysis of variance across all exposure groups identified significant expression changes in 1079 transcripts compared to time-matched controls (Table S3). Entrez or Ensdart IDs were identified for 935 of these in the Ensembl zebrafish genome assembly (Zv9). Unsupervised bidirectional clustering of all experimental groups indicated a strong developmental time point effect, and revealed unique gene expression patterns in response to the three PAHs. At 24 hpf, DBT and PYR exposure groups clustered closely, while BAA induced a strikingly different expression pattern (Figs. 3A, D). DBT and PYR exposure groups also clustered at 48 hpf, but with distinct separation from the 24 hpf samples and with a notably larger group of down-regulated transcripts. The expression profile induced by BAA at 48 hpf clustered more closely with 24 hpf BAA than with the other 48 hpf PAH samples (Fig. 3A). At 24 hpf, DBT, PYR and BAA exposures induced significant changes in 357, 67 and 38 transcripts, respectively. As reflected in the heatmap, more transcripts were differentially expressed at 48 hpf, but relative quantities of differentially expressed transcripts were maintained; DBT induced changes in 656, PYR in 191 and BAA in 107 transcripts (Figs. 3B-D). Fifteen genes that were significantly differentially regulated by at least one of the PAHs were selected for qRT-PCR validation of the differential regulatory patterns observed in the array. PAH- and time-dependent expression changes were confirmed for the majority of genes examined (Table S4). Fold change values were similar between the microarray and qRT-PCR for most genes, demonstrating good reliability of the microarray for identifying meaningful changes in gene expression induced by PAH exposure. *mstnb*, which was identified as a significantly decreased transcript at 24 hpf, was not decreased in BAA-exposed samples analyzed by qRT-PCR. Upon further investigation, however, we identified 5 probes on the Agilent array that target *mstnb*, only one of which identified a significant expression difference (p value 0.04). This suggested nonspecificity of that probe for *mstnb*, or potentially differential splicing. As others have reported previously,

we observed lower correlation between the microarray and qRT-PCR for down-regulated transcripts; qRT-PCR identified fewer changes that met statistical significance ($p < 0.05$), but trends in regulation were consistent (Morey et al. 2006).

For each PAH, we compared transcripts significantly differentially expressed at 24 hpf with transcripts that were significant at 48 hpf. These experimental time points encompass a period of rapid development in zebrafish, during which fin morphogenesis begins, the circulatory system forms, tactile sensitivity and swimming behavior are initiated, and pigment develops (Kimmel et al. 1995). Developmental progression is reflected in the stronger influence of time point than PAH structure in the bidirectional clustering (Fig. 3A).

While 95 transcripts were misexpressed by DBT at both 24 and 48 hpf, they represented only 27% of the 24 hpf significant gene set, and did not include the most highly misexpressed transcripts from either time point. The most differentially expressed transcripts across both time points were *acana*, *ankrd1b* and *hspb11* (Fig. 3B). *ankrd1b* and *hspb11* are both involved in myogenesis; *hspb11* is specifically expressed in muscle pioneers, up-regulated by intracellular calcium, and involved in muscle fiber organization in developing zebrafish (Kluver et al. 2011; Kojic et al. 2011). Similar to DBT, the expression of only a few transcripts were significant impacted by PYR at both 24 and 48 hpf. The two genes differentially expressed >2 fold were *tnfb*, a member of the tumor necrosis factor family of proinflammatory cytokines, and *zgc:153258* (Fig. 3C). These robust responses, conserved over time, represent potential biomarkers of exposure to the individual PAHs. The transcripts with the largest fold changes, however, were not consistent over time, which suggested that separate analysis at each time point could provide better insight into mechanisms driving responses to DBT and PYR exposure.

The BAA transcriptional profile is consistent from 24 to 48 hpf, and distinct from DBT and PYR-induced changes.

In contrast to DBT and PYR, 45% of transcripts differentially expressed by BAA at 24 hpf were also significant at 48 hpf, and those with the largest fold changes were conserved between time points (Fig. 3D). The most highly misexpressed genes at 24 hpf were *cyp1a*, *cyp1b1*, *cyp1c1*, *cyp1c2*, *ahrra* and *foxq1l*. All of these genes were elevated and, along with *sult6b1* and *ctgfb*, remained elevated at 48 hpf. The *cyp1* genes and *aryl-hydrocarbon*

receptor repressor (*ahrra*) are well-known targets of AHR, while *sult6b1* is a recently identified sulfotransferase that could potentially be involved in BAA metabolism. Together, these genes represent a consistent signature of the transcriptional response to BAA exposure in zebrafish embryos from 24 to 48 hpf.

We conducted between-PAH comparisons separately at 24 and 48 hpf to identify significant transcripts unique to each PAH exposure. Though BAA exposure affected the smallest number of transcripts, they were highly induced and formed a distinct cluster (Fig. 3D) that overlapped minimally with the DBT and PYR transcriptional profiles. Only 7 of the significant genes in the 24 hpf BAA exposure group were similarly differentially expressed in response to DBT or PYR. At 48 hpf, the BAA expression pattern remained distinct, where only 27 of the 107 differentially expressed transcripts were similarly regulated by another PAH. In additions, we observed no correlation between the transcriptional response induced by BAA and either of the other PAHs at 24 or 48 hpf ($r^2 < 0.2$), using linear regression analysis of all significant transcripts. The entire set of significant BAA transcripts was therefore used for analysis of pathways and biological functions disrupted by BAA exposure at 24 and 48 hpf (discussed below).

DBT and PYR induce a similar dose-dependent transcriptional profile.

Common patterns in gene expression between DBT and PYR exposure groups are apparent in the heatmap in Figure 3A at both time points; however, the magnitude of the PYR-induced transcriptional response is visibly lower. This trend was reflected in the linear regression analysis of PYR vs. DBT log2 expression values, where a strong positive correlation was observed at 24 hpf (Fig. 4A, union of DBT and PYR significant transcripts, $r^2 = 0.77$, $p < 0.001$). The regression slope, however, demonstrated that DBT-induced expression changes were on average 1.6 fold greater than PYR-induced changes in these transcripts. This apparent dose-effect suggested that solubility and uptake were the primary drivers of differential transcriptional responses between these compounds, rather than unique molecular targets. The dose-effect is supported by the body burden data; DBT body burdens were 3.4 times higher than PYR body burdens in the 25 μM exposure cohorts at 24 hpf. This trend persisted with a similar correlation at 48 hpf ($r^2 = 0.647$, $p< 0.001$), wherein DBT on average induced 1.75-fold greater expression changes than PYR (Fig. 4B)

and DBT body burdens were 1.8 times greater than PYR. We found that comparing the PAHs following ANOVA analysis exaggerated transcriptional profile differences, because many PYR-induced changes did not reach statistical significance ($p < 0.05$ compared to control). Because of the significant correlation in responses, we employed a direct statistical comparison of DBT and PYR to better define the conserved transcriptional response, as well as identify transcripts with meaningful expression differences between the two groups.

Direct pairwise comparison of DBT and PYR log2 FC values at 24 hpf identified 343 similarly expressed transcripts, and only 42 that were significantly different ($p < 0.05$). At 48 hpf, 139 were significantly different, while 572 transcripts were similar between the two PAHs. Because of the overwhelming conservation of response, functional analysis was performed using the set of similarly expressed genes at each time point to identify biological processes disrupted by DBT and PYR exposure. We focused further mechanistic analysis on the DBT-PYR vs. the BAA responses.

Disruption of ion transport, muscle function, and metabolism by DBT and PYR at 24 hpf

Of the 343 transcripts representing the conserved DBT and PYR responses at 24 hpf, approximately 70% were under-expressed compared to control. 308 had sufficient annotation, which translated to 256 unique DAVID IDs. Fatty acid biosynthesis, ion transport, skeletal muscle contraction, steroid biosynthesis and oxoacid metabolism were the most enriched of the 12 significant biological processes identified by DAVID functional analysis, which together depict wide disruption of molecular signaling by 24 hpf (Table 4). We used the MetaCore Statistical Interactome tool to identify major transcription factors predicted to regulate significant genes in this dataset. JUN, RELA, SP1, PPARA, RXRA, ESR1, ESR2, and NR3C1 (glucocorticoid receptor) were predicted to regulate the largest sets of differentially expressed transcripts (Table S5). Transcripts were up- and down-regulated in approximately equal numbers, and there was considerable overlap of the predicted targets of these transcription factors. The extensive molecular responses to these PAHs highlighted a complex network of regulatory processes involved in normal embryo development that were unlikely to be mediated through one primary transcription factor but rather were responsive to chemical-induced perturbations such as oxidative stress, inflammation, altered metabolism and disruption of ion balance and cardiac function.

The ion transport biological process contained the largest number of misregulated transcripts at 24 hpf (Table 3) and is discussed further below as a common biological response to all PAH exposures in this study. Ion balance is important for muscle development and function, which was also significantly affected by DBT and PYR at 24 hpf. Transcripts in the skeletal muscle contraction and muscle cell development biological processes were primarily under-expressed, and included myoglobin, which is also required for angiogenesis in zebrafish (Table 3)(Vlecken et al. 2009). Members of the ion transport cluster may interact with these transcripts or themselves be important for muscle and cardiac function in the context of zebrafish development.

Genes involved in fatty acid biosynthesis, steroid biosynthesis and oxoacid metabolism were also primarily underexpressed (Table 3). A notable exception was *cholesterol 25-hydroxylase* (*ch25h*), which encodes a cholesterol metabolizing enzyme involved in the inflammatory response, and was elevated >4 fold by both DBT and PYR (Park and Scott 2010). The functions of these genes and their roles during development have yet to be characterized in zebrafish, but together they implicate disruption of metabolic processes.

Transcription factors RELA and JUN are predicted to regulate renin-angiotensin system-related genes misexpressed in DBT and PYR exposed embryos.

In contrast to the previously-discussed biological processes, the majority of genes associated with negative regulation of cell proliferation (Table 3) have known roles in zebrafish development, and many are predicted downstream targets of the NF-κB family member RELA (Table S5; *bdnf*, *tnfrsf9a*, *zgc:114127*, *agt*, *msxe*, *tnfb*) as well as JUN (Table S5; *cx43*, *smad3b*, *tnfrsf9a*, *agt*, *tnfb*). These processes are likely mediated through multiple interacting transcription factors. Of the 15 transcription factors that were significant at 24 hpf, RELA and JUN were predicted to regulate the most highly induced genes in the DBT-PYR dataset, *agt* and *tnfb*, as well as many other genes involved in the significant biological processes. The inflammatory cytokine *tnfb* is one of two *TNF* homologs in zebrafish, both of which are highly induced in larvae in response to LPS stimulation (Wiens and Glenney 2011). Angiotensinogen (AGT) is the precursor of angiotensin (ANG II), a potent regulator of blood pressure and water homeostasis in the renin-angiotensin signaling (RAS) network (Wu et al. 2011). Transcription of *AGT* is induced by glucocorticoids through the

glucocorticoid receptor, as well as by TNF and other inflammatory cytokines via activation of NF-κB (Brasier and Li 1996). ANG II induces *AGT* transcription in a positive feedback loop involving NF-κB, and also activates JUN via JNK signaling in cardiac myocytes and vascular smooth muscle cells (Brasier and Li 1996; Brasier et al. 2000). As an initiator of tissue inflammation, TNF activates NF-κB, induces inflammatory and anti-apoptotic gene expression in a cell-type dependent manner, and also activates JUN via JNK (Tian et al. 2005). In this study, DBT and PYR exposure led to increased expression of *tnfb* and *complement component 7*, along with macrophage-related genes *mpeg1* and *mst1*, all of which are involved in innate inflammatory response. Both *Tnf* and *Agt* are expressed in the myocardium of rats following ischemia, remodel ATP-dependent calcium channels, and have been implicated in atherosclerosis and hypertension (Isidoro Tavares et al. 2009). In developing rat embryos, activation of angiotensin receptors with exogenous ANG II disrupts cardiac looping (Price et al. 1997). These two genes, in combination with the significant enrichment of other targets of RELA, JUN, and the glucocorticoid receptor, suggest that inflammatory response and RAS signaling may play a role in the cascade of effects observed in response to DBT and PYR exposure. Cardiovascular functions of the RAS system are conserved in teleosts (Le Mevel et al. 2008), and though not yet explored in embryonic zebrafish, the RAS system has been identified as important for fetal cardiovascular response, body fluid balance, and neuroendocrine regulation, and may be involved in fetal programming of hypertension later in life (Mao et al. 2009).

We created a map of key predicted transcription factors, including RELA and JUN, and their downstream targets that were significantly misregulated in the three PAH exposure groups at 24 or 48 hpf (Fig. 5). A substantial number of transcripts, including *TNF* and *AGT*, are predicted to be regulated by both transcription factors, but RELA is predicted to regulate the largest number of genes that were induced by DBT/PYR exposure at 24 or 48 hpf. While RELA was also identified as a significant transcriptional regulator of BAA genes (discussed below), the DBT-PYR and BAA exposure networks overlapped with only 10 RELA targets (Fig. 5, purple). Fig. 5 therefore highlights the distinct nature of RELA regulatory roles in the toxicity pathways of different PAH structures.

Developmental processes in DBT and PYR-exposed embryos are widely misregulated at 48 hpf.

By 48 hpf, 572 transcripts were differentially expressed in DBT and PYR embryos compared to controls, 478 of which were annotated. DAVID functional analysis identified 21 biological processes that were significantly affected by DBT and PYR exposure; oxoacid metabolic process was the most enriched functional cluster, but was composed of different genes than at 24 hpf (Table 4). Many of the most significant processes misregulated at 48 hpf were directly related to embryonic development, and the regionalization, neurogenesis and central nervous system development functions together highlight widespread disruption of nervous system development (Table 4). Thirty-five transcription factors were predicted to regulate significantly enriched groups of genes within this dataset; JUN, PPARA, RELA, RXRAa and SP1 were significant at both 24 and 48 hpf (Table S5). NR3C1 and ER, which were significant at 24 hpf, were no longer enriched at 48 hpf, whereas CREB1, P53, YY1 and TBP became significant with the largest numbers of misregulated downstream targets. The breadth of expression changes at 48 hpf is not restricted to a singular toxicity pathway, but rather encompasses substantial network perturbations consistent with aberrant embryonic development. These and the many other misexpressed transcripts may result from a cascade of processes downstream of the genes disrupted at 24 hpf, but also reflect the vast molecular changes that occur in a normally developing zebrafish between these two time points.

Biological functions of BAA-misregulated genes are consistent with AHR-dependent toxicity.

Human or mouse homologues were available for 29 of the 38 transcripts significantly misregulated by BAA at 24 hpf, which translated to 19 unique DAVID IDs. Several genes, including *cyp1a*, were represented by multiple probes within this significant transcript set. Functional analysis of genes misregulated by BAA at 24 hpf identified two biological processes, hormone metabolism and tissue development, that were significantly enriched within this dataset (Table 4). Metabolic process genes were up-regulated, and included well-known biomarkers of AHR activation such as *cyp1a*, as well as *si:dkey-94e7.2*, a predicted homolog of retinol dehydrogenase 11 (*RDH11*). Expression of genes involved in tissue development was also primarily increased (Table 3), likely via AHR signaling. A *foxq1* homolog was induced by BAA, and based on the probe sequence we identified the transcript as *foxq1b*, an AHR-dependent TCDD-inducible gene expressed in zebrafish jaw primordium (Planchart and Mattingly 2010). Tissue development genes *ptn* and *ctgfb* (Table 3) are not

known to be directly regulated by the AHR, but may be important mediators of AHR-dependent developmental toxicity; *ctgfb* was also induced in developing jaws of zebrafish exposed to TCDD (Xiong et al. 2008).

Transcription factor prediction identified AHR as significant at 24 hpf, along with its dimerization partner, ARNT, and C/EBPδ (Table S5). The large fold changes in a relatively small number of significant transcripts suggest that BAA interacts with one primary transcription factor at 24 hpf, and the transcriptional profile supports previous demonstration of AHR-dependent toxicity induced by BAA (Incardona et al. 2006).

BAA transcriptional response indicates oxidative and metabolic stress at 48 hpf

The BAA transcriptional response expanded to 107 misexpressed transcripts at 48 hpf, 99 of which were sufficiently annotated. Though the *cyp1* genes remained the most strongly elevated, they were joined by *ahrra*, *wfikkn1*, and *cathepsin L.1* (*ctsl.1*), which was elevated 4-fold. *Ctsl.1* encodes a widely expressed protease important for blood pressure regulation, and was recently identified as dioxin-responsive (Mbewe-Campbell et al. 2012). DAVID functional annotation clustering of the 70 unique targets identified eight significantly enriched biological functions (Table 4). Genes involved in hormone metabolism again formed a significant cluster, which included two phase 2 metabolizing enzymes, *ugt1b5* and *ugt1b7*, along with the *cyp1* transcripts. Transcripts associated with cation transport, in contrast, were not known AHR targets, and are discussed further within the ion transport response common to all three PAHs. The cellular homeostasis transcript group was composed of antioxidant-related genes (*gsr*, *prdx1*, and *zgc:92066*, a homolog of *FTMT*), and transcripts involved in blood pressure regulation and chemokine signaling that are not known to be direct targets of AHR (Table 4).

Vascular development genes are misexpressed in BAA-exposed embryos.

Genes involved in vasculature development were over-represented among transcripts affected by BAA at 48 hpf. They included chemokine receptor *cxcr4a,* which had increased expression, and its ligand, *cxcl12b*, which was under-expressed. Interestingly, this expression pattern was also observed in a microarray analysis of TCDD-induced transcriptional changes in zebrafish jaw, suggesting misregulated chemokine signaling may indeed be involved in AHR-mediated toxicity in the developing embryo (Xiong et al. 2008).

cxcr4a is required for arterial-venous network formation and is expressed in response to low blood flow and in unperfused blood vessels in developing zebrafish (Bussmann et al. 2011). Other vasculature development genes included *connexin 39.4* (*cx39.4*), *connective tissue growth factor b* (*ctgfb*), *c-fos induced growth factor* (*figf,* previously *vegf-d*) and *TCDD-inducible poly(ADP-ribose) polymerase* (*tiparp*) (Table 4). *atp2a2a* (reduced expression) is not annotated as a vascular development gene, but is required for heart looping in zebrafish (Ebert et al. 2005). Together these transcriptional changes convey disruption of vascular development and circulatory system function. This is in agreement with blood pressure misregulation and endothelial dysfunction in rats developmentally exposed to benzo(a)pyrene, another PAH known to induce AHR signaling (Jules et al. 2012).

RELA is a significant transcription factor in the BAA regulatory network

Transcription factor analysis at 48 hpf predicted involvement of multiple transcription factors (Table S5). AHR was interestingly no longer significant, though its dimerization partner ARNT was predicted to regulate a significantly enriched cluster of genes. SP1, TP53, CREB1 and RELA were upstream of the largest number of genes misregulated by BAA at 48 hpf (Table S5). RELA interacts directly with AHR and is an important regulator of inflammatory immune and oxidative stress responses (Tian et al. 1999). The RELA and AHR regulatory networks are displayed in Figure 5, which shows that the AHR regulates a set of genes that were distinct to the BAA exposures and were not predicted to be under direct regulation by RELA. The large number of genes downstream of RELA, however, which includes some of the most highly misexpressed genes such as *CTGF* and *CTSL.1*, suggests RELA may play an important role in the BAA toxicity pathway (Figure 5).

Differential affinities for the AHR result in few transcripts common to PAH exposure

The few genes similarly misregulated by all three PAHs represent potential general biomarkers of PAH exposure. At 24 hpf, only 5 transcripts were similarly affected by all 3 PAHs. The most highly elevated probes (approximately 3-fold for all PAHs), A_15_P477220 and A_15_P247256, both target ESTs that are not yet annotated in the zebrafish V9 genome. Decreased transcripts at 24 hpf included *slco5a1*, a solute carrier organic transporter family member, and a non-specific probe. By 48 hpf, 23 transcripts were similarly expressed in response to all three PAHs. The most elevated genes across all three PAHs were *cyp1a*,

cyp1b1, *wfikkn1*, *LOC794658* (similar to chrm3), *s100z* and *cxcr4a*. The largest decreases were observed in *g0s2*, *kif20a*, *cfdl*, and two uncharacterized genes, *zgc:171318* and *zgc:153311*. Though the molecular toxicity pathways of BAA and DBT/PYR are, on the whole, very different, these genes highlight some commonalities.

The most commonly used biomarker of AHR activation, *cyp1a*, was elevated by all three PAHs at 48 hpf. However, DBT and PYR only induced 1.2 and 2.1 fold changes, respectively, whereas BAA induced *cyp1a* 34.5-fold. The minimal and delayed *cyp1a* induction suggests that it occurs via metabolites or very weak AHR activation by DBT and PYR. Barron and colleagues reported the potency of BAA as an AHR agonist as 519 times greater than PYR, and though DBT was not analyzed, 3-ring PAHs included in the study were less potent than PYR or were inactive in assay systems (Barron et al. 2004). Though Cyp1a protein expression is induced by PYR exposure in zebrafish embryos, Incardona et al. reported a markedly different expression pattern than was observed with BAA, and suggested Cyp1a metabolism and hepatic toxicity were drivers of the developmental effects (Incardona et al. 2006). DBT, in contrast, has been reported to induce developmental toxicity via disruption of early cardiac function, as well as act as a Cyp1a inhibitor (Incardona et al. 2004; Wassenberg et al. 2005). In light of these proposed different mechanisms of action, the overlap of transcripts misregulated by DBT and PYR at both 24 and 48 hpf in this study is striking. Indeed, the different malformations observed in DBT and PYR-treated embryos, despite similar molecular response profiles, may be a result of metabolic processes that are more active after the 48 hpf time point employed in this study. The marked effects of DBT exposure on axis formation, which were not observed in response to PYR, could also be explained by the dramatic differences in PAH body burden at these equivalent exposure concentrations. Signaling that directs axis formation occurs early in development; the uptake of DBT was relatively rapid, whereas the lower solubility and uptake of PYR potentially did not achieve a threshold concentration to induce such effects.

Disruption of ion transport and calcium signaling is common across all PAHs

Ion transport and homeostatic processes were misregulated by all three PAHs in this study, though the significant transcripts in the DBT-PYR response are largely different from those affected by BAA. All three exposures induced differential expression of genes involved in

calcium homeostasis, suggesting that calcium signaling plays a role in PAH-induced developmental toxicity, as has been shown previously in dioxin-exposed zebrafish at 48 hpf (Alexeyenko et al. 2010). In a separate study of TCDD effects on heart development, transcriptional changes related to calcium homeostasis preceded the development of cardiac malformations in zebrafish, suggesting that they may be causal for malformations rather than simply a result of reduced blood flow (Carney et al. 2006). Though early calcium influx is a well-known response to several AHR ligands, the dependence of this response on AHR binding and the consequence within the developmental context are unknown.

PAHs have previously been shown to increase intracellular calcium through protein tyrosine kinases, inhibiting SERCA activity, and activating RYR receptors, though the intensity and duration of the response is dependent on PAH and cell type (Archuleta et al. 1993; Krieger et al. 1995; Gao et al. 2005). All three PAHs in our study increased transcription of calcium binding protein *s100z*. The S100 family of EF-hand calcium binding proteins regulates a diverse range of cellular functions in a calcium-dependent manner, and is associated with many pathological conditions including inflammation, atherosclerosis, diabetes, and neurodegeneration (Hermann et al. 2012). Future investigation of the dependence of these transcriptional changes on AHR signaling will provide insight into whether they represent a common mechanism, or are induced via different molecular responses to the PAHs in our study

Differential AHR activation results in distinct RELA regulatory responses to PAH exposures

RELA was a predicted transcriptional regulator of both the BAA and DBT-PYR toxicological responses. Despite this, there was little overlap in the transcriptional networks (Figure 5). Differential AHR activation can explain the AHR gene battery that was uniquely induced by BAA at 24 hpf. However, a large portion of the RELA network expressed in response to DBT and PYR was not affected by BAA exposure (Figure 5). This difference could potentially be explained by dose. BAA is the least soluble in water, and body burden was an order of magnitude lower that the other PAHs. We therefore cannot exclude the possibility that BAA would activate the DBT-PYR transcriptional network at an equivalent internal concentration. Body burdens of DBT and PYR-exposed embryos are within the range reported to induce toxicity through mechanisms classified under “nonpolar narcosis”, such

as interference with lipid fluidity and membrane function (Vanwezel and Opperhuizen 1995). The general pattern of narcotic response, including lost sense of balance, response to stimuli, and reduced ventilation frequency, is not applicable to the early developmental stages of embryos analyzed in this study. However, DBT concentrations in embryos averaged 3.4 μmol/g at 24 hpf, and PYR reached 2.9 μmol/g by 48 hpf following exposure to 25 μM waterborne concentrations. Toxicity from nonpolar narcosis has been reported to occur at 2-8 μmol/g body weight, depending on the compound and organism (Vanwezel and Opperhuizen 1995). The data presented here characterize the extensive molecular response to these relatively high internal concentrations, and our analysis identified RELA as a significant mediator in the DBT-PYR transcriptional network. Interestingly, though BAA toxicity was induced by a lower body burden concentration of 0.12 μmol/g at 48 hpf, network analysis also identified RELA as a significant regulator of BAA-induced transcriptional changes. This suggests that RELA involvement in PAH toxicity is modulated by both AHR activation and PAH concentration. Future studies with multiple PAHs would be useful for identifying whether transcriptional networks identified here are differentially induced by diverse PAH structures.

Transcriptional responses to PAH exposures are conserved across species

We compared the profiles of genes differentially regulated by three PAHs in the developing zebrafish embryo and identified disrupted biological processes that overlap notably with studies in other model systems. The genes differentially regulated by BAA were consistent with previous reports of AHR activation by this PAH, and many of them were identified in array studies with other known AHR ligands in zebrafish. All three PAHs misregulated genes important in vasculature development and cardiac function. This has been observed in BaP-exposed rats, as well as in previous studies of fish exposed to a number of PAHs (Incardona et al. 2009; Incardona et al. 2011; Huang et al. 2012; Jules et al. 2012). Oxidative stress was a component of the toxic response, as has also been reported previously, and we observed differential regulation of immune-related genes, particularly by DBT and PYR. Though fewer studies have examined PAHs that are not strong AHR agonists, PAHs that do not induce CYP1A have similarly been observed to induce inflammatory cytokines in cells in culture (Suresh et al. 2009; Ovrevik et al. 2010). PAHs are known immunotoxicants in fish, with well-established effects on lymphocytes (Krieger et al. 1994; Reynaud and Deschaux

2006). The gene expression changes observed in this study, however, primarily represent innate immune responses, as the adaptive immune system is not mature until weeks 4-6 of development (Meeker and Trede 2008). We therefore would not expect to see substantial overlap between the genes observed in this study and others conducted with tissues from adult organisms. Nevertheless, calcium binding and immune response were identified as important differentially expressed gene clusters in human macrophage leukemia cells exposed to diverse PAHs in vitro, and metal ion binding and transport were the most significant biological processes associated with occupational PAH exposure in peripheral blood of coke-oven workers (Wan et al. 2008; Wu et al. 2011). Chronic PAH exposure in coke-oven workers has also been associated with altered immunological parameters, including increased TNFα in serum, as well increased markers of lipid peroxidation and oxidative stress (Jeng et al. 2011). Increased malondialdehyde and decreased reduced glutathione were similarly observed in bronchial asthma patients, and correlated with blood phenanthrene levels, providing further evidence of PAH-induced oxidative stress in human populations (Suresh et al. 2009).

We identified multiple potential biomarkers of individual PAHs over time, as well as genes commonly misregulated by PAHs with differential AHR affinity. Many of the significant biological processes disrupted in this study, such as ion homeostasis, have been observed previously in other models, and provide insight into fundamental molecular pathways that are sensitive to PAH exposure and conserved between organ systems and species. Further investigation of these pathways in response to more structurally diverse PAHs in the environment will be invaluable to understanding the hazard potential of PAH exposure during development.

Acknowledgements

We are grateful to Cari Buchner, Carrie Barton and the staff at the Sinnhuber Aquatic Research Laboratory for fish husbandry expertise. Bradley Stewart processed the microarrays at the University of Wisconsin McArdle Laboratory of Cancer Research Microarray Facility. We would also like to thank Tanguay laboratory members, in particular Jane La Du, Michael Simonich and Sean Bugel, for their feedback and critical review of the manuscript. This work was supported by the NIEHS Superfund Research Program P42 ES016465, Core Center Grant P30 ES000210 and the NIEHS Training Grant T32 ES007060.

Pacific Northwest National Laboratory is a multi-program national laboratory operated by Battelle Memorial Institute for the DOE under contract number DE-AC05-76RLO1830.

References

Alexeyenko, A., D. M. Wassenberg, et al. (2010). "Dynamic zebrafish interactome reveals transcriptional mechanisms of dioxin toxicity." PLoS One **5**(5): e10465.

Andreasen, E. A., J. M. Spitsbergen, et al. (2002). "Tissue-specific expression of AHR2, ARNT2, and CYP1A in zebrafish embryos and larvae: effects of developmental stage and 2,3,7,8-tetrachlorodibenzo-p-dioxin exposure." Toxicol Sci **68**(2): 403-419.

Archuleta, M. M., G. L. Schieven, et al. (1993). "7,12-Dimethylbenz[a]anthracene activates protein-tyrosine kinases Fyn and Lck in the HPB-ALL human T-cell line and increases tyrosine phosphorylation of phospholipase C-gamma 1, formation of inositol 1,4,5-trisphosphate, and mobilization of intracellular calcium." Proc Natl Acad Sci U S A **90**(13): 6105-6109.

Barron, M. G., R. Heintz, et al. (2004). "Relative potency of PAHs and heterocycles as aryl hydrocarbon receptor agonists in fish." Mar Environ Res **58**(2-5): 95-100.

Benjamini, Y. and Y. Hochberg (1995). "Controlling the False Discovery Rate - a Practical and Powerful Approach to Multiple Testing." Journal of the Royal Statistical Society Series B-Methodological **57**(1): 289-300.

Binder, R. L. and J. J. Stegeman (1984). "Microsomal electron transport and xenobiotic monooxygenase activities during the embryonic period of development in the killifish, Fundulus heteroclitus." Toxicol Appl Pharmacol **73**(3): 432-443.

Bostrom, C. E., P. Gerde, et al. (2002). "Cancer risk assessment, indicators, and guidelines for polycyclic aromatic hydrocarbons in the ambient air." Environ Health Perspect **110 Suppl 3**: 451-488.

Brasier, A. R., M. Jamaluddin, et al. (2000). "Angiotensin II induces gene transcription through cell-type-dependent effects on the nuclear factor-kappaB (NF-kappaB) transcription factor." Mol Cell Biochem **212**(1-2): 155-169.

Brasier, A. R. and J. Li (1996). "Mechanisms for inducible control of angiotensinogen gene transcription." Hypertension **27**(3 Pt 2): 465-475.

Burstyn, I., H. Kromhout, et al. (2005). "Polycyclic aromatic hydrocarbons and fatal ischemic heart disease." Epidemiology **16**(6): 744-750.

Bussmann, J., S. A. Wolfe, et al. (2011). "Arterial-venous network formation during brain vascularization involves hemodynamic regulation of chemokine signaling." Development **138**(9): 1717-1726.

Carney, S. A., J. Chen, et al. (2006). "Aryl hydrocarbon receptor activation produces heart-specific transcriptional and toxic responses in developing zebrafish." Mol Pharmacol **70**(2): 549-561.

Choi, H., W. Jedrychowski, et al. (2006). "International studies of prenatal exposure to polycyclic aromatic hydrocarbons and fetal growth." Environ Health Perspect **114**(11): 1744-1750.

Ciganek, M., J. Neca, et al. (2004). "A combined chemical and bioassay analysis of traffic-emitted polycyclic aromatic hydrocarbons." Sci Total Environ **334-335**: 141-148.

Collins, J. F., J. P. Brown, et al. (1998). "Potency equivalency factors for some polycyclic aromatic hydrocarbons and polycyclic aromatic hydrocarbon derivatives." Regul Toxicol Pharmacol **28**(1): 45-54.

Detmar, J., M. Y. Rennie, et al. (2008). "Fetal growth restriction triggered by polycyclic aromatic hydrocarbons is associated with altered placental vasculature and AhR-dependent changes in cell death." Am J Physiol Endocrinol Metab **295**(2): E519-530.

Durant, J. L., W. F. Busby, Jr., et al. (1996). "Human cell mutagenicity of oxygenated, nitrated and unsubstituted polycyclic aromatic hydrocarbons associated with urban aerosols." Mutat Res **371**(3-4): 123-157.

Ebert, A. M., G. L. Hume, et al. (2005). "Calcium extrusion is critical for cardiac morphogenesis and rhythm in embryonic zebrafish hearts." Proc Natl Acad Sci U S A **102**(49): 17705-17710.

EPA, U. (2012). "Integrated Risk Information System." from http://www.epa.gov/iris/.

Gao, J., A. A. Voss, et al. (2005). "Ryanodine receptor-mediated rapid increase in intracellular calcium induced by 7,8-benzo(a)pyrene quinone in human and murine leukocytes." Toxicol Sci **87**(2): 419-426.

Guengerich, F. P. (2000). "Metabolism of chemical carcinogens." Carcinogenesis **21**(3): 345-351.

Hansch, C., Albert, L., Hoekman, D. (1995). Exploring QSAR: Volume 2: Hydrophobic, Electronic, and Steric Constants, American Chemical Society.

Hecht, S. S., S. G. Carmella, et al. (2010). "Analysis of phenanthrene and benzo[a]pyrene tetraol enantiomers in human urine: relevance to the bay region diol epoxide hypothesis of benzo[a]pyrene carcinogenesis and to biomarker studies." Chem Res Toxicol **23**(5): 900-908.

Hermann, A., R. Donato, et al. (2012). "S100 calcium binding proteins and ion channels." Front Pharmacol **3**: 67.

Hertz-Picciotto, I., H. Y. Park, et al. (2008). "Prenatal exposures to persistent and non-persistent organic compounds and effects on immune system development." Basic Clin Pharmacol Toxicol **102**(2): 146-154.

Huang da, W., B. T. Sherman, et al. (2009). "Systematic and integrative analysis of large gene lists using DAVID bioinformatics resources." Nat Protoc **4**(1): 44-57.

Huang, L., C. Wang, et al. (2012). "Benzo[a]pyrene exposure influences the cardiac development and the expression of cardiovascular relative genes in zebrafish (Daniorerio) embryos." Chemosphere **87**(4): 369-375.

Incardona, J. P., M. G. Carls, et al. (2009). "Cardiac arrhythmia is the primary response of embryonic Pacific herring (Clupea pallasi) exposed to crude oil during weathering." Environ Sci Technol **43**(1): 201-207.

Incardona, J. P., M. G. Carls, et al. (2005). "Aryl hydrocarbon receptor-independent toxicity of weathered crude oil during fish development." Environ Health Perspect **113**(12): 1755-1762.

Incardona, J. P., T. K. Collier, et al. (2004). "Defects in cardiac function precede morphological abnormalities in fish embryos exposed to polycyclic aromatic hydrocarbons." Toxicol Appl Pharmacol **196**(2): 191-205.

Incardona, J. P., H. L. Day, et al. (2006). "Developmental toxicity of 4-ring polycyclic aromatic hydrocarbons in zebrafish is differentially dependent on AH receptor isoforms and hepatic cytochrome P4501A metabolism." Toxicol Appl Pharmacol **217**(3): 308-321.

Incardona, J. P., T. L. Linbo, et al. (2011). "Cardiac toxicity of 5-ring polycyclic aromatic hydrocarbons is differentially dependent on the aryl hydrocarbon receptor 2 isoform during zebrafish development." Toxicol Appl Pharmacol **257**(2): 242-249.

Isidoro Tavares, N., P. Philip-Couderc, et al. (2009). "Angiotensin II and tumour necrosis factor alpha as mediators of ATP-dependent potassium channel remodelling in post-infarction heart failure." Cardiovasc Res **83**(4): 726-736.

Jeng, H. A., C. H. Pan, et al. (2011). "Polycyclic aromatic hydrocarbon-induced oxidative stress and lipid peroxidation in relation to immunological alteration." Occup Environ Med **68**(9): 653-658.

Jules, G. E., S. Pratap, et al. (2012). "In utero exposure to benzo(a)pyrene predisposes offspring to cardiovascular dysfunction in later-life." Toxicology.

Jung, K. H., J. H. Noh, et al. (2011). "Molecular signature for early detection and prediction of polycyclic aromatic hydrocarbons in peripheral blood." Environ Sci Technol **45**(1): 300-306.

Kimmel, C. B., W. W. Ballard, et al. (1995). "Stages of embryonic development of the zebrafish." Dev Dyn **203**(3): 253-310.

Kluver, N., L. Yang, et al. (2011). "Transcriptional response of zebrafish embryos exposed to neurotoxic compounds reveals a muscle activity dependent hspb11 expression." PLoS One **6**(12): e29063.

Kojic, S., D. Radojkovic, et al. (2011). "Muscle ankyrin repeat proteins: their role in striated muscle function in health and disease." Crit Rev Clin Lab Sci **48**(5-6): 269-294.

Krieger, J. A., J. L. Born, et al. (1994). "Persistence of calcium elevation in the HPB-ALL human T cell line correlates with immunosuppressive properties of polycyclic aromatic hydrocarbons." Toxicol Appl Pharmacol **127**(2): 268-274.

Krieger, J. A., D. R. Davila, et al. (1995). "Inhibition of sarcoplasmic/endoplasmic reticulum calcium ATPases (SERCA) by polycyclic aromatic hydrocarbons in HPB-ALL human T cells and other tissues." Toxicol Appl Pharmacol **133**(1): 102-108.

Le Mevel, J. C., F. Lancien, et al. (2008). "Central cardiovascular actions of angiotensin II in trout." Gen Comp Endocrinol **157**(1): 27-34.

Lee, M. S., S. Magari, et al. (2011). "Cardiac autonomic dysfunction from occupational exposure to polycyclic aromatic hydrocarbons." Occup Environ Med **68**(7): 474-478.

Mandrell, D., L. Truong, et al. (2012). "Automated zebrafish chorion removal and single embryo placement: optimizing throughput of zebrafish developmental toxicity screens." J Lab Autom **17**(1): 66-74.

Mao, C., L. Shi, et al. (2009). "Development of fetal brain renin-angiotensin system and hypertension programmed in fetal origins." Prog Neurobiol **87**(4): 252-263.

Mathew, R., J. A. McGrath, et al. (2008). "Modeling polycyclic aromatic hydrocarbon bioaccumulation and metabolism in time-variable early life-stage exposures." Environ Toxicol Chem **27**(7): 1515-1525.

Mbewe-Campbell, N., Z. Wei, et al. (2012). "Genes and environment: novel, functional polymorphism in the human cathepsin L (CTSL1) promoter disrupts a xenobiotic response element (XRE) to alter transcription and blood pressure." J Hypertens.

Meeker, N. D. and N. S. Trede (2008). "Immunology and zebrafish: spawning new models of human disease." Dev Comp Immunol **32**(7): 745-757.

Menzie, C. A., B. B. Potocki, et al. (1992). "Exposure to Carcinogenic Pahs in the Environment." Environmental Science & Technology **26**(7): 1278-1284.

Morey, J. S., J. C. Ryan, et al. (2006). "Microarray validation: factors influencing correlation between oligonucleotide microarrays and real-time PCR." Biol Proced Online **8**: 175-193.

Naumova, Y. Y., S. J. Eisenreich, et al. (2002). "Polycyclic aromatic hydrocarbons in the indoor and outdoor air of three cities in the U.S." Environ Sci Technol **36**(12): 2552-2559.

Nebert, D. W., T. P. Dalton, et al. (2004). "Role of aryl hydrocarbon receptor-mediated induction of the CYP1 enzymes in environmental toxicity and cancer." J Biol Chem **279**(23): 23847-23850.

Nebert, D. W., A. L. Roe, et al. (2000). "Role of the aromatic hydrocarbon receptor and [Ah] gene battery in the oxidative stress response, cell cycle control, and apoptosis." Biochem Pharmacol **59**(1): 65-85.

Nikolsky, Y., E. Kirillov, et al. (2009). "Functional analysis of OMICs data and small molecule compounds in an integrated "knowledge-based" platform." Methods Mol Biol **563**: 177-196.

Ovrevik, J., V. M. Arlt, et al. (2010). "Differential effects of nitro-PAHs and amino-PAHs on cytokine and chemokine responses in human bronchial epithelial BEAS-2B cells." Toxicol Appl Pharmacol **242**(3): 270-280.

Park, K. and A. L. Scott (2010). "Cholesterol 25-hydroxylase production by dendritic cells and macrophages is regulated by type I interferons." J Leukoc Biol **88**(6): 1081-1087.

Petersen, G. I. and P. Kristensen (1998). "Bioaccumulation of lipophilic substances in fish early life stages." Environmental Toxicology and Chemistry **17**(7): 1385-1395.

Pfaffl, M. W. (2001). "A new mathematical model for relative quantification in real-time RT-PCR." Nucleic Acids Res **29**(9): e45.

Planchart, A. and C. J. Mattingly (2010). "2,3,7,8-Tetrachlorodibenzo-p-dioxin upregulates FoxQ1b in zebrafish jaw primordium." Chem Res Toxicol **23**(3): 480-487.

Polidori, A., J. Kwon, et al. (2010). "Source proximity and residential outdoor concentrations of PM(2.5), OC, EC, and PAHs." J Expo Sci Environ Epidemiol **20**(5): 457-468.

Price, R. L., W. Carver, et al. (1997). "The effects of angiotensin II and specific angiotensin receptor blockers on embryonic cardiac development and looping patterns." Dev Biol **192**(2): 572-584.

Ramesh, A., S. A. Walker, et al. (2004). "Bioavailability and risk assessment of orally ingested polycyclic aromatic hydrocarbons." Int J Toxicol **23**(5): 301-333.

Ramirez, N., A. Cuadras, et al. (2011). "Risk assessment related to atmospheric polycyclic aromatic hydrocarbons in gas and particle phases near industrial sites." Environ Health Perspect **119**(8): 1110-1116.

Reimers, M. J., J. K. La Du, et al. (2006). "Ethanol-dependent toxicity in zebrafish is partially attenuated by antioxidants." Neurotoxicol Teratol **28**(4): 497-508.

Ren, A., X. Qiu, et al. (2011). "Association of selected persistent organic pollutants in the placenta with the risk of neural tube defects." Proc Natl Acad Sci U S A **108**(31): 12770-12775.

Rennie, M. Y., J. Detmar, et al. (2011). "Vessel tortuousity and reduced vascularization in the fetoplacental arterial tree after maternal exposure to polycyclic aromatic hydrocarbons." Am J Physiol Heart Circ Physiol **300**(2): H675-684.

Reynaud, S. and P. Deschaux (2006). "The effects of polycyclic aromatic hydrocarbons on the immune system of fish: a review." Aquat Toxicol **77**(2): 229-238.

Saeed, A. I., V. Sharov, et al. (2003). "TM4: a free, open-source system for microarray data management and analysis." Biotechniques **34**(2): 374-378.

Shannon, P., A. Markiel, et al. (2003). "Cytoscape: a software environment for integrated models of biomolecular interaction networks." Genome Res **13**(11): 2498-2504.
Shi, Z., N. Dragin, et al. (2010). "Organ-specific roles of CYP1A1 during detoxication of dietary benzo[a]pyrene." Mol Pharmacol **78**(1): 46-57.
Suresh, R., A. Shally, et al. (2009). "Assessment of association of exposure to polycyclic aromatic hydrocarbons with bronchial asthma and oxidative stress in children: A case control study." Indian J Occup Environ Med **13**(1): 33-37.
Tian, B., D. E. Nowak, et al. (2005). "A TNF-induced gene expression program under oscillatory NF-kappaB control." BMC Genomics **6**: 137.
Tian, Y., S. Ke, et al. (1999). "Ah receptor and NF-kappaB interactions, a potential mechanism for dioxin toxicity." J Biol Chem **274**(1): 510-515.
Tilton, S. C., T. L. Tal, et al. (2012). "Bioinformatics resource manager v2.3: an integrated software environment for systems biology with microRNA and cross-species analysis tools." BMC Bioinformatics **13**(1): 311.
Truong, L., S. L. Harper, et al. (2011). "Evaluation of embryotoxicity using the zebrafish model." Methods Mol Biol **691**: 271-279.
Van Metre, P. C. and B. J. Mahler (2005). "Trends in hydrophobic organic contaminants in urban and reference lake sediments across the United States, 1970-2001." Environ Sci Technol **39**(15): 5567-5574.
Van Metre, P. C. and B. J. Mahler (2010). "Contribution of PAHs from coal-tar pavement sealcoat and other sources to 40 U.S. lakes." Sci Total Environ **409**(2): 334-344.
Vanwezel, A. P. and A. Opperhuizen (1995). "Narcosis Due to Environmental-Pollutants in Aquatic Organisms - Residue-Based Toxicity, Mechanisms, and Membrane Burdens." Critical Reviews in Toxicology **25**(3): 255-279.
Vlecken, D. H., J. Testerink, et al. (2009). "A critical role for myoglobin in zebrafish development." Int J Dev Biol **53**(4): 517-524.
Wan, B., J. W. Yarbrough, et al. (2008). "Structure-related clustering of gene expression fingerprints of thp-1 cells exposed to smaller polycyclic aromatic hydrocarbons." SAR QSAR Environ Res **19**(3-4): 351-373.
Wassenberg, D. M., A. L. Nerlinger, et al. (2005). "Effects of the polycyclic aromatic hydrocarbon heterocycles, carbazole and dibenzothiophene, on in vivo and in vitro CYP1A activity and polycyclic aromatic hydrocarbon-derived embryonic deformities." Environ Toxicol Chem **24**(10): 2526-2532.
Wiens, G. D. and G. W. Glenney (2011). "Origin and evolution of TNF and TNF receptor superfamilies." Dev Comp Immunol **35**(12): 1324-1335.
Wu, C., H. Lu, et al. (2011). "Molecular and Pathophysiological Features of Angiotensinogen: A Mini Review." N Am J Med Sci (Boston) **4**(4): 183-190.
Wu, M. T., T. C. Lee, et al. (2011). "Whole genome expression in peripheral-blood samples of workers professionally exposed to polycyclic aromatic hydrocarbons." Chem Res Toxicol **24**(10): 1636-1643.
Xiong, K. M., R. E. Peterson, et al. (2008). "Aryl hydrocarbon receptor-mediated down-regulation of sox9b causes jaw malformation in zebrafish embryos." Mol Pharmacol **74**(6): 1544-1553.

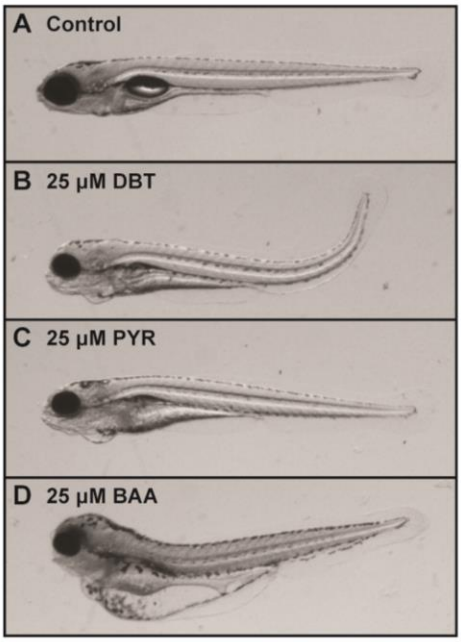


Figure 2-1 PAHs induce abnormalities in developing zebrafish

Representative images of 120 hpf larvae after exposure to (A) 1% DMSO control, (B) 25 µM DBT, (C) 25 µM PYR, and (D) 25 µM BAA from 6 to 48 hpf.

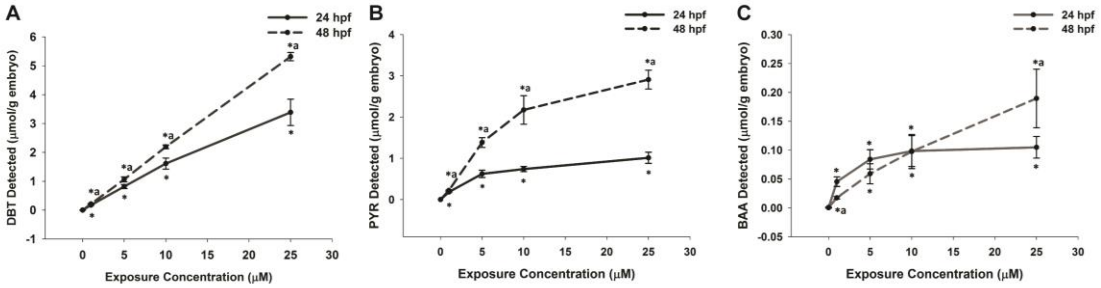


Figure 2-2 Body burdens of PAH detected in embryos

PAH was detected in embryos exposed to (A) DBT, (B) PYR, and (C) BAA from 6 to 24 (solid lines) or 48 (dashed lines) hpf. *Significantly different than time-matched DMSO control (Mann-Whitney rank sum test, $p < 0.05$). [a]Significant difference between 48 and 24 hpf samples at the same exposure concentration (Mann-Whitney rank sum test, $p < 0.05$).

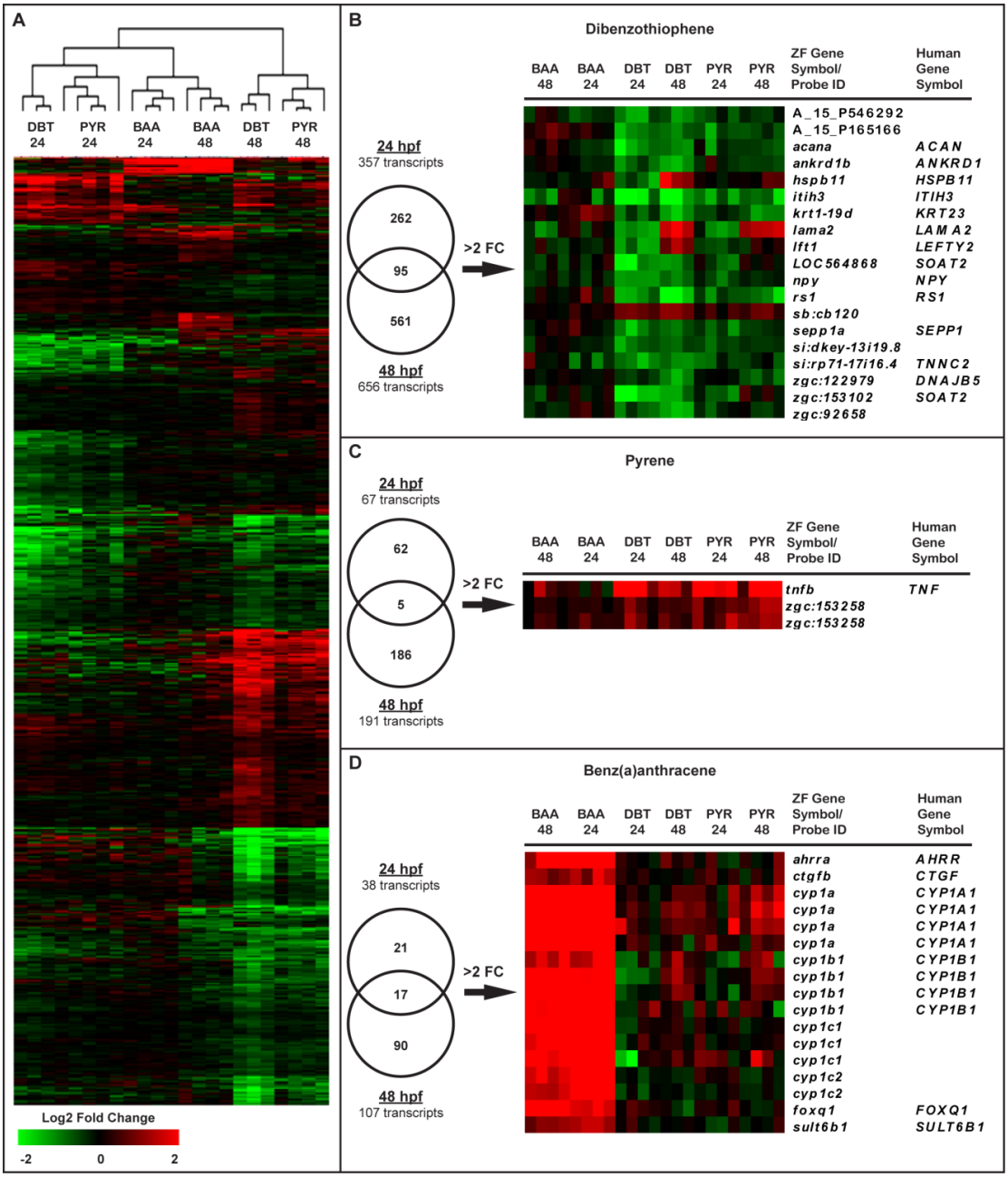


Figure 2-3 Differentially expressed transcripts in PAH-exposed embryos

A) Bidirectional hierarchical clustering heatmap of log2 fold change (FC) values of all 1079 genes significantly differentially expressed compared to control (One-way ANOVA with 5% FDR, adjusted p value < 0.05). Comparison of significant genes between 24 and 48 hpf for (B) DBT, (C) PYR and (D) BAA exposure groups is shown by Venn diagram. Heatmap enlargements show transcripts differentially expressed (adjusted p value < 0.05, >2-FC) at both time points for each PAH.

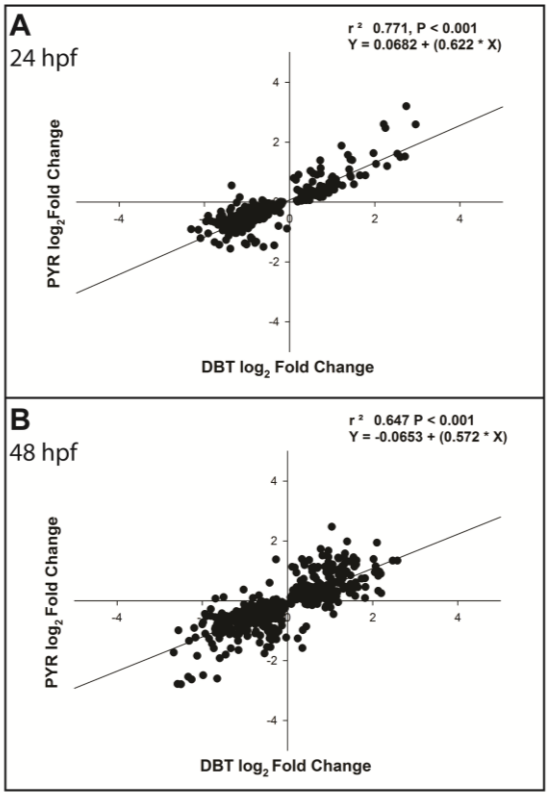


Figure 2-4 Direct comparison of PYR and DBT expression values

Comparison of gene expression between PYR and DBT treatment groups at 24 and 48 hpf. Linear regressions of log2 FC values for the union of transcripts significantly ($p<0.05$) misregulated by DBT or PYR compared to control ($n = 712$). Linear associations at (A) 24 hpf and (B) 48 hpf were both significant ($p < 0.001$).

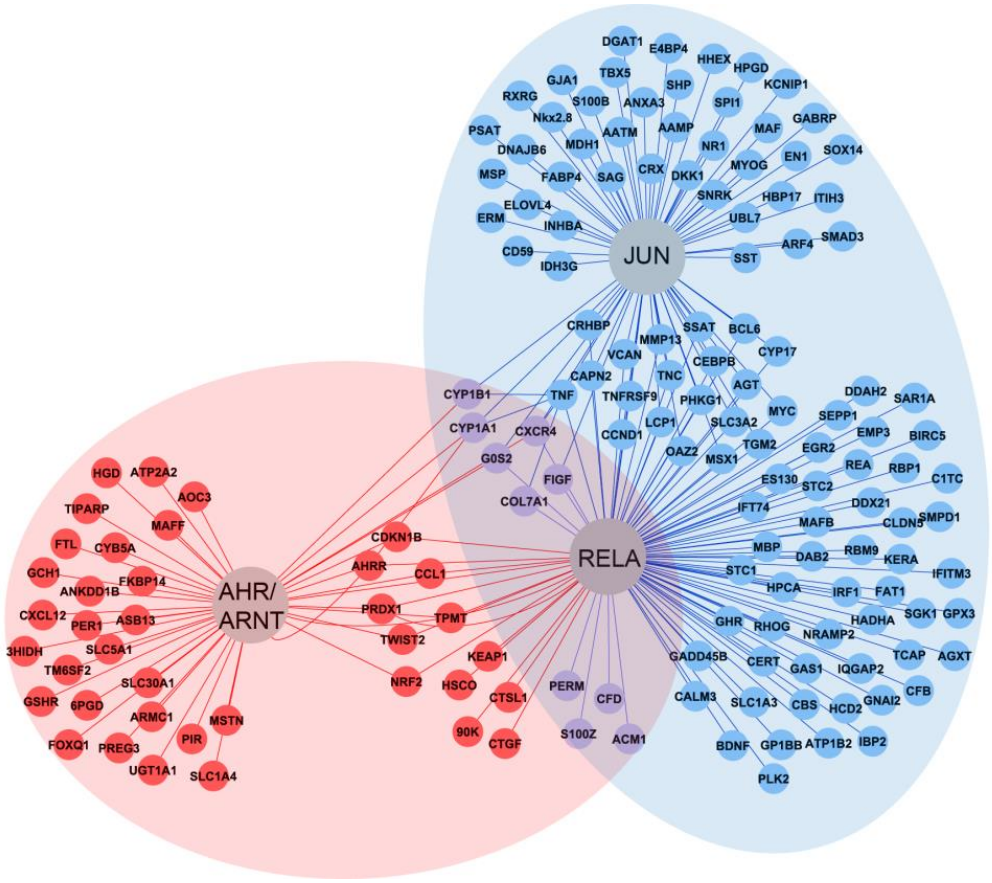


Figure 2-5 Comparison of AHR, RELA and JUN regulatory networks

Networks of transcripts under regulatory control of AHR, RELA and JUN, that were misregulated in response to BAA (red), or DBT/PYR (blue) exposure. Transcripts that overlapped between the two PAH networks are represented in purple. Significant transcripts ($p<0.05$) from both the 24 and 48 hpf time points were combined to create the regulatory network.

Table 2-1 Developmental abnormalities in PAH-exposed embryos

Mean percentage of embryos (95 percentile) with malformations observed at 120 hpf following exposure to 25 μM BAA, DBT, PYR or DMSO control from 6 to 48 hpf. * Significantly different than DMSO Control, $p < 0.05$, one-way ANOVA with Dunnett's post hoc test.

	Effect							
Treatment	**Mortality**	**Axis**	**Eye**	**Jaw**	**Pericardial Edema**	**Snout**	**Yolk Sac**	**Any Malformation**
Control	4 (1, 13)	3.1 (1, 10)	4.2 (1, 16)	7.3 (2, 21)	4.2 (1, 13)	4.2 (1, 15)	4.2 (1, 15)	11 (5, 22)
DBT	3.3 (0, 58)	44.8 (9, 87)*	15.5 (1, 78)	75.9 (21, 97)*	55.2 (12, 92)*	19 (2, 76)*	15.5 (1, 75)	83.3 (39, 97)*
PYR	5 (0, 57)	12.3 (1, 61)	5.3 (0, 72)	98.3 (33, 100)*	68.4 (19, 95)*	22.8 (2, 79)*	36.8 (4, 88)*	98.3 (33, 100)*
BAA	1.7 (0, 67)	8.5 (1, 55)	10.2 (0, 74)	81.4 (25, 98)*	74.6 (23, 97)*	32.2 (4, 85)*	54.2 (9, 94)*	85 (41, 98)*

Table 2-2 Comparison of malformations induced by PAH treatments

P values of Tukey's all pairwise post hoc test are displayed for each comparison, malformations that were significantly different between treatment groups are shaded ($p < 0.05$).

	Effect							
Test	**Mortality**	**Axis**	**Eye**	**Jaw**	**Pericardial Edema**	**Snout**	**Yolk Sac**	**Any Malformation**
DBT v Control	8.53E-01	4.30E-06	1.41E-01	2.70E-06	2.50E-06	4.66E-02	1.01E-01	7.20E-09
PYR v Control	7.09E-01	6.02E-02	6.53E-01	1.50E-04	3.10E-08	2.22E-02	1.87E-03	2.10E-05
BAA v Control	9.47E-01	1.83E-01	3.31E-01	8.20E-07	4.60E-09	3.44E-03	3.10E-05	6.10E-09
DBT v PYR	9.31E-01	1.91E-03	4.84E-01	1.75E-01	4.63E-01	9.27E-01	1.67E-01	1.85E-01
BAA v DBT	8.93E-01	5.10E-04	8.34E-01	8.83E-01	1.80E-01	4.59E-01	8.26E-03	9.80E-01
BAA v PYR	7.23E-01	8.07E-01	7.93E-01	2.58E-01	8.22E-01	6.87E-01	3.78E-01	2.21E-01

Table 2-3 Biological functions affected by PAH exposure at 24 hpf

Significantly enriched biological functions identified by DAVID analysis of all transcripts differentially regulated (adjusted p value < 0.05) by BAA exposure or by DBT and PYR at 24 hpf. E score: overall cluster enrichment score, %: percentage of total gene list involved in functional cluster, p-values determined by modified Fischer's Exact test (EASE score).

	Biological Process	GO Term	Downregulated genes	Upregulated genes	E Score	%	P Value
BAA	hormone metabolic process	GO:0042445		*cyp1a, cyp1b1, cyp1c1, cyp1c2, si:dkey-94e7.2*	2.06	15.79	5.12E-03
	tissue development	GO:0009888	*mstnb*	*foxq1l, ptn, ctgfb*	1.21	21.05	2.77E-02
DBT and PYR	fatty acid biosynthetic process	GO:0006633	*elovl6, fads2, ptgds, si:ch73-131e21.5, tpi1b*	*ch25h, elovl7a*	2.67	3.02	6.10E-04
	ion transport	GO:0006811	*atp2a1l, cpt1b, gabra1, grin1b, KCNAB1, kcnip1b, kcnip3, LOC100004247, rhbg, sfxn4, si:ch211-195b13.1, si:ch211-221p4.4, slc24a5, zgc:101827, zgc:113361, zgc:158296*	*LOC571584, si:ch211-244h7.4, slc22a18, slc31a1, tmem38b, zgc:162356, zgc:162495*	2.32	8.30	7.86E-03
	skeletal muscle contraction	GO:0003009	*homer1, mb, si:rp71-17i16.4, tnni2b.2*		2.18	1.51	1.10E-03
	steroid biosynthetic process	GO:0006694	*cyp17a1, hmgcs1, hsd17b7, lss, nsdhl, rdh8l*	*ch25h, dhcr7*	2.14	3.02	9.43E-04
	oxoacid metabolic process	GO:0043436	*acsf3, cpt1b, elovl6, fabp11b, fads2, ghra, hibadhb, mdh1b, ptgds, rbp1a, rnpep, si:ch73-131e21.5, tpi1b, tyrp1b, zgc:113076, zgc:154046*	*ch25h, elovl7a, mthfd1*	1.93	7.17	1.27E-02
	intermediate filament organization	GO:0045109	*dnajb6b, krt1-19d, krt23, nefm*		1.80	1.13	6.71E-03
	negative regulation of cell proliferation	GO:0008285	*bdnf, cd9a, cx43, smad3b, tnfrsf9a, wfdc1, zgc:114127, zgc:158296*	*agt, msxe, notch2, tbx16, tnfb*	1.77	4.91	1.67E-02
	muscle cell development	GO:0055001	*homer1, LOC796577, myoz1a, zgc:158296*	*myog*	1.71	1.89	1.89E-02
	sterol biosynthetic process	GO:0016126	*hmgcs1, lss, nsdhl*	*ch25h, dhcr7*	1.56	1.89	5.49E-03

Table 2-3 (Continued)

	Biological Process	GO Term	Downregulated genes	Upregulated genes	E Score	%	P Value
DBT and PYR	cellular amide metabolic process	GO:0043603	*ghra, hibadhb, mdh1b, tpi1b, zgc:113076*		1.41	1.89	2.64E-02
	monosaccharide catabolic process	GO:0046365	*hibadhb, mdh1b, pfkma, tpi1b, zgc:162337*		1.28	1.89	3.16E-02
	regulation of erythrocyte differentiation	GO:0045646		*inhbaa, mafba, spi1l*	1.09	1.13	4.65E-02

Table 2-4 Biological functions affected by PAH exposure at 48 hpf

Significantly enriched biological functions identified by DAVID analysis of all transcripts differentially regulated (adjusted p value < 0.05) in BAA exposed embryos, and common transcripts disrupted by DBT and PYR at 48 hpf. E score: overall cluster enrichment score, %: percentage of total gene list involved in functional cluster, p-values determined by modified Fischer's Exact test (EASE score).

	Biological Process	GO Term	Downregulated Genes	Upregulated Genes	E Score	%	P Value
BAA	cellular homeostasis	GO:0019725	*edn2, cxcl12b, atp2a2a*	*slc30a1, cxcr4a, zgc:92066, gsr, prdx1, ccl1, ccr9a*	2.75	14.29	4.50E-04
	chemotaxis	GO:0006935	*cxcl12b, edn2*	*ccr9a, cxcr4a, ccl1*	2.50	7.14	2.18E-03
	hormone met. process	GO:0042445	*lrata*	*ugt1b5, ugt1b7, cyp1b1, cyp1c2, cyp1a, cyp1c1*	2.16	5.71	1.26E-02
	tetrapyrrole met. process	GO:0033013	-	*zgc:77234, ugt1b5, ugt1b7, cyp1a*	1.92	4.29	1.25E-02
	vasculature development	GO:0001944	*cx39.4, cxcl12b*	*ctgfb, cxcr4a,figf, tiparp*	1.89	8.57	1.01E-02
	H_2O_2 met. process	GO:0042743	-	*cyp1a, prdx1*	1.59	4.29	5.65E-03
	cation transport	GO:0006812	*armc1l, atp2a2a, cx39.4, slc5a1*	*cdkn1bl, slc30a1, zgc:92066*	1.32	10.00	3.83E-02
	organ development	GO:0048513	*atp2a2a, col7a1, cx39.4, cxcl12b, edn2, prl, slc5a1*	*cdkn1bl, ctgfb, cxcr4a, cyp1a, figf, foxq1, foxf2a, tiparp, ved*	1.29	21.43	4.12E-02
DBT and PYR	oxoacid metabolic process	GO:0043436	*acaa1, acadl, adipor1a, adipor1b, agxt2l1, agxta, agxtb, amt, cpt1b, elovl4b, glsa, got2a, hadha, hadhb, idh3b, LOC565975, lta4h, mdh1b, padi2, rbp2b, rnpep, sc5dl, sgpl1, zgc:113076, zgc:136850, zgc:154046*	*aspg, cbsb, cyp1a, cyp26c1, gldc, npc1, phgdh, ppat, psat1, slc1a3a*	4.72	8.35	2.66E-05
	embryonic development ending in birth or egg hatching	GO:0009792	*ift52, ric8a, zfpm2b*	*capn2b, cebpb, col4a3bp, cyp1a, eng1b, evx1, foxa, gas1b, gata2a, hoxa2b, hoxa4a, hoxb2a, hoxc1a, hoxc6b, msxe, nkx2.7, pax1a, phgdh, si:ch211-204c21.1, tcap, tgfbr1a*	3.61	5.90	1.01E-04
	regionalization	GO:0003002	*egr2b, ift52, neurod*	*cyp26c1, egr2a, eng1b, evx1, foxa, gas1b, hhex, hoxa2b, hoxa4a, hoxb2a, hoxc4a, hoxc6b, pax1a, tcap, tgfbr1a*	3.38	4.18	2.75E-04

Table 2-4 (Continued)

	Biological Process	GO Term	Downregulated Genes	Upregulated Genes	E Score	%	P Value
DBT and PYR	neurogenesis	GO:0022008	*bdnf, clic5, crx, egr2b, gnao1b, LOC799290, mbp, mbpa, neurod, otx5, rab3aa, rnd1, spon2b, vcanb*	*ascl1a, btg4, cebpb, cxcr4a, egr2a, eng1b, epha2, evx1, foxa, gas1b, gata2a, gdf7, her15.1, hoxa2b, mag, nr2f6b, phgdh, slc1a3a, tgfbr1a, unc5b, uts1*	2.77	7.62	3.27E-03
	embryonic organ development	GO:0048568	*clic5, neurod, zfpm2b*	*cebpb, gas1b, gata2a, hoxa2b, hoxa4a, hoxb2a, hoxc4a, myca, otop1, tcap, tgfbr1a*	2.43	3.44	2.40E-03
	positive regulation of macromolecule metabolic process	GO:0010604	*crx, egr2b, fkbp1ab, ift52, klf2a, LOC570917, maf, neurod, npas4, otx5, psmd4b, psmd7, psmd8, rxrga, tnni2b.2, zfpm2b, zgc:110116*	*ascl1a, cask, cebpb, cebpg, egr2a, evx1, foxa, gata2a, gdf7, her15.1, hhex, hoxa2b, im:7162084, irf11, myca, pth1a, sox19a, tgfbr1a, tnfb, uts1, vgll2b, zgc:158781*	2.35	9.34	2.19E-03
	negative regulation of cell communication	GO:0010648	*gnai2, hcrt, rgs11, zgc:136569, bcl6ab*	*cyp26c1, dkk1b, gas1b, hhex, im:7162084, npc1, onecut1, rgs4*	1.99	3.44	1.01E-02
	cellular component morphogenesis	GO:0032989	*bbs7, bdnf, clic5, cryaa, egr2b, rab3aa, spon2b, vcanb*	*bcl6ab, col4a3bp, cxcr4a, egr2a, gas1b, gdf7, hoxa2b, LOC796577, onecut1, sfrp5, si:ch211-204c21.1, slc1a3a, tcap, unc5b*	1.93	5.16	9.16E-03
	central nervous system development	GO:0007417	*egr2b, faim2, gnao1b, LOC799290, mbpa, mbpa, neurod, sepp1a, sh3gl2*	*ascl1a, cxcr4a, cyp26c1, dkk1b, egr2a, eng1b, evx1, foxa, gas1b, gata2a, hhex, hoxa2b, hoxb2a, msxe, nkx2.7, phgdh*	1.87	5.41	1.27E-02
	hormone met. process	GO:0042445	*lrata, rbp2b*	*crhbp, cyp1a, cyp1b1, cyp26c1, scarb1,*	1.82	1.97	1.51E-02
	organ morphogenesis	GO:0009887	*agc1, bbs7, clic5, crx, cryaa, cryaa, fkbp1ab, ift52, neurod, otx5, sgpl1, six6a*	*cmlc1, gas1b, hhex, hoxa2b, hoxa4a, hoxb2a, hoxc4a, im:7162084, msxe, myca, otop1, tcap, tgfbr1a, tnfb, zgc:158781*	1.79	6.39	1.62E-02
	fatty acid oxidation	GO:0019395	*acaa1, adipor1a, adipor1b, cpt1b, hadha, hadhb, zgc:154046*		1.66	1.47	7.21E-03

Table 2-4 (Continued)

	Biological Process	GO Term	Downregulated Genes	Upregulated Genes	E Score	%	P Value
DBT and PYR	muscle tissue dev.	GO:0060537	*fkbp1ab, LOC100536295, rxrga, zfpm2b*	*cmlc1, LOC796577, nkx2.7, tcap, vgll2b*	1.65	2.21	2.45E-02
	reg. of transmission of nerve impulse	GO:0051969	*bdnf, cspg5b, egr2b, gnai2, hcrt, rab3aa, zgc:136569*	*egr2a, slc1a3a, tnfb, uts1*	1.58	2.46	2.56E-02
	pos. reg. of cellular process	GO:0048522	*bdnf, crx, egr2b, fkbp1ab, gnai2, hcrt, ift52, klf2a, LOC556700, LOC570917, maf, mfge8a, neurod, npas4, otx5, pnp4b, psmd4b, psmd7, psmd8, rxrga, si:ch211-135f11.1, tnni2b.2, trim35, zfpm2b, zgc:100906, zgc:110116, zgc:110680*	*ascl1a, bcl6ab, cask, cebpb, cebpg, cyp1a, egf, egr2a, evx1, flt4, foxa, GAS1 (3 of 3), gas1b, gata2a, gdf7, her15.1, hhex, hoxa2b, im:7162084, irf11, LOC794658, myca, ncs1a, nkx2.7, onecut1, plk2b, pth1a, scarb1, si:dkey-24p1.4, slc1a3a, sox19a, sst1.1, tgfbr1a, tnfb, uts1, vgll2b, zgc:154093, zgc:158781, zgc:85939*	1.57	15.5	2.29E-02
	oxidoreduction coenzyme met. process	GO:0006733	*coq3, idh3b, idh3g, itgb1bp3, mdh1b, pgls, pnp4b, zgc:113076*		1.55	1.72	3.73E-03
	pos. reg. of catalytic activity	GO:0043085	*gadd45bb, gnai2, gnao1b, gng13b, hcrt, psmd4b, psmd7, psmd8, ptplad1, rgn, si:ch211-135f11.1, zgc:110116*	*cmlc1, cxcr4a, egf, LOC794658, myca, npr3, pth1a, scarb1, slc11a2, tgfbr1a, tnfb*	1.52	5.9	2.22E-02
	pyridine nucleotide metabolic process	GO:0019362	*idh3b, itgb1bp3, mdh1b, pgls, pnp4b, zgc:113076*		1.44	1.47	9.14E-03
	cellular amino acid metabolic process	GO:0006520	*agxt2l1, agxta, agxtb, amt, glsa, got2a, padi2*	*aspg, cbsb, gldc, phgdh, ppat, psat1, slc1a3a*	1.43	3.19	2.33E-02
	cell morph. involved in differentiation	GO:0000904	*bdnf, clic5, cryaa, egr2b, rab3aa, spon2b, vcanb*	*cxcr4a, egr2a, gas1b, gdf7, hoxa2b, si:ch211-204c21.1, slc1a3a, unc5b*	1.38	3.44	3.54E-02
	ear development	GO:0043583	*bdnf, clic5*	*gas1b, her15.1, hoxa2b, myca, otop1, tcap*	1.35	1.97	2.53E-02

Table 2-S1 Primer sequences used for qRT-PCR

Ensembl Transcript ID	Zebrafish Gene Symbol	Forward primer	Reverse Primer
ENSDART00000010918	*agt*	TGACGGACACACAGTTTTAC	GTTGCTTCAGGTTGAAATGC
ENSDART00000105896	*atp2a1l*	AGCAGTTCATTCGTTACCTG	AGAACAACCAGCCAGAAATC
ENSDART00000077511	*ccr9a*	GCATGTTGGTATTTGAAGCC	CTGTGTCCGACATAACAGAG
ENSDART00000066439	*ch25h*	ACCACAAATACACATCCACC	TCATTCAAAGTGCAGTGTCC
ENSDART00000017756	*ctsl.1*	GGACTCCTACCCCTATGAAG	ATAACCAACAGCCAGAACAC
ENSDART00000038200	*cyp1a*	TGCCGATTTCATCCCTTTCC	AGAGCCGTGCTGATAGTGTC
ENSDART00000099870	*cyp1b1*	CTGCATTGATTTCCGAGACGTG	CACACTCCGTGTTGACAGC
ENSDART00000019953	*cyp1c1*	AGTGGCACAGTCTACTTTGAGAG	TCGTCCATCAGCACTCAG
ENSDART00000016487	*cyp1c2*	GTGGTGGAGCACAGACTAAG	TTCAGTATGAGCCTCAGTCAAAC
ENSDART00000103784	*edn2*	CCAGGATCAGCTAGAGAGAG	ATTTCACTGGTGTGGAAGAG
ENSDART00000109464	*g0s2*	ATAACCACCGACAAACAAGG	AGCATGTCAAAGTCTGGTTC
ENSDART00000100386	*mstnb*	AAGAGGACGATGAACATGC	GATCGTATTCGGTGTCTTCC
ENSDART00000025669	*slc16a9b*	TCCCTGTCACCAAGAACTAC	TGAAGTAAAACGCCAGATCG
ENSDART00000130131	*sult6b1*	GTGGGTTTAACTGGATGGTG	GAGACCACTGTGTCTTTCG
ENSDART00000017569	*tnfb*	GTCCTACAGCACCATTTACC	ATTCAGTGCACAACTCTCAC

Table 2-S2 Percent recovery by GC-MS for PAH body burden studies

Percent recovery GC-MS method of PAH detection in zebrafish embryos, calculated from laboratory control samples spiked with BAA, DBT or PYR in DMSO.

Spike (nmoles)	Average Detected (nmoles)	Percent Recovery	Standard Deviation
BAA 24 hpf			
5	4.5	89.1	3.7
25	22.1	88.6	5.6
50	47.2	94.4	3.8
125	118.6	94.8	4.9
DBT 24 hpf			
5	5.1	101.4	2.5
25	25.0	100.1	1.7
50	49.9	99.9	3.8
125	134.5	107.6	2.6
PYR 24 hpf			
5	5.5	109.7	4.0
25	29.1	116.4	10.3
50	61.6	123.1	5.2
125	156.7	125.4	10.6
BAA 48 hpf			
5	4.6	91.1	1.1
25	20.1	80.3	1.5
50	44.1	88.2	3.0
125	111.3	89.0	2.5
DBT 48 hpf			
5	5.3	105.7	3.1
25	26.0	103.8	3.3
50	51.2	102.4	4.7
125	136.17	108.94	2.94
PYR 48 hpf			
5	5.1	102.3	6.1
25	25.8	103.4	3.7
50	56.0	111.9	4.2
125	144.7	115.8	5.2

Table 2-S4 Gene expression values detected by microarray and qRT-PCR

Mean log2 fold change and B-H adjusted p-values of differentially regulated transcripts from the microarray, compared with log2 fold change (mean ± SD) detected with qRT-PCR. [a]Significantly different from vehicle control (One-way ANOVA with Tukey's post hoc test and 5% FDR, p< 0.05) [b]Significantly different from vehicle control (One-way ANOVA with Tukey's post hoc test, p< 0.05).

		24 hpf						48 hpf					
Zebrafish Gene Symbol	**Human Gene Symbol**	**DBT *Array* log_2 FC**	**DBT *QPCR* log_2 FC**	**PYR *Array* log_2 FC**	**PYR *QPCR* log_2 FC**	**BAA *Array* log_2 FC**	**BAA *QPCR* log_2 FC**	**DBT *Array* log_2 FC**	**DBT *QPCR* log_2 FC**	**PYR *Array* log_2 FC**	**PYR *QPCR* log_2 FC**	**BAA *Array* log_2 FC**	**BAA *QPCR* log_2 FC**
agt	*AGT*	2.6[a]	2.7 ± 0.7[b]	1.5	2.1 ± 1.3	-0.0	0.1 ± 0.9	---	---	---	---	---	---
atp2a1l	*ATP2A1*	-1.1[a]	-1.8 ± 0.7	-0.6	-0.9 ± 0.8	-0.1	0.1 ± 0.9	---	---	---	---	---	---
ccr9a	*CCR9*	0.04	0.4 ± 1.0	0.2	0.8 ± 0.5	0.7	1.7 ± 0.5	-0.2	0.2 ± 0.5	-0.1	-0.2 ± 0.5	1.8[a]	1.3 ± 0.3[a]
ch25h	*CH25H*	2.2	2.7 ± 1.3[b]	2.6[a]	3.0 ± 1.1[b]	0.1	1.0 ± 0.5	---	---	---	---	---	---
ctsl.1	*CTSL1*	---	---	---	---	---	---	0.5	-0.2 ± 0.4	-0.1	-0.4 ± 0.1	2.1[a]	1.7 ± 0.2[b]
cyp1a	*CYP1A1*	0.6	-0.0 ± 0.5	0.7	0.5 ± 0.5	4.1[a]	4.8 ± 0.4[b]	0.3	0.8 ± 0.2	1.1	1.4 ± 0.5[b]	5.1[a]	6.6 ± 0.3[b]
cyp1b1	*CYP1B1*	0.2	-0.8 ± 0.2	0.3	-0.5 ± 0.5	2.8[a]	2.4 ± 0.3[b]	-0.2	0.6 ± 0.3	0.0	0.1 ± 0.6	2.5[a]	2.6 ± 0.3[b]
cyp1c1		-0.8	-0.3 ± 0.4	0.5	-0.1 ± 0.5	2.5[a]	2.2 ± 0.4[b]	0.2	-0.2 ± 0.2	0.7	-0.2 ± 0.3	4.7[a]	4.1 ± 0.4[b]
cyp1c2		-0.2	-0.3 ± 0.2	0.3	0 ± 0.2	1.7[a]	1.6 ± 0.3[b]	-0.4	-0.2 ± 0.3	-0.2	-0.3 ± 0.3	2.5[a]	3.1 ± 0.3[b]
g0s2	*G0S2*	-1.3[a]	-1.6 ± 0.4[b]	-0.8	-0.5 ± 0.3	-0.2	0.1 ± 0.2	-0.2	-0.4 ± 0.5	-1.0[a]	-0.8 ± 0.4	-1.1[a]	-0.6 ± 0.2
mstnb	*MSTN*	-1.8[a]	-0.7 ± 0.1	-0.6	-0.2 ± 0.3	-1.5[a]	-0.3 ± 0.2	---	---	---	---	---	---
si:ch211-202b2.2	*EDN2*	---	---	---	---	---	---	-0.0	-0.0 ± 0.1	-0.3	0.0 ± 0.3	-0.9[a]	-0.8 ± 0.2[b]
slc16a9b	*SLC16A9*	3.0[a]	3.3 ± 0.3[b]	2.6[a]	2.9 ± 0.4[b]	0.2	0.3 ± 0.8	---	---	---	---	---	---
sult6b1	*SULT6B1*	0.1	0.1 ± 0.1	-0.2	0.2 ± 0.3	1.0[a]	1.0 ± 0.2	0.0	-0.3 ± 0.3	0.0	-0.3 ± 0.2	1.4[a]	1.0 ± 0.2[b]
tnfb	*TNF*	2.7[a]	2.9 ± 1.0[b]	3.2[a]	3.2 ± 0.6[b]	0.6	0.0 ± 0.2	1.0	0.8 ± 0.2	2.5[a]	2.3 ± 0.7[b]	0.1	-0.2 ± 0.2

Table 2-S5 Predicted transcription factors for PAH responses

Transcription factors identified as significantly over-connected (p<0.05) to genes differentially expressed (p<0.05) in response to PAH exposure at 24 and 48 hpf. Significance was calculated by hypergeometric distribution in MetaCore. Actual: Number of genes in the dataset that interact with the transcription factor, Expected: Number of genes in the dataset predicted to interact with the transcription factor based on total number of interactions on the Agilent platform calculated as mean value for hypergeometic distribution, Ratio: Connectivity ratio (Actual/Expected), p-value: Probability to have the given value of Actual or higher (FDR adjusted p-value <0.05)

	24 hpf								48 hpf							
Network Object Name	**BAA Actual**	**BAA Expected**	**BAA Ratio**	**BAA p-value**	**DBT-PYR Actual**	**DBT-PYR Expected**	**DBT-PYR Ratio**	**DBT-PYR p-value**	**BAA Actual**	**BAA Expected**	**BAA Ratio**	**BAA p-value**	**DBT-PYR Actual**	**DBT-PYR Expected**	**DBT-PYR Ratio**	**DBT-PYR p-value**
AHR	4.00	0.51	7.78	1.53E-03												
TFAP2A									10.00	2.65	3.77	2.81E-04				
ARNT	4.00	0.13	30.03	8.38E-06					5.00	0.69	7.29	6.08E-04				
CEBPD	3.00	0.19	15.68	8.74E-04					6.00	0.95	6.31	3.66E-04				
JUN					19.00	9.07	2.09	1.95E-03					25.00	13.87	1.80	3.29E-03
CREB1									14.00	5.14	2.73	4.69E-04	55.00	30.53	1.80	1.31E-05
CUX1									6.00	0.60	9.97	2.95E-05				
EGR2													9.00	3.10	2.90	3.81E-03
EN1					4.00	0.33	12.21	2.58E-04								
ERG													8.00	1.38	5.81	5.67E-05
ESR1					37.00	19.46	1.90	1.12E-04								
ESR2					11.00	2.89	3.81	1.51E-04								
FOXO1													15.00	3.91	3.83	8.20E-06
FOSL2													6.00	1.13	5.32	7.97E-04
NR3C1					26.00	12.96	2.01	5.73E-04	10.00	3.09	3.24	9.31E-04				
GLI1													7.00	1.50	4.66	6.80E-04
HBP1													3.00	0.19	15.97	5.68E-04
HES1					6.00	1.19	5.05	1.16E-03								
HOXB1													3.00	0.19	15.97	5.68E-04
IRF4													6.00	1.44	4.17	2.95E-03
JUND													12.00	3.82	3.14	4.38E-04
KLF4													14.00	5.54	2.53	1.35E-03
KLF5													6.00	0.81	7.37	1.23E-04
NR1H3													9.00	2.69	3.34	1.45E-03
MYEF2													2.00	0.06	31.94	9.78E-04

Table 2-S5 (Continued)

	24 hpf								48 hpf							
Network Object Name	**BAA Actual**	**BAA Expected**	**BAA Ratio**	**BAA p-value**	**DBT-PYR Actual**	**DBT-PYR Expected**	**DBT-PYR Ratio**	**DBT-PYR p-value**	**BAA Actual**	**BAA Expected**	**BAA Ratio**	**BAA p-value**	**DBT-PYR Actual**	**DBT-PYR Expected**	**DBT-PYR Ratio**	**DBT-PYR p-value**
NFATC4					4.00	0.45	8.88	9.40E-04								
NFE2L2									7.00	1.19	5.89	1.77E-04				
REST													20.00	9.99	2.00	2.55E-03
NR4A2					5.00	0.76	6.60	8.84E-04								
OTX2													6.00	1.38	4.36	2.34E-03
TP53													42.00	27.11	1.55	3.05E-03
PITX2													8.00	1.66	4.82	2.22E-04
PPARA					13.00	2.50	5.20	1.25E-06					13.00	3.82	3.40	1.14E-04
PKNOX1													3.00	0.28	10.65	2.22E-03
PGR													17.00	6.07	2.80	1.28E-04
RARA													10.00	3.69	2.71	3.90E-03
RELA					24.00	11.39	2.11	4.65E-04	16.00	3.81	4.20	7.49E-07	35.00	17.41	2.01	6.65E-05
RORA					10.00	2.46	4.07	1.76E-04								
RXRA					10.00	2.68	3.73	3.59E-04					11.00	4.10	2.68	2.71E-03
SF1													9.00	2.38	3.78	5.89E-04
SIX1					5.00	0.53	9.39	1.60E-04								
SIX4					3.00	0.20	14.65	9.16E-04								
SOX5													11.00	3.29	3.35	4.39E-04
SOX6													5.00	0.72	6.94	6.19E-04
SP1					74.00	48.31	1.53	5.79E-05	24.00	11.95	2.01	3.48E-04	112.00	73.86	1.52	1.38E-06
SP3									9.00	1.97	4.56	1.45E-04				
STAT5A													13.00	4.10	3.17	2.34E-04
TBP													27.00	15.03	1.80	2.40E-03
TCF7L2													17.00	6.67	2.55	3.88E-04
TWIST2													2.00	0.06	31.94	9.78E-04
UBTF													2.00	0.06	31.94	9.78E-04
YY1													30.00	15.56	1.93	4.52E-04

Chapter 3 - AHR2 mutant reveals functional diversity of aryl hydrocarbon receptors in zebrafish

Britton C. Goodale[1], Jane K. La Du[1], William H. Bisson[2], Derek B. Janszen[3], Katrina M. Waters[3] and Robert L. Tanguay[1*]

1. Department of Environmental and Molecular Toxicology, the Environmental Health Sciences Center, Oregon State University, Corvallis, OR, USA

2. Pharmaceutical Biochemistry Group, School of Pharmaceutical Sciences, University of Geneva, Geneva, Switzerland

3. Computational Biology and Bioinformatics Group, Pacific Northwest National Laboratory, Richland, WA, USA

Published in PLOS ONE

7(1): e29346. doi:10.1371/journal.pone.0029346

Abstract

The aryl hydrocarbon receptor (AHR) is well known for mediating the toxic effects of TCDD and has been a subject of intense research for over 30 years. Current investigations continue to uncover its endogenous and regulatory roles in a wide variety of cellular and molecular signaling processes. A zebrafish line with a mutation in *ahr2* (*ahr2*^{hu3335}), encoding the AHR paralogue responsible for mediating TCDD toxicity in zebrafish, was developed via Targeting Induced Local Lesions IN Genomes (TILLING) and predicted to express a non-functional AHR2 protein. We characterized AHR activity in the mutant line using TCDD and leflunomide as toxicological probes to investigate function, ligand binding and CYP1A induction patterns of paralogues AHR2, AHR1A and AHR1B. By evaluating TCDD-induced developmental toxicity, mRNA expression changes and CYP1A protein in the AHR2 mutant line, we determined that *ahr2*^{hu3335} zebrafish are functionally null. *In silico* modeling predicted differential binding of TCDD and leflunomide to the AHR paralogues. AHR1A is considered a non-functional pseudogene as it does not bind TCCD or mediate *in vivo* TCDD toxicity. Homology modeling, however, predicted a ligand binding conformation of AHR1A with leflunomide. AHR1A-dependent CYP1A immunohistochemical expression in the liver provided *in vivo* confirmation of the *in silico* docking studies. The *ahr2*^{hu3335} functional knockout line expands the experimental power of zebrafish to unravel the role of the AHR during development, as well as highlights potential activity of the other AHR paralogues in ligand-specific toxicological responses.

Introduction

The aryl hydrocarbon receptor (AHR), while best known for its role as an environmental sensor and mediator of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) toxicity, has captured attention in recent years with a growing body of research elucidating its endogenous functions. As a member of the bHLH-per-ARNT-sim(PAS) family of proteins, the AHR is a transcriptional regulator containing two evolutionarily-conserved domains: a basic helix-loop-helix (bHLH) domain, which enables binding to aromatic hydrocarbon-responsive elements (AHREs), and a PAS domain, consisting of two 51- amino acid imperfect repeats (PAS-A and PAS-B), responsible for dimerization, ligand binding and interaction with other proteins (Fukunaga et al. 1995; Fukunaga and Hankinson 1996). Originally discovered for its role in modulating TCDD sensitivity in mice, the AHR binds a wide variety of ligand structures, including polycyclic and halogenated aromatic hydrocarbons (PAH and HAHs). Ligand binding induces disassociation from a cytoplasmic protein complex and translocation to the nucleus where the AHR heterodimerizes with the aryl hydrocarbon nuclear translocator (ARNT) (Nebert et al. 1975; Schmidt and Bradfield 1996; Denison and Nagy 2003). The AHR-ARNT heterodimer, along with other transcriptional enhancers, binds to AHREs and activates transcription of CYP1A, as well as NQO1, ALHD3A1, UGT1A6 and many other genes involved in metabolism, oxidative stress response and cell signaling (Nebert et al. 2000; Sartor et al. 2009). The role of the AHR in mediating toxicity of environmental contaminant exposure has been extensively studied (reviewed in (Gu et al. 2000; Nebert et al. 2004; Kerkvliet 2009)), and mechanism of action in immune, reproductive, developmental and other toxicological responses remain active areas of investigation. The diversity of physiological systems impacted by AHR activation and its crosstalk with other regulatory pathways support the notion that endogenous functions for the receptor likely preceded its role as an environmental sensor (Puga et al. 2009).

TCDD binding activity of the AHR is conserved among vertebrates. Substitutions in critical residues produce variation in ligand affinity, which underlies differences in TCDD sensitivity between species, inbred mouse strains, and wild fish populations (Nebert et al. 1975; Ema et al. 1994; Hahn 2002; Wirgin et al.). Structural comparisons of receptors provide information necessary for risk assessment extrapolation between species, as well as insight into receptor evolution (Hahn et al. 2006). In addition, *in silico* modeling of the

AHR has emerged as a powerful screening tool for potential AHR ligands (Bisson et al. 2009; Murray et al.).

Developing fish embryos are extremely sensitive to AHR-mediated planar hydrocarbon toxicity and hold a number of experimental advantages including development external to the mother, ease of observation, and genetic tractability. As such, zebrafish are a valuable model for investigation of developmental signaling processes in the context of xenobiotic exposures (Billiard et al. 2002; Carney et al. 2006). In teleosts, genome-wide duplication events have resulted in co-orthologs for many mammalian genes. While some gene duplicates have become non-functional, others have been evolutionarily conserved via the partitioning of functions between paralogues (Postlethwait et al. 2004). Three AHR isoforms have been identified in zebrafish: AHR1A, AHR1B, and AHR2 (Tanguay et al. 1999; Andreasen et al. 2002; Hahn 2002; Karchner et al. 2005). Numerous studies with known AHR ligands, however, have identified AHR2 as the primary mediator of early life stage toxicological effects in zebrafish (Prasch et al. 2003; Teraoka et al. 2003; Antkiewicz et al. 2006). Antisense oligonucleotide (morpholino) knockdown of AHR2 affords almost complete resistance to TCDD-induced developmental toxicity, and prevents the inhibitory effects of AHR ligands on epimorphic regeneration (Prasch et al. 2003; Mathew et al. 2006). Toxicity of many other HAHs and PAHs is also primarily dependent on AHR2. While AHR1B does bind TCDD, it is less sensitive to activation by TCDD than AHR2 (Karchner et al. 2005; Antkiewicz et al. 2006; Billiard et al. 2006). In contrast, AHR1A does not bind TCDD and is deficient in transactivation activity (Andreasen et al. 2002; Karchner et al. 2005). Beyond functioning as xenobiotic sensors, the zebrafish AHR paralogues are proposed to serve endogenous functions that have yet to be elucidated.

Recent studies have highlighted endogenous roles for the AHR in a complex array of immune system, cell cycle regulatory, reproductive and developmental processes (Peterson et al. 1993; Hernandez-Ochoa et al. 2009; Kerkvliet 2009; Matsumura et al. 2009; Singh et al. 2009). AHR knockout mouse strains developed by three different groups illustrate the importance of the AHR in normal liver development and immune function, and continue to expand understanding of the receptor's role in both toxicological responses and normal physiology (Fernandez-Salguero et al. 1995; Schmidt et al. 1996; Lahvis et al. 2005). A

functional zebrafish AHR2 knockout line will allow for investigation of the biological functions of the receptor throughout the zebrafish lifespan, and will eliminate the concern of incomplete knockdown that can occur with morpholinos. Complete loss of AHR2 activity in a zebrafish line will also enable functional analysis of the other two receptors, which has to date been experimentally difficult. As the primary mediator of TCDD toxicity, we proposed AHR2 as a target of great value to the zebrafish community for Targeting Induced Local Lesions IN Genomes (TILLING). Here we describe characterization of AHR function in the first TILLING-indentified AHR2 mutant zebrafish. We report loss of AHR2 function in a mutant AHR2 line, and present evidence of ligand- and tissue-specific activation and function of AHR1A and AHR1B.

Results

Generation of a functionally null AHR2 zebrafish line

The *ahr2*^{hu3335} line was established, upon request, by the Hubrecht institute from a TILLING-identified founder with a TTG to TAG point mutation in residue Leu534, resulting in a premature stop codon in the transactivation domain of AHR2 (Figure 1). While the bHLH and PAS domains are predicted to remain intact in the truncated protein, the transactivation domain of zebrafish AHR2 is required for transcriptional activation (Andreasen et al. 2002). In addition, the premature stop codon location is > 55 nucleotides upstream of an exon-exon boundary, likely rendering the mutant AHR2 mRNA a target of nonsense-mediated mRNA decay, which will be further discussed below (Wittkopp et al. 2009).

ahr2 ^{hu3335} zebrafish survived to adulthood with no consistently observed abnormalities during development. Jaw, gill and fin malformations were observed in adult fish, but did not appear to cause significant morbidity or mortality (Figure 2). The fins of *ahr2*^{hu3335} adult zebrafish are damaged compared to their *ahr2*$^{+}$ clutch mates, a characteristic which persisted in the offspring of wild-type 5D-outcrossed *ahr2*$^{hu3335/+}$ zebrafish (Figure 2A, B). Visible jaw malformations in *ahr2*^{hu3335} adults prompted us to investigate bone structure using non-destructive microCt scanning. MicroCt imaging revealed structural differences in the neurocrania of an *ahr2*^{hu3335} and an aged-matched wild-type strain 5D adult zebrafish, including a striking extension of the ethmoid and mandibular regions (Figure 2C, D)

(Cubbage and Mabee 1996). Further, the dentary, maxilla and premaxilla of the *ahr2*^{hu3335} zebrafish had notably different structure, creating an extended mandible. Other bones, such as the orbitals and supraorbitals, appeared smaller in the *ahr2*^{hu3335} zebrafish, which may be an artifact of scanning reduced bone thickness compared to the wild type (Cubbage and Mabee 1996).

In comparison to their *ahr*$^{+}$ and *ahr*$^{+/hu3335}$ siblings, spawning activity of *ahr2*^{hu3335} homozygous crosses was less robust and egg fertilization rates were low (50-75%). As is discussed further in regard to developmental toxicity assays, pericardial edema and jaw malformations occurred with higher incidence in some of the *ahr2*^{hu3335} clutches. Sporadic spawning activity of *ahr2*^{hu3335} homozygous crosses and successful in vitro fertilization demonstrated that the *ahr2*^{hu3335} mutation does not prevent reproductive function in this line. Irregular spawning, however, suggests deficits in reproductive physiology or behavior.

ahr2^{hu3335} embryos are resistant to TCDD-induced developmental toxicity

To assess AHR2 function in the *ahr2*^{hu3335} strain, we compared developmental toxicity of TCDD in the *ahr2*^{hu3335} mutants to *ahr2*$^{+}$ embryos. Exposure to 0.1, 1 or 10 nM TCDD resulted in a concentration-dependent increase in axis malformations and pericardial edema observed at 120 hpf in the *ahr2*$^{+}$ embryos (Figure 3A, C). Of the fifteen endpoints evaluated, TCDD concentration was significantly correlated with increases in yolk sac and pericardial edemas, and axis, eye, snout, jaw and trunk malformations (Table 2). Mortality, touch response, fin, pigment, brain, circulatory, somite and otic malformations were not significant responses in either fish line. *ahr2*^{hu3335} embryos were resistant to TCDD-dependent malformations, and the responses of *ahr2*$^{+}$ and *ahr2*^{hu3335} embryos to TCDD exposure were significantly different from each other (Table 2). Background pericardial edema and jaw malformations were observed in *ahr2*^{hu3335} embryos but were not TCDD-dependent.

mRNA expression indicates the ahr2^{hu3335} mutation abrogates AHR2 function

We evaluated mRNA expression to further assess AHR2 function, and observed a 16-fold difference in AHR2 transcript abundance between *ahr2*$^{+}$ and *ahr2*^{hu3335} embryos (Figure 4A). This supports the hypothesis that AHR2 mRNA is degraded in the *ahr2*^{hu3335} line. We next examined AHR2-dependent gene expression to determine whether the point mutation

perturbs expression of downstream transcriptional targets. Expression of CYP1A, CYP1B1, CYP1C1, CYP1C2, AHR1A and AHR1B transcripts were not significantly different between *ahr2*^{hu3335} and *ahr2*$^{+}$ embryos (Figure 4A).

To further confirm the lack of AHR2 functionality, we investigated mRNA expression changes in response to TCDD, which induces AHR2-dependent expression of a number of mRNAs at 48 hpf (Jonsson et al. 2007). Developmental TCDD exposure induced robust expression of CYP1A, CYP1C1 and CYP1C2 mRNA at 48 hpf in *ahr2*$^{+}$ embryos relative to vehicle-treated controls (Figure 4B). As expected in the absence of a functional AHR2, mRNA expression was not significantly elevated in *ahr2*^{hu3335} embryos exposed to TCDD.

AHR1A predicted to bind leflunomide but not TCDD

We recently reported a homology model that has been used to predict binding affinity of potential ligands to the human, mouse and zebrafish AHRs (O'Donnell et al.). In order to investigate differential function of the zebrafish AHR paralogues, we tested TCDD and a known AHR ligand with a non-classical structure, leflunomide, in a series of molecular docking studies. Sequence alignment of the mouse and zebrafish AHR-PASB domains produced identities of 65.1% (zfAHR1A), 78.5% (zfAHR1B) and 70.5 % (zfAHR2). High similarity between the three isoforms at the primary and predicted tertiary structural levels was also noted, with 74.3% (AHR2/1B) and 69.9% (AHR1B/1A) identity. TCDD and leflunomide were docked into zebrafish AHR1A-, AHR1B-, and AHR2-LBD homology models. TCDD docked in AHR2 and AHR1B with predicted binding energies of -3.97 kcal/mol and -4.86 kcal/mol, respectively, but was unable to dock in AHR1A (Table 3, Figure 5A-B). Leflunomide was also able dock in AHR2 and AHR1B, with predicted binding energies of -2.13 kcal/mol and -1.97 kcal/mol, respectively (Table 3). Interestingly, in contrast to TCDD, leflunomide docked into AHR1A, but in a unique orientation (Bisson et al. 2009) (Table 3, Figure 5E).

AHR1A possesses specific residues that play potential roles in TCDD insensitivity (Karchner et al. 2005). Key residues characterized in the mouse AHR-LBD influencing TCDD binding are conserved in zebrafish AHR2 and AHR1B, which are both TCDD sensitive (Pandini et al. 2007; Bisson et al. 2009; Pandini et al. 2009). In AHR1A, residues His296, Ala386 and Gln388 have been substituted with Tyr296, Thr386 and His388 (Karchner et al. 2005). The

side chains of these residues cause both decreased volume and altered polarity of the AHR1A binding pocket, in comparison to AHR2, AHR1B, as well as mouse and human AHRs (26). TCDD docking is consequently not possible in AHR1A, which has been confirmed both *in vitro* and *in vivo* (Andreasen et al. 2002; Karchner et al. 2005). Homology modeling predicted that leflunomide, however, is able to dock in AHR1A with a unique orientation not found in human, mouse, or zebrafish AHR1B and AHR2 isoforms (O'Donnell et al.). As shown previously, leflunomide docks in AHR2 and AHR1B with a hydrogen bond (HB) interaction between the nitrogen atom of the isoxazole ring of the ligand and the OH of the side chain of Thr294 ((O'Donnell et al.), Figure 5C, D). Here we employed the homology model to examine AHR1A interaction with leflunomide for the first time, and discovered that the leflunomide docking position is flipped and a double HB interaction between the nitrogen and oxygen of the isoxazole ring of the ligand and the side chain of Thr354 is formed (Figure 5E). A binding energy of -2.19 kcal/mol was predicted which is in the range calculated for the other two isoforms (Table 3). Based on these data, we predicted that leflunomide would be a functional AHR1A ligand.

CYP1A protein induction patterns are ligand- and AHR isoform-dependent

We used immunohistochemical analysis of CYP1A protein expression as a biomarker of AHR activation to investigate *in vivo* AHR ligand binding patterns in TCDD and leflunomide-exposed larvae. Exposure to 1 nM TCDD from 6-24 hpf induces AHR2-dependent CYP1A expression in a number of tissues, including the heart, liver and enteric tract, with the predominant expression in the vascular endothelium of larvae (Figure 6A) (Andreasen et al. 2002). We focused our evaluation of AHR function on the most robust CYP1A induction patterns, which were observed in vasculature and liver (Andreasen et al. 2002; Carney et al. 2004). As predicted by the qRT-PCR results, CYP1A protein expression in TCDD-exposed *ahr2*^{hu3335} larvae was limited to faint vascular expression, just above background, in all embryos examined (Figure 6B). Exposure to 10 nM TCDD, which induced severe malformations and robust CYP1A expression in wild-type embryos, did not notably increase CYP1A expression in *ahr2*^{hu3335} larvae (data not shown).

To confirm the predicted binding of leflunomide to all three zebrafish AHRs *in vivo*, we examined CYP1A induction in *ahr2*^{hu3335} larvae exposed to 10 μM leflunomide from 48-72

hpf. In comparison to wild type larvae, vascular CYP1A expression was drastically reduced in leflunomide-exposed *ahr2*^{hu3335} larvae (Figure 6C, D). In contrast to TCDD exposure, however, AHR2-independent CYP1A expression was observed in the developing livers of leflunomide-exposed *ahr2*^{hu3335} larvae (Figure 6D). This expression pattern persisted in larvae exposed until 120 hpf, with vascular expression remaining low and liver expression increasing, likely due to growth that occurs from 72-120 hpf (data not shown).

Based on molecular docking studies (Figure 5), we hypothesized that leflunomide induced CYP1A in *ahr2*^{hu3335} larvae via activation of the other AHR homologs, and utilized splice-blocking morpholinos to transiently knock down AHR1A and AHR1B separately and in combination. We conducted immunohistochemical analysis of CYP1A expression at 72 hpf to capture the window of morpholino efficacy, which was confirmed with PCR using primers flanking the target sites (Supplemental Figure 1). As the liver is small at 72 hpf, we employed double-staining with a hepatocyte nuclear factor 4α (HNF4α) antibody to confirm the presence of hepatocytes (Dong et al. 2007) (data not shown). CYP1A expression in AHR1B morpholino-injected *ahr2*^{hu3335} larvae persisted in the liver (Figure 6E), but was notably absent in the vasculature. In contrast, injection of the AHR1A morpholino in *ahr2*^{hu3335} embryos blocked leflunomide-induced expression of CYP1A in the liver, while faint vascular expression remained. When co-injected, the AHR1A and AHR1B morpholinos blocked all CYP1A expression in leflunomide-exposed *ahr2*^{hu3335} larvae (Figure 6F). When expression of all 3 AHR isoforms was eliminated, CYP1A expression in leflunomide-exposed embryos was indistinguishable from vehicle-exposed controls (Figure 6G).

Discussion

ahr2^{hu3335} zebrafish, homozygous for a point mutation in *ahr2*, survive to adulthood and are functional AHR2 knockouts by all measures tested. The premature stop codon in residue 534 is predicted to result in a non-functional protein due to its truncated transactivation domain. Though we cannot exclude the possibility that some biological activity of a potential cryptic protein remains, we saw no evidence to support its presence. Analysis of *ahr2*^{hu3335} mRNA levels suggests that the mutant AHR2 transcript is at least partially degraded and the truncated protein may be present only at very low levels, if at all.

The *ahr2*^{hu3335} adult zebrafish exhibit notable fin and skeletal differences compared to wild type. We also observed a higher background of developmental abnormalities in *ahr2*^{hu3335} larvae. These phenotypes may not necessarily be due to the mutation; reduced spawning and small clutch sizes of *ahr2*^{hu3335} zebrafish limited the selection of embryos for experiments, whereas large wild type clutches allow for precise selection of high-quality embryos. Studies in both AHR-deficient and AHR ligand-treated mice provide strong evidence of an endogenous role of the receptor in female reproductive physiology. Deficiencies in maintaining pregnancy and surviving lactation have been reported in AHR knockout mice (Abbott et al. 1999), and disruption of AHR function alters ovarian development, folliculogenesis, steroid hormone synthesis, ovulation and possibly reproductive senescence (Hernandez-Ochoa et al. 2009). In keeping with AHR knockout mouse models, *ahr2*^{hu3335} zebrafish are capable of producing viable embryos, but exhibit decreased reproductive success. It is important to note, however, that other ENU-induced mutations throughout the genome of this fish line could be responsible for observed phenotypic abnormalities. Zebrafish TILLING mutants require multiple outcrosses to reduce undesired mutations to background levels. Because outcrosses of the *ahr2*^{hu3335} line were in progress at the time of this study, it is premature to attribute all phenotypic abnormalities observed in *ahr2*^{hu3335} homozygotes to the mutation in *ahr2*. Decreased reproductive capacity of homozygous mutants, as well as fin and jaw abnormalities may represent interesting models of endogenous AHR function and certainly warrant further investigation if they persist in the mutant line following further outcrosses.

In the present study, we used TCDD as a tool to investigate AHR2 function in the *ahr2*^{hu3335} line. We found that *ahr2*^{hu3335} embryos were resistant to TCDD-induced developmental toxicity at concentrations that cause severe malformations in *ahr2*$^{+}$ embryos. *ahr2*^{hu3335} embryos treated with 10nM TCDD showed few signs of morbidity at 120 hpf. Transient AHR2 knockdown delays, but does not prevent, TCDD-induced mortality (Prasch et al. 2003). Therefore it would be interesting to examine longer-term effects of TCDD exposure in future experiments with the *ahr2*^{hu3335} line.

The most well-known biomarker of AHR activation is the induction of CYP1A expression. Among the suite of cytochrome P450 metabolizing enzymes in zebrafish, CYP1A, CYP1B1,

CYP1C1 and CYP1C2 are elevated in response to AHR agonist exposure (Jonsson et al. 2007). In agreement with our developmental toxicity data, no elevation in CYP1A, CYP1C1 or CYP1C2 expression was observed in TCDD-exposed *ahr2*^{hu3335} embryos. Taken together, these data support the concept that AHR2 is not functional in this line. The notable, but statistically insignificant, increase in CYP1A expression following TCDD treatment in *ahr2*^{hu3335} embryos is likely due to TCDD activation of AHR1B, as further discussed below.

While the dependence of CYP1A activation by TCDD on AHR2 is well-established, studies with PAHs in zebrafish embryos have revealed diverse CYP1A expression patterns dependent on other AHR isoforms (Incardona et al. 2005; Incardona et al. 2006). This study represents the first time that an *in silico*-based modeling approach was utilized to investigate ligand binding by all three receptors. Molecular docking with TCDD predicted that both AHR1B and AHR2, but not AHR1A, would bind TCDD due to substitutions in the binding pocket. In contrast to TCDD, *in silico* modeling with leflunomide predicts favorable binding energies for all three zebrafish AHR isoforms. Interestingly, leflunomide docked into AHR1A with a different predicted conformation than in the other two receptors, but with equivalent affinity. This finding is particularly intriguing, as AHR1A is incapable of binding classical AHR ligands (Karchner et al. 2005), is deficient in transactivation activity (Andreasen et al. 2002), and therefore was once considered non-functional.

We confirmed the AHR modeling results *in vivo* using CYP1A protein expression as a biomarker of AHR activation. In keeping with our mRNA expression and *in silico* modeling studies, TCDD-exposed *ahr2*^{hu3335} larvae were largely devoid of CYP1A protein expression observed in TCDD-exposed *ahr2*$^{+}$ larvae. Leflunomide also induces strong vascular CYP1A protein expression in *ahr2*$^{+}$ larvae, but unlike with TCDD, the *ahr2*^{hu3335} embryos exhibited striking leflunomide-induced CYP1A expression in the liver. This finding is in agreement with the modeling results. To tease apart AHR isoform-dependence of the residual CYP1A expression, we transiently knocked down the receptors individually and in combination in *ahr2*^{hu3335} larvae. We found AHR1B-dependent vascular induction and AHR1A-dependent liver induction of CYP1A expression. Knockdown of AHR1A and AHR1B in combination prevented all CYP1A induction. Taken together, these data suggest that, contrary to

previous observations with TCDD, all three AHR isoforms are involved in leflunomide-induced CYP1A expression in zebrafish larvae.

These data demonstrate that there are concrete differences in ligand binding activity of the zebrafish AHRs, and that AHR1A is not a pseudogene as previously proposed, but rather has affinity for different ligand structures. While residual CYP1A expression has been observed in TCDD-treated AHR2-morphants, it was faint and vascular in nature, attributable to incomplete knockdown (Prasch et al. 2003). Our immunohistochemical results with the *ahr2*^{hu3335} line suggest that mild vascular expression of CYP1A is induced via AHR1B, and can be effectively knocked down to background with morpholino injection. AHR1A-dependent CYP1A expression is seemingly incongruous with previous investigation of AHR1A function *in vitro,* but the lack of a known AHR1A ligand limited previous efforts. The AHR1A-dependent CYP1A expression pattern we observed here is consistent with the reported AHR1A mRNA expression in the liver (Andreasen et al. 2002).

Putative AHR1A ligands could be identified with further *in silico* modeling; work by Incardona and colleagues also offers clues with several PAHs that induce CYP1A expression independently of AHR2 (Incardona et al. 2005; Incardona et al. 2006; Incardona et al.). Pyrene induced liver expression of CYP1A in an AHR1A-dependent manner (Incardona et al. 2006), and more recently retene-induced CYP1A expression was shown to be incompletely dependent on AHR2 (Scott et al.). Here, we offer further evidence that AHR1A is a functional receptor *in vivo*, though the transactivation requirements for this receptor remain to be elucidated. *In vitro* data with AHR chimera proteins suggest that transactivation requirements of AHR1A differ from those of AHR2 (Andreasen et al. 2002).

The presence of three apparently functional aryl hydrocarbon receptors in zebrafish raises several interesting questions: How do these receptors differ? What functions have led to their evolutionary conservation? And to what extent do the AHR1 receptors need to be considered in toxicological studies in zebrafish? While the presence of multiple AHRs certainly complicates study of receptor function in fish, subfunction partitioning among isoforms presents a unique opportunity to unravel the many physiological functions of the AHR that are conserved among vertebrates (Postlethwait et al. 2004). As summarized in Table 4, the studies presented here add to a body of research demonstrating significant

differences in receptor expression, ligand binding, and mRNA induction activity. With respect to transcript localization, AHR2 is widely distributed through most organs investigated in adult zebrafish, while AHR1A is mainly expressed in the liver, and to a lesser extent in the heart, kidney and swim bladder (Andreasen et al. 2002). AHR1B expression has yet to be fully characterized, but our CYP1A IHC results suggest that the isoform is widely distributed, but is expressed at much lower levels than AHR2. The subfunction partitioning of these receptors is not strictly locational. Overlapping expression of AHR2 and AHR1A has been previously described, and we also noted overlap in AHR2- and AHR1-dependent CYP1A expression patterns (Andreasen et al. 2002; Karchner et al. 2005). A cell or tissue-level analysis may reveal more subtle localization differences, as has been implied in differential PAH-induced CYP1A patterns in endocardial and myocardial tissue (Incardona et al. 2006; Incardona et al.). Little is yet known about the endogenous function of these receptors and their downstream transcriptional targets. If expression of AHR1A and AHR1B is limited, it may be difficult to detect significant changes in their transcriptional targets in whole embryo homogenate. As we have shown here, however, the *ahr2*^{hu3335} line will ease the study of the other two receptors by removing the overpowering transcriptional changes induced through AHR2. The three receptors together present an intriguing opportunity to unravel multiple regulatory functions that may be conserved in the mammalian AHR.

This is the first report of CYP1A induction dependent on all three of the zebrafish AHRs. However, toxicity mediated through the AHR1 receptors has not, as of yet, been documented. Pyrene liver toxicity was reduced with AHR1A knockdown, but AHR2 knockdown prevented the majority of the chemical's developmental effects (Incardona et al. 2006). In the case of TCDD and other similarly-structured HAHs, the small binding pocket of AHR1A prevents it from having a role in ligand-induced toxicological effects. AHR1A and AHR1B receptors may hold little importance in toxicological studies with these compounds. Indeed the studies presented here support the large body of previous research indicating that TCDD-induced early life stage toxicity is mediated through AHR2. Though some CYP1A and other downstream target induction may occur via AHR1B, any developmental abnormalities caused by this pathway are more subtle than those investigated to date. The possibility remains, however, that AHR1B may play a role in later life stage impacts of

TCDD. These data warrant further investigation of the AHR isoforms with structurally diverse, less-well studied compounds. Ultimately, further bioinformatic and modeling efforts with zebrafish and mammalian AHRs could help determine the best model for human AHR activity, taking into account both ligand binding and receptor expression characteristics.

This was the first time that all three AHR isoforms were knocked down in developing zebrafish. Our findings suggest that, consistent with mammalian literature, AHR function is not required to complete development (Schmidt et al. 1996; Gonzalez and Fernandez-Salguero 1998). Without full histological evaluations of the AHR1Amo/AHR1Bmo/*ahr2*^{hu3335} larvae at 120hpf, we cannot exclude non-lethal malformations, particularly hepatic abnormalities, which have been reported in AHR knockout mice (Schmidt et al. 1996; Lahvis et al. 2005). It may not be possible to fully answer the question of whether the AHR paralogues are required for hepatic development in zebrafish with the tools employed here, as the liver undergoes significant development after 72hpf, when morpholino efficacy is in decline. We therefore present the *ahr2*^{hu3335} line as a valuable resource available to the zebrafish research community, and suggest that development of both AHR1A and AHR1B (already requested by the research community) mutant lines would further extend the power of this model for investigating both the endogenous and ligand-mediated roles of the AHR in developing vertebrates.

Materials and Methods

Zebrafish lines and embryos

Adult zebrafish were housed according to Institutional Animal Care and Use Committee protocols at Oregon State University on a recirculating system with water temperature of 28±1°C and a 14 h light/10 h dark schedule. Zebrafish embryos carrying a point mutation in *ahr2* (*ahr2*^{hu3335} strain) were requested and generously provided by the Hubrecht Institute. The *ahr2*^{hu3335} line was identified from a library of *N*-ethyl-*N*-nitrosourea (ENU)-mutagenized zebrafish using the TILLING method as previously described (Wienholds et al. 2003). Offspring of heterozygous *ahr2*^{hu3335} carriers were raised to adulthood at the Sinnhuber Aquatic Research Laboratory, and genotyped for the *ahr2*^{hu3335} point mutation withDNA isolated from fin clips (Wienholds et al. 2003). PCR amplification was performed

with genomic DNA and *ahr2* gene-specific primers (Table 1), the product was purified using a QIAquick PCR purification kit (Qiagen) and sequenced with an ABI 3730 capillary sequencer at the Center for Genome Research and Biocomputing at Oregon State University. Homozygous carriers of the T -> A point mutation in residue 534 (Figure 1) were identified to create an *ahr2*^{hu3335} population. Because the TILLING method relies on random mutagenesis, mutant lines of interest carry other mutations throughout the genome. F1 fish are predicted to carry 3000-6000 mutations and multiple outcrosses are necessary to reduce off-target mutations (Moens et al. 2008). *ahr2*^{hu3335} carriers were outcrossed to thewild type 5D (*ahr2*$^{+/+}$) line, and homozygous mutants were identified from an incross of their progeny. The *ahr2*^{hu3335} mutant line has been maintained with subsequent outcrosses on the wild type 5D background, which was also used for all *ahr2*$^{+}$ control experiments in our laboratory.

All developmental toxicity experiments were conducted with fertilized embryos obtained from group spawns of adult zebrafish as described previously (Reimers et al. 2006). Embryos used in experiments are defined as homozygous (*ahr2*^{hu3335}), heterozygous (*ahr2*$^{hu3335/+}$) or wild-type (*ahr2*$^{+}$) for the point mutation in AHR2.

MicroCt imaging

Micro computed tomography (μCT) was used for nondestructive three-dimensional imaging of zebrafish heads. The fish were scanned using a Scanco μCT40 scanner (Scanco Medical AG, Basserdorf, Switzerland) at 45 kVp, 177 mA, and a voxel size of 12 x 12 x 12 mm. The heads were imaged at threshold settings of 140 (scale 0 – 1000).

Homology modeling, molecular docking and binding energy calculations

Molecular Modeling of zebrafish AHR2, AHR1B and AHR1A isoforms was conducted as described previously (17). Briefly, the homology models of mouse, human, rat and zebrafish AHR-LBD (ligand binding domains) were built using the NMR resolved structure of the PAS domain of human hypoxia-inducible-factor 2α as the 3D-template. Models were then refined in the internal coordinates with Molsoft ICM v3.5-1p. Molecular docking of TCDD and Leflunomide ligands and binding energy calculation were performed as reported (Bisson et al. 2009).

Chemical exposures and developmental toxicity assessment

TCDD (99.2% purity in DMSO, Cambridge Isotope Laboratories) and leflunomide (Sigma-Aldrich) were dissolved in DMSO. All exposures were conducted in E2 embryo medium with staged embryos (Kimmel et al. 1995). Embryos were batch exposed to 0.1, 1, 10 nM TCDD or 0.1% DMSO vehicle control in 2 mL embryo medium in glass vials from 6-24 hours post fertilization (hpf). Embryos were then rinsed 4X with embryo media and transferred to plastic dishes to develop until the indicated experimental time points. Embryo homogenate for mRNA expression analysis was collected at 48 hpf, and developmental toxicity of TCDD exposure was assessed by visually inspecting embryos at 120 hpf for malformations as previously described (Truong et al.) with three biological replicates. Developmental toxicity assay data were analyzed by fitting a 2 parameter logistic regression model to the concentration-response data for each malformation. Significance of the TCDD concentration-response curve was calculated for each fish line. Differential responses were assessed with a t-test to compare the parameters from the *ahr2*$^{+}$ model to those from the *ahr2*^{hu3335} model. No adjustment for multiplicity was made. R software v12.0 (2010) was used for these analyses.

For leflunomide exposures, embryos were transferred into individual wells of a 96-well plate and exposed to 10 μM leflunomide or 0.1% DMSO control in 100 μl embryo medium from 48-72 hpf, when they were humanely euthanized and fixed for immunohistochemistry analysis.

Total RNA isolation and reverse transcription

For qRT-PCR studies, 20 embryos per treatment group were homogenized in TRIzol (Invitrogen) and stored at -80°C until use. Total RNA was isolated via phenol/guanidine isothiocyanate/chloroform separation. For morpholino splice-blocking confirmation, 15 embryos were homogenized in RNAzol (Molecular Research Center) for total RNA isolation. RNA was quantified using a SynergyMx microplate reader (Biotek) with the Gen5 Take3 module to calculate 260/280 O.D. ratios. Superscript III First-Strand Synthesis (Invitrogen) was used with oligo(dT) primer to reverse transcribe cDNA from total RNA.

Quantitative RT-PCR

Relative abundance of AHR1A, AHR1B, AHR2, CYP1A, CYP1B1, CYP1C1 and CYP1C2 mRNA transcripts were assessed in whole embryo homogenate. Gene-specific primers (MWG Operon) are listed in Table 1. All qRT-PCR assays were performed in 20 µl reactions consisting of 10 µl Power SYBR Green PCR master mix (Applied Biosystems), 0.4 ul each primer, 9.2 ul H_2O and 50 ng equivalents of cDNA. Amplification (Step One Plus, Applied Biosystems) was performed with cycling parameters as follows: 95°C for 10 min; 40 cycles of 95°C for 15 s, 60°C for 1 min; 95°C for 15 sec and 60°C for 1 min. A melt curve was performed at 3° increments to assess for multiple products.

qRT-PCR analysis was performed with StepOne Software v2.1 (Applied Biosystems) using the ΔΔCt method with genes of interest normalized to β-actin (Livak and Schmittgen 2001). Three independent biological replicates were assessed and statistically analyzed by comparing *ahr2*^{hu3335} to *ahr2*$^{+}$ or TCDD-treated to control with a Student's t-test using Graphpad Prism 5.01 software (Graphpad Software Inc. La Jolla,CA).

Morpholino injection

Splice-blocking morpholinos designed against AHR1A and AHR1B were purchased from Gene Tools (Philomath, OR). The AHR1A splice-blocking morpholino (AHR1Amo, 5' CTTTTGAAGTGACTTTTGGCCCGCA 3') was described previously (Incardona et al. 2006) and was tagged on the 3' end with fluorescein. We designed a morpholino to target the exon7/intron7 boundary of AHR1B (AHR1Bmo, 5' ACACAGTCGTCCATGATTACTTTGC 3'). A standard control morpholino from Gene Tools (cmo, 5' CCTCTTACCTCAGTTACAATTTATA 3') was used as a negative control. *ahr2*^{hu3335} embryos were injected at the 1-2 cell stage with approximately 2 nl of 1.5 mM morpholino dissolved in ultrapure water with 0.5% phenol red. For AHR1Amo +AHR1Bmo co-injections, the final concentration of each morpholino was 0.83 mM. Embryos were allowed to develop in fish water and screened for successful morpholino incorporation with fluorescein visualization at 24 hpf. mRNA mis-splice was confirmed with PCR primers flanking the target sites at 24 and 72 hpf (AHR1A and AHR1B-mo primers Table 1).

Immunohistochemistry (IHC)

Wild-type strain 5D and *ahr2*^{hu3335} embryos treated with 1nM TCDD (or 0.1% DMSO control) from 6-24 hpf were fixed at 120 hpf in 4% paraformaldehyde (J.T. Baker) overnight

at 4°C. Leflunomide treated embryos (48-72 hpf) were fixed at 72 hpf to capture the window of morpholino efficacy. Mouse α fish CYP1A monoclonal (1:500 dilution, Biosense laboratories) and goat α human HNF4α polyclonal (1:100 dilution, Santa Cruz Biotechnology) primary antibodies were used. Secondary antibodies consisted of Alexafluor® 546 rabbit α mouse IgG (H+L) (1:1000) and Alexafluor® 488 donkey α goat IgG (H+L) (1:1000) (Molecular Probes, Eugene, OR). Immunohistochemistry was performed as previously described (Mathew et al. 2006). Briefly, whole fixed embryos were permeabilized with 0.005% trypsin on ice for 10 min, washed 3X with PBST and post-fixed in 4% paraformaldehyde for 10 min. Samples were blocked for 1h in 10% normal goat serum (single labeling) or BlockAid (double labeling) (Invitrogen). Samples were incubated with primary antibodies overnight at 4°C, followed by 4 30min washes in PBST and incubation with secondary antibody overnight at 4°C. At least 8 embryos per treatment group were imaged by epi-fluorescence microscopy using a Zeiss Axiovert 200M microscope with 5X and 10X objectives.

Acknowledgments

This work was supported by the NIEHS Environmental Health Sciences SRP grant Project #3 P42 ES016465, Core Center Grant P30 ES00210 and the NIEHS Training Grant T32 ES7060 to RLT. We gratefully acknowledge Edwin Cuppen at the Hubrecht Institute for the *ahr2*^{hu3335} line and the European Commission Consortium Integrated Project "ZF-MODELS-Zebrafish Models for Human Development and Disease", Contract No. LSHG-CT-2003-503496. We would like to thank the Skeletal Biology Laboratory at OSU for the microCt analysis, and the Center for Genome Research and Biocomputing at OSU for sequencing support. We are grateful to Cari Buchner, Carrie Barton, Brittany McCauslin, Jessika Dobson and the staff at the Sinnhuber Aquatic Research Laboratory for fish husbandry expertise and to members of the Tanguay laboratory for their helpful feedback and critical review of the manuscript.

References

(2010). R: A language and environment for statistical computing. R. D. C. Team. Vienna, Austria.

Abbott, B. D., J. E. Schmid, et al. (1999). "Adverse reproductive outcomes in the transgenic Ah receptor-deficient mouse." Toxicol Appl Pharmacol **155**(1): 62-70.

Andreasen, E. A., M. E. Hahn, et al. (2002). "The zebrafish (Danio rerio) aryl hydrocarbon receptor type 1 is a novel vertebrate receptor." Mol Pharmacol **62**(2): 234-249.

Andreasen, E. A., J. M. Spitsbergen, et al. (2002). "Tissue-specific expression of AHR2, ARNT2, and CYP1A in zebrafish embryos and larvae: effects of developmental stage and 2,3,7,8-tetrachlorodibenzo-p-dioxin exposure." Toxicol Sci **68**(2): 403-419.

Antkiewicz, D. S., R. E. Peterson, et al. (2006). "Blocking expression of AHR2 and ARNT1 in zebrafish larvae protects against cardiac toxicity of 2,3,7,8-tetrachlorodibenzo-p-dioxin." Toxicol Sci **94**(1): 175-182.

Billiard, S. M., M. E. Hahn, et al. (2002). "Binding of polycyclic aromatic hydrocarbons (PAHs) to teleost aryl hydrocarbon receptors (AHRs)." Comp Biochem Physiol B Biochem Mol Biol **133**(1): 55-68.

Billiard, S. M., A. R. Timme-Laragy, et al. (2006). "The role of the aryl hydrocarbon receptor pathway in mediating synergistic developmental toxicity of polycyclic aromatic hydrocarbons to zebrafish." Toxicol Sci **92**(2): 526-536.

Bisson, W. H., D. C. Koch, et al. (2009). "Modeling of the aryl hydrocarbon receptor (AhR) ligand binding domain and its utility in virtual ligand screening to predict new AhR ligands." J Med Chem **52**(18): 5635-5641.

Carney, S. A., R. E. Peterson, et al. (2004). "2,3,7,8-Tetrachlorodibenzo-p-dioxin activation of the aryl hydrocarbon receptor/aryl hydrocarbon receptor nuclear translocator pathway causes developmental toxicity through a CYP1A-independent mechanism in zebrafish." Mol Pharmacol **66**(3): 512-521.

Carney, S. A., A. L. Prasch, et al. (2006). "Understanding dioxin developmental toxicity using the zebrafish model." Birth Defects Res A Clin Mol Teratol **76**(1): 7-18.

Cubbage, C. C. and P. M. Mabee (1996). "Development of the cranium and paired fins in the zebrafish Danio rerio (Ostariophysi, cyprinidae)." Journal of Morphology **229**(2): 121-160.

Denison, M. S. and S. R. Nagy (2003). "Activation of the aryl hydrocarbon receptor by structurally diverse exogenous and endogenous chemicals." Annu Rev Pharmacol Toxicol **43**: 309-334.

Dong, P. D., C. A. Munson, et al. (2007). "Fgf10 regulates hepatopancreatic ductal system patterning and differentiation." Nat Genet **39**(3): 397-402.

Ema, M., N. Ohe, et al. (1994). "Dioxin binding activities of polymorphic forms of mouse and human arylhydrocarbon receptors." J Biol Chem **269**(44): 27337-27343.

Fernandez-Salguero, P., T. Pineau, et al. (1995). "Immune system impairment and hepatic fibrosis in mice lacking the dioxin-binding Ah receptor." Science **268**(5211): 722-726.

Fukunaga, B. N. and O. Hankinson (1996). "Identification of a novel domain in the aryl hydrocarbon receptor required for DNA binding." J Biol Chem **271**(7): 3743-3749.

Fukunaga, B. N., M. R. Probst, et al. (1995). "Identification of functional domains of the aryl hydrocarbon receptor." J Biol Chem **270**(49): 29270-29278.

Gonzalez, F. J. and P. Fernandez-Salguero (1998). "The aryl hydrocarbon receptor: studies using the AHR-null mice." Drug Metab Dispos **26**(12): 1194-1198.

Gu, Y. Z., J. B. Hogenesch, et al. (2000). "The PAS superfamily: sensors of environmental and developmental signals." Annu Rev Pharmacol Toxicol **40**: 519-561.

Hahn, M. E. (2002). "Aryl hydrocarbon receptors: diversity and evolution." Chem Biol Interact **141**(1-2): 131-160.

Hahn, M. E., S. I. Karchner, et al. (2006). "Unexpected diversity of aryl hydrocarbon receptors in non-mammalian vertebrates: insights from comparative genomics." J Exp Zool A Comp Exp Biol **305**(9): 693-706.

Hernandez-Ochoa, I., B. N. Karman, et al. (2009). "The role of the aryl hydrocarbon receptor in the female reproductive system." Biochem Pharmacol **77**(4): 547-559.

Incardona, J. P., M. G. Carls, et al. (2005). "Aryl hydrocarbon receptor-independent toxicity of weathered crude oil during fish development." Environ Health Perspect **113**(12): 1755-1762.

Incardona, J. P., H. L. Day, et al. (2006). "Developmental toxicity of 4-ring polycyclic aromatic hydrocarbons in zebrafish is differentially dependent on AH receptor isoforms and hepatic cytochrome P4501A metabolism." Toxicol Appl Pharmacol **217**(3): 308-321.

Incardona, J. P., T. L. Linbo, et al. (2011). "Cardiac toxicity of 5-ring polycyclic aromatic hydrocarbons is differentially dependent on the aryl hydrocarbon receptor 2 isoform during zebrafish development." Toxicol Appl Pharmacol **257**(2): 242-249.

Jonsson, M. E., M. J. Jenny, et al. (2007). "Role of AHR2 in the expression of novel cytochrome P450 1 family genes, cell cycle genes, and morphological defects in developing zebra fish exposed to 3,3',4,4',5-pentachlorobiphenyl or 2,3,7,8-tetrachlorodibenzo-p-dioxin." Toxicol Sci **100**(1): 180-193.

Karchner, S. I., D. G. Franks, et al. (2005). "AHR1B, a new functional aryl hydrocarbon receptor in zebrafish: tandem arrangement of ahr1b and ahr2 genes." Biochem J **392**(Pt 1): 153-161.

Kerkvliet, N. I. (2009). "AHR-mediated immunomodulation: the role of altered gene transcription." Biochem Pharmacol **77**(4): 746-760.

Kimmel, C. B., W. W. Ballard, et al. (1995). "Stages of embryonic development of the zebrafish." Dev Dyn **203**(3): 253-310.

Lahvis, G. P., R. W. Pyzalski, et al. (2005). "The aryl hydrocarbon receptor is required for developmental closure of the ductus venosus in the neonatal mouse." Mol Pharmacol **67**(3): 714-720.

Livak, K. J. and T. D. Schmittgen (2001). "Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method." Methods **25**(4): 402-408.

Mathew, L. K., E. A. Andreasen, et al. (2006). "Aryl hydrocarbon receptor activation inhibits regenerative growth." Mol Pharmacol **69**(1): 257-265.

Matsumura, F., A. Puga, et al. (2009). "Biological functions of the arylhydrocarbon receptor: beyond induction of cytochrome P450s. Introduction to this special issue." Biochem Pharmacol **77**(4): 473.

Moens, C. B., T. M. Donn, et al. (2008). "Reverse genetics in zebrafish by TILLING." Brief Funct Genomic Proteomic **7**(6): 454-459.

Murray, I. A., C. A. Flaveny, et al. (2011). "Suppression of cytokine-mediated complement factor gene expression through selective activation of the Ah receptor with 3',4'-dimethoxy-alpha-naphthoflavone." Mol Pharmacol **79**(3): 508-519.

Nebert, D. W., T. P. Dalton, et al. (2004). "Role of aryl hydrocarbon receptor-mediated induction of the CYP1 enzymes in environmental toxicity and cancer." J Biol Chem **279**(23): 23847-23850.

Nebert, D. W., J. R. Robinson, et al. (1975). "Genetic expression of aryl hydrocarbon hydroxylase activity in the mouse." J Cell Physiol **85**(2 Pt 2 Suppl 1): 393-414.

Nebert, D. W., A. L. Roe, et al. (2000). "Role of the aromatic hydrocarbon receptor and [Ah] gene battery in the oxidative stress response, cell cycle control, and apoptosis." Biochem Pharmacol **59**(1): 65-85.

O'Donnell, E. F., K. S. Saili, et al. (2010). "The anti-inflammatory drug leflunomide is an agonist of the aryl hydrocarbon receptor." PLoS One **5**(10).

Pandini, A., M. S. Denison, et al. (2007). "Structural and functional characterization of the aryl hydrocarbon receptor ligand binding domain by homology modeling and mutational analysis." Biochemistry **46**(3): 696-708.

Pandini, A., A. A. Soshilov, et al. (2009). "Detection of the TCDD binding-fingerprint within the Ah receptor ligand binding domain by structurally driven mutagenesis and functional analysis." Biochemistry **48**(25): 5972-5983.

Peterson, R. E., H. M. Theobald, et al. (1993). "Developmental and reproductive toxicity of dioxins and related compounds: cross-species comparisons." Crit Rev Toxicol **23**(3): 283-335.

Postlethwait, J., A. Amores, et al. (2004). "Subfunction partitioning, the teleost radiation and the annotation of the human genome." Trends Genet **20**(10): 481-490.

Prasch, A. L., H. Teraoka, et al. (2003). "Aryl hydrocarbon receptor 2 mediates 2,3,7,8-tetrachlorodibenzo-p-dioxin developmental toxicity in zebrafish." Toxicol Sci **76**(1): 138-150.

Puga, A., C. Ma, et al. (2009). "The aryl hydrocarbon receptor cross-talks with multiple signal transduction pathways." Biochem Pharmacol **77**(4): 713-722.

Reimers, M. J., J. K. La Du, et al. (2006). "Ethanol-dependent toxicity in zebrafish is partially attenuated by antioxidants." Neurotoxicol Teratol **28**(4): 497-508.

Sartor, M. A., M. Schnekenburger, et al. (2009). "Genomewide analysis of aryl hydrocarbon receptor binding targets reveals an extensive array of gene clusters that control morphogenetic and developmental programs." Environ Health Perspect **117**(7): 1139-1146.

Schmidt, J. V. and C. A. Bradfield (1996). "Ah receptor signaling pathways." Annu Rev Cell Dev Biol **12**: 55-89.

Schmidt, J. V., G. H. Su, et al. (1996). "Characterization of a murine Ahr null allele: involvement of the Ah receptor in hepatic growth and development." Proc Natl Acad Sci U S A **93**(13): 6731-6736.

Scott, J. A., J. P. Incardona, et al. (2011). "AhR2-mediated, CYP1A-independent cardiovascular toxicity in zebrafish (Danio rerio) embryos exposed to retene." Aquat Toxicol **101**(1): 165-174.

Singh, K. P., F. L. Casado, et al. (2009). "The aryl hydrocarbon receptor has a normal function in the regulation of hematopoietic and other stem/progenitor cell populations." Biochem Pharmacol **77**(4): 577-587.

Tanguay, R. L., C. C. Abnet, et al. (1999). "Cloning and characterization of the zebrafish (Danio rerio) aryl hydrocarbon receptor." Biochim Biophys Acta **1444**(1): 35-48.

Teraoka, H., W. Dong, et al. (2003). "Induction of cytochrome P450 1A is required for circulation failure and edema by 2,3,7,8-tetrachlorodibenzo-p-dioxin in zebrafish." Biochem Biophys Res Commun **304**(2): 223-228.

Truong, L., S. L. Harper, et al. (2011). "Evaluation of embryotoxicity using the zebrafish model." Methods Mol Biol **691**: 271-279.

Wienholds, E., F. van Eeden, et al. (2003). "Efficient target-selected mutagenesis in zebrafish." Genome Res **13**(12): 2700-2707.

Wirgin, I., N. K. Roy, et al. (2011). "Mechanistic basis of resistance to PCBs in Atlantic tomcod from the Hudson River." Science **331**(6022): 1322-1325.

Wittkopp, N., E. Huntzinger, et al. (2009). "Nonsense-mediated mRNA decay effectors are essential for zebrafish embryonic development and survival." Mol Cell Biol **29**(13): 3517-3528.

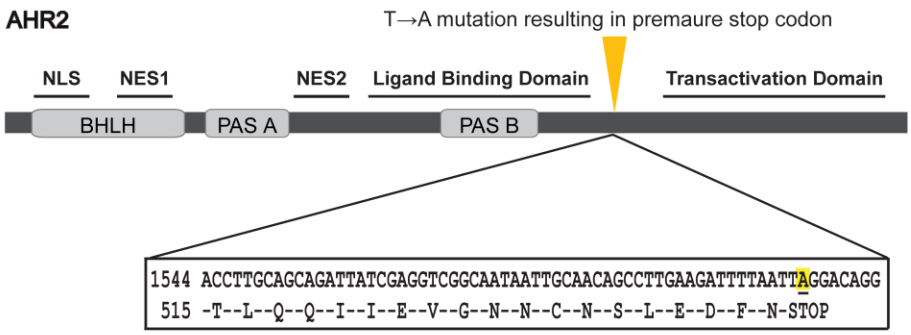


Figure 3-1 Schematic diagram of predicted AHR2 protein in *ahr2*^{hu3335} zebrafish

The *ahr2*^{hu3335} zebrafish line has a T -> A point mutation in residue 534, resulting in a premature stop codon in the transactivation domain of the protein. The predicted truncated protein contains the ligand binding, DNA binding and ARNT binding domains, but lacks the transactivation domain previously shown to be essential for a functional AHR2 protein (Hahn 1998; Andreasen et al. 2002). NLS: nuclear localization signal, NES1: nuclear export signal 1, NES2: nuclear export signal 2.

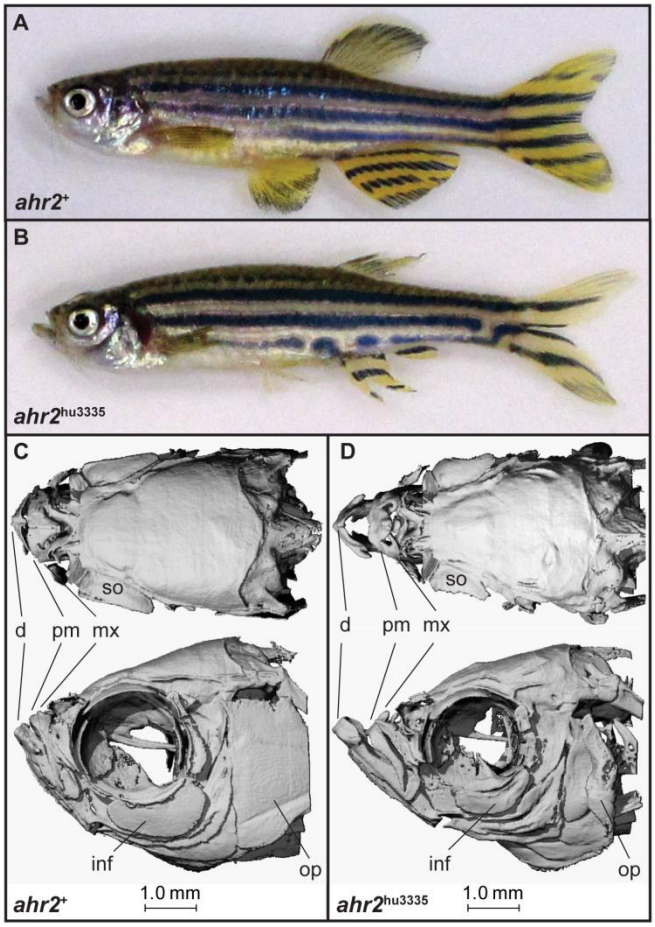


Figure 3-2 Fin and skeletal abnormalities in *ahr2*^{hu3335} zebrafish

A-B) Brightfield and (**C-D)** microCt imaging of adult *ahr2*$^{+}$ and *ahr2*^{hu3335}zebrafish. Notable differences were observed in the dentate (d), premaxilla (pm), maxilla (mx), supraorbital (so), infraorbital 3(inf) and operculum (op).

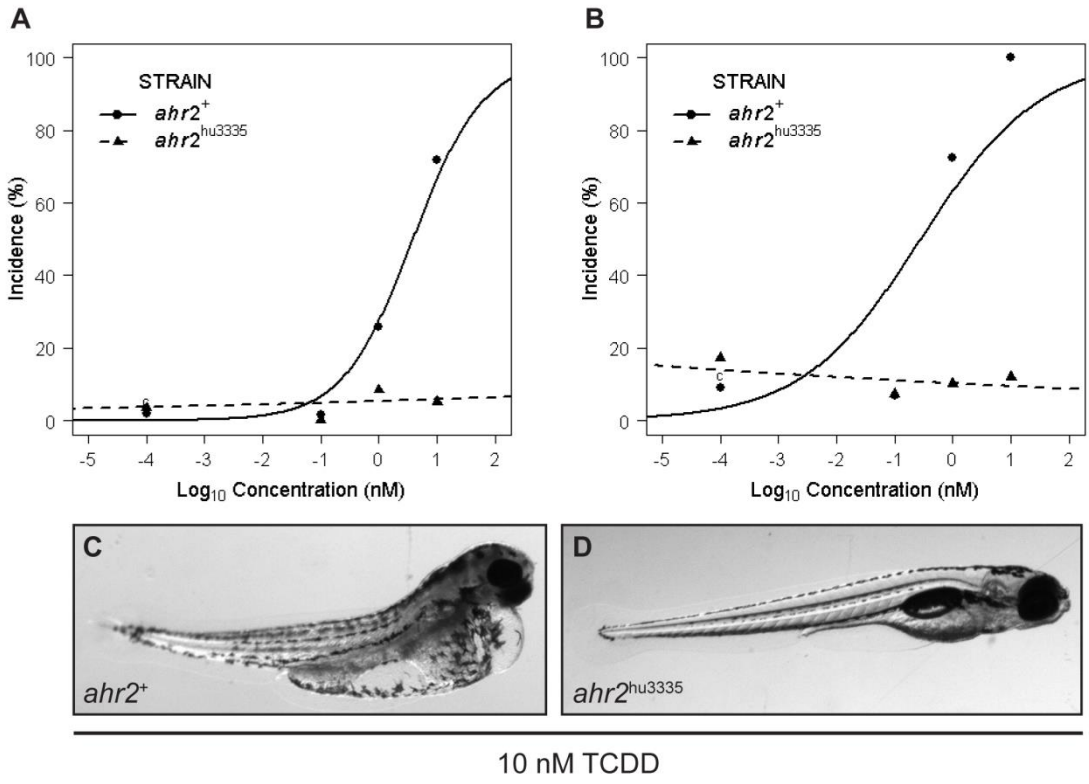


Figure 3-3 *ahr2*^{hu3335} embryos are resistant to TCDD developmental abnormalities

A) Percent of embryos with axis malformations and **B)** percent incidence pericardial edema at 120 hpf in embryos treated with 0, 0.1, 1 or 10 nM TCDD from 6-24 hpf. Vehicle control groups (c, 0.1% DMSO) are displayed at 10^{-4} for graphing purposes. Data represent three independent experiments with 20 embryos per treatment group. **C)** Representative image of *ahr2*$^{+}$ and **(D)** *ahr2*^{hu3335} embryos developmentally exposed to 10 nM TCDD at 120 hpf.

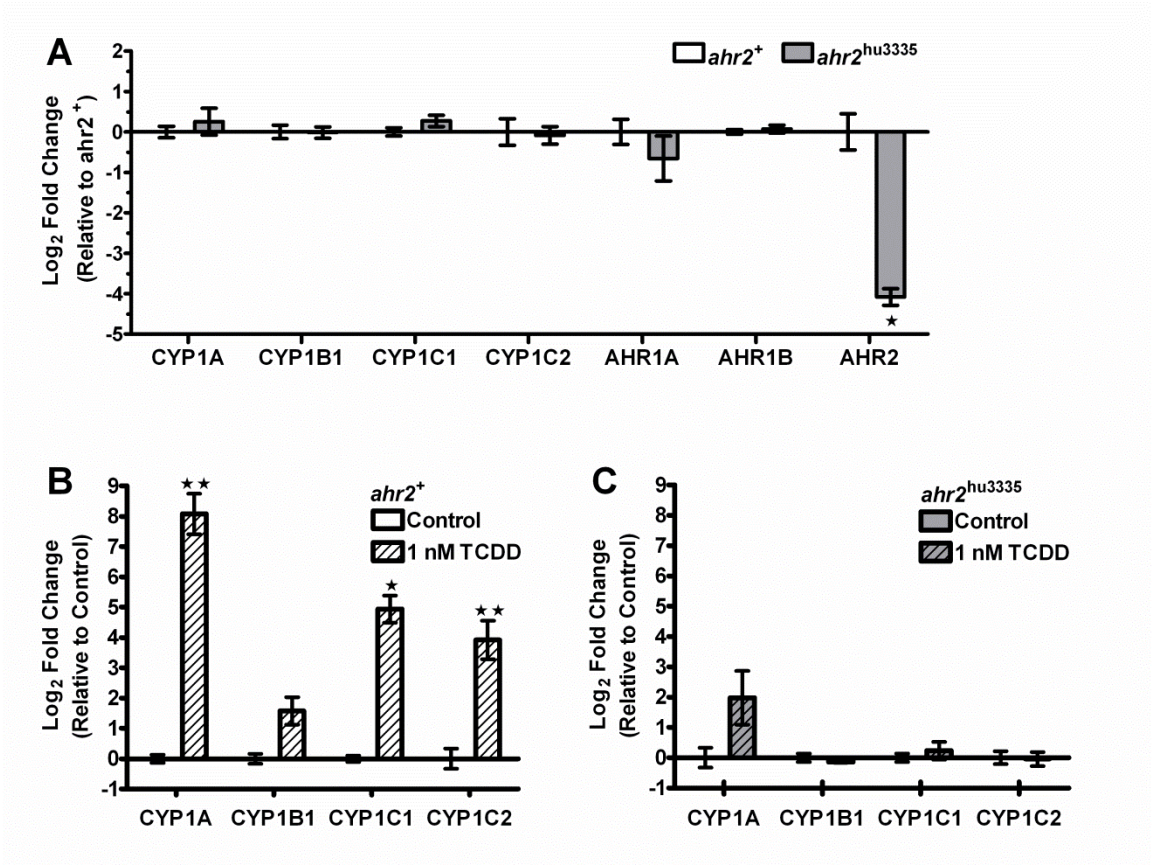


Figure 3-4 *ahr2*^{hu3335} embryos are resistant to TCDD-induced CYP induction

A) Comparative analysis of AHR1A, AHR1B, CYP1A, CYP1B, CYP1C1 and CYP1C2 mRNA expression in wild-type 5D and *ahr2*^{hu3335} mutant embryos at 48hpf . ΔΔCt values were calculated by comparing sample ΔCt values (normalized to β-actin) to the mean *ahr2*$^{+}$ ΔCt for each gene. Data were analyzed by paired student's t-test, * $p < .05$. **B)** Developmental exposure (6-24 hpf) to 1nM TCDD induced significant CYP1A, CYP1C1 and CYP1C2 expression at 48 hpf in *ahr2*$^{+}$ embryos. Data is shown normalized to vehicle-treated controls and was analyzed with paired student's t-test, *$p < .05$, ** $p < .01$. **C)** Developmental exposure to 1nM TCDD did not induce significant mRNA expression changes in *ahr2*^{hu3335} embryos. While CYP1A was elevated, the difference was not significant (paired student's t-test treated vs. vehicle control.

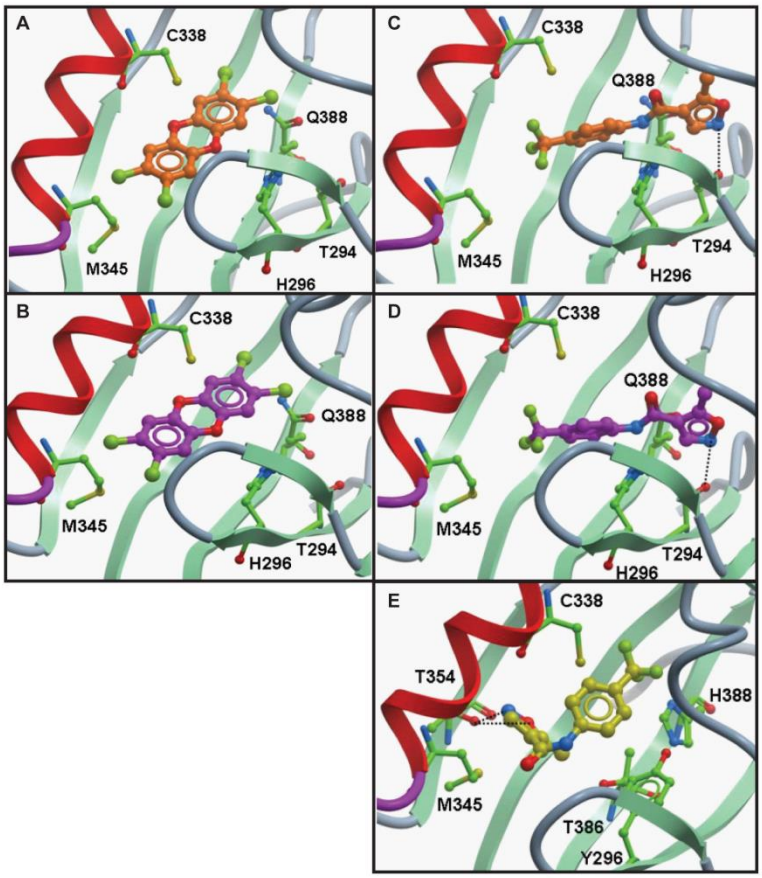


Figure 3-5 Molecular docking of TCDD and Leflunomide in zebrafish AHR isoforms

A) TCDD docking orientation in zebrafish AHR2- and **B**) AHR1B-LBD homology model binding pocket (ICM v3.5-1n, Molsoft). **C)** Leflunomide docking orientation into AHR2-, **D)** AHR1B- and **E)** AHR1A homology model binding pockets. The residues are displayed as sticks and colored by atom type with the carbon atoms in green. The protein backbone is displayed as ribbon and colored by secondary structure. The ligand is displayed as sticks and colored by atom

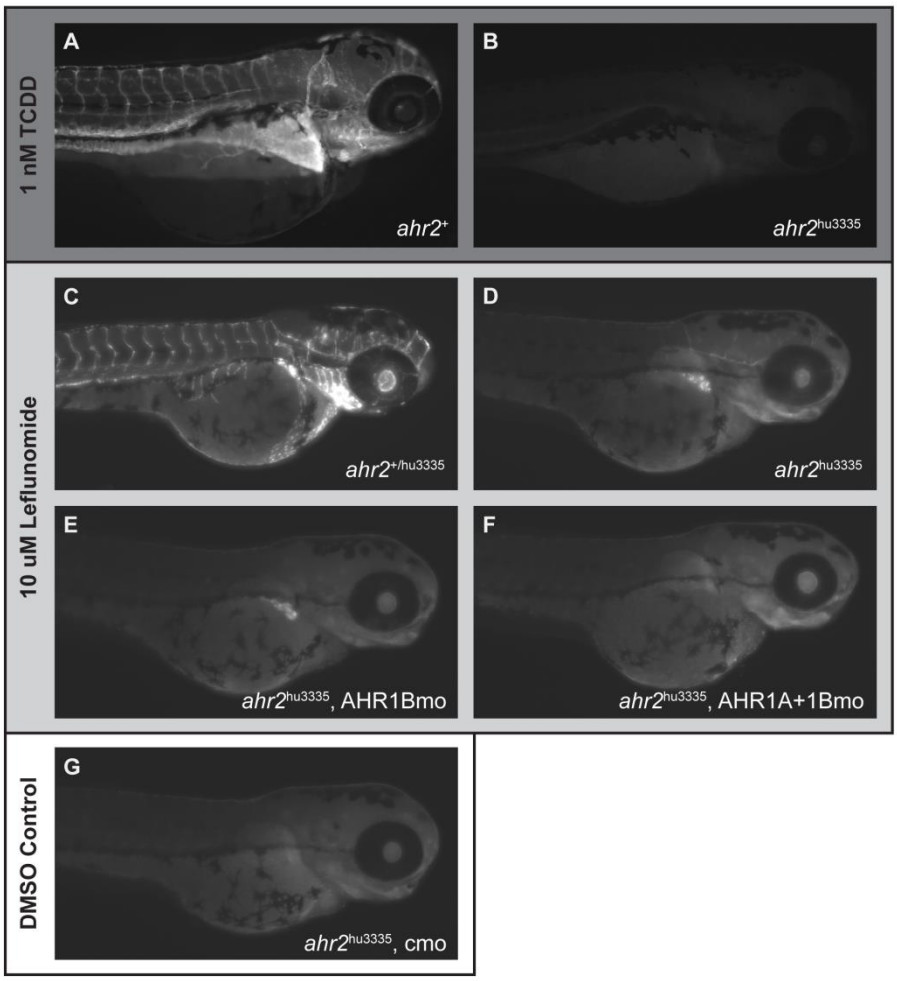


Figure 3-6 CYP1A protein expression patterns

CYP1A expression at 120 hpf in **(A)** *ahr2*$^{+}$ and **(B)** *ahr2*^{hu3335} larvae following exposure to 1 nM TCDD from 6-24 hpf. **C)** Leflunomide-induced CYP1A expression at 72 hpf in wild-type and **(D)** *ahr2*^{hu3335} mutants. **E)** Leflunomide-induced CYP1A expression in AHR1B-morphant *ahr2*^{hu3335} larvae and **F)** larvae co-injected with AHR1A and AHR1B of morpholinos. **(G)** DMSO control. TCDD-exposed embryos were IHC processed side-by-side and imaged at 120 hpf using the same exposure settings and a single focal plane. Leflunomide-exposed embryos and DMSO control were processed side-by-side and imaged at 72 hpf using the same exposure times; images were created from a z-stack of 10 15.4uM slices centered on the liver.

Table 3-1 Primer sequences for PCR experiments

mo- morpholino mis-splice detection **mut**- mutant point mutation detection

Target	Forward Primer (5'- 3')	Reverse Primer (5'- 3')
AHR1A	CGCAAAAGGAGGAAACCTGTC	CCTGTAGCAAAAATTCCCCCT
AHR1B	GGTTTGTCGTCAAACAACAGTAACCACG	CCACCAACACAAAGCCATTAAGAGCCTG
AHR1B-mo	CTTTGTGTGTCGTTTCCGATGCC	GCACAGTAGAGCATATCAGCTGC
AHR2	TGGACTAGATCAGACAACCC	GAAGAGGGAGAGTCATTGTG
AHR2-mut	TATTGCTAGGCAGAGAGCAC	GATGTCTTCTGTGATGATTTCG
CYP1A	TGCCGATTTCATCCCTTTCC	AGAGCCGTGCTGATAGTGTC
CYP1B1	CTGCATTGATTTCCGAGACGTG	CACACTCCGTGTTGACAGC
CYP1C1	AGTGGCACAGTCTACTTTGAGAG	TCGTCCATCAGCACTCAG
CYP1C2	GTGGTGGAGCACAGACTAAG	TTCAGTATGAGCCTCAGTCAAAC
β-ACTIN	AAGCAGGAGTACGATGAGTC	TGGAGTCCTCAGATGCATTG

Table 3-2 Concentration responses for developmental effects

Effect	p-value of *ahr2*$^{+}$ TCDD concentration-response	p-value of *ahr2*^{hu3335} TCDD concentration-response	p- value of *ahr2*$^{+}$ *ahr2*^{hu3335} differential response
yolk sac edema	< 0.0001	0.7181	0.0004
axis	< 0.0001	0.2754	0.0006
eye	< 0.0001	1.0000	0.0005
snout	< 0.0001	0.6706	0.0004
jaw	< 0.0001	0.8632	0.0011
pericardial edema	< 0.0001	0.0848	0.0002

Table 3-3 Predicted binding energy values for zebrafish AHR2, AHR1B and AHR1A (kcal/mol), ND – unable to dock

	AHR2	AHR1B	AHR1A
TCDD	-3.97	-4.86	ND
Leflunomide	-2.13	-1.97	-2.19

Table 3-4 Summary of zebrafish AHR ligand binding, activity and expression

Receptor	Receptor mRNA expression in adult zebrafish	In vitro TCDD binding and activity	Homology model predicted ligand binding		Dominant receptor-dependent CYP1A protein induction pattern (larval)
			TCDD	*Leflunomide*	
AHR1A	heart, swimbladder, liver, kidney(Andreasen et al. 2002)	N(Andreasen et al. 2002; Karchner et al. 2005)	N	Y	liver
AHR1B	NA	Y(Karchner et al. 2005)	Y	Y	vasculature
AHR2	brain, heart, muscle, swimbladder, liver, gill, skin, eye, kidney, fin(Andreasen et al. 2002)	Y(Andreasen et al. 2002; Karchner et al. 2005)	Y	Y	liver, vasculature

Chapter 4 - Ahr-dependent developmental toxicity and differential transcriptional profiles induced by 4-ring oxygenated PAHs

Goodale, B.C.[1], Tilton, S.C.[2], Sullivan, C.M.[1,3],Waters, K.M2. and Tanguay, R.L[1].

1.Department of Environmental and Molecular Toxicology, the Environmental Health Sciences Center, Oregon State University, Corvallis, OR,

2.Computational Biology and Bioinformatics, Pacific Northwest National Laboratory, Richland, WA

3. Center for Genome Research and Biocomputing, Oregon State University, Corvallis, OR

Formatted for submission to Toxicological Sciences

Abstract

Polycyclic aromatic hydrocarbons (PAHs) are common air pollutants and combustion byproducts that exhibit diverse mutagenic, carcinogenic, proinflammatory and teratogenic properties. Unsubstituted (parent) PAHs are defined by three or more fused benzene rings; oxygen-substituted PAHs (OPAHs) are also formed during combustion, as well as via phototoxidation and biological degradation of parent PAHs. Despite their prevalence both in contaminated industrial sites and in urban air, their mechanisms of action in biological systems are relatively understudied. Like parent PAHs, OPAHs exhibit structure-dependent mutagenic activities and differential activation of the aryl hydrocarbon receptor (AHR). In the canonical AHR signaling pathway, the AHR translocates to the nucleus upon ligand binding, dimerizes with the aryl hydrocarbon receptor nuclear translocator (ARNT), and binds to DNA response elements to activate transcription of a suite of downstream genes that include cytochrome p450 phase 1 metabolizing enzymes (CYP1A, CYP1B1). In a screen of diverse OPAHs for developmental toxicity in zebrafish, 4-ring OPAHs benzanthrone (BEZO) and benz(a)anthracene-7,12-dione (7,12-B[a]AQ) induced similar morphological aberrations and markers of oxidative stress, but only 7,12-B[a]AQ significantly induced cyp1a expression. We investigated the role of the AHR in mediating the toxicity of BEZO and 7,12-B[a]AQ, and found that knockdown of *ahr2* rescued developmental effects caused by both compounds. Using comparative RNA-seq, we show that BEZO induces expression of xenobiotic metabolizing genes directly regulated by AHR with distinctively lower potency than 7,12-B[a]AQ. The much larger majority of significantly-induced transcripts, including genes that regulated redox-homeostasis, were affected similarly by these two OPAHs. Biological functions and transcription factors predicted to regulate the genes significantly misexpressed by BEZO and 7,12-B[a]AQ suggest that the AHR interacts differentially with other transcription factors and coactivators to mediate the developmental toxicity caused by these compounds.

Introduction

Polycyclic aromatic hydrocarbons (PAHs) are major components of combustion emissions and contaminants at hazardous waste sites. Automobiles, wood burning, coal-based energy production and other combustion processes produce both parent (unsubstituted) PAHs, consisting of multiple fused carbon rings, as well as a variety of substituted derivatives. PAHs are associated with both the gaseous and ultrafine particulate fractions of urban air, can accumulate in the lungs when inhaled, and are considered major carcinogenic components of combustion emissions (Bostrom et al. 2002; Ramirez et al. 2011). Oxygenated, nitrated, and methylated PAHs also form from parent PAHs through photo-oxidation (via ozone and hydroxyl radical) reactions as well as biotic metabolism in the environment (Yu 2002; Lundstedt et al. 2007). The EPA has identified 16 PAHs on its priority pollutant list, based on their prevalence at Superfund sites and potential health effects (EPA 2012). Parent PAHs are routinely measured in order to estimate total PAH contamination levels and potential hazard, but degradation products, such as oxygenated PAHs (OPAHs) are less frequently accounted for. As detection methods have improved and standards are more widely available, recent studies showed that OPAHs are present in PAH-contaminated environmental samples, and it is expected that they may be present at higher concentrations than parent PAHs (Lundstedt et al. 2006; Layshock et al. 2010; Walgraeve et al. 2010). Despite this, little data is available about their toxicity. Like a number of parent PAHs, some OPAHs are mutagenic (Durant et al. 1996; Gurbani et al. 2013). Based on the varied and structure-dependent toxicological effects of unsubstituted PAHS, which include developmental toxicity mediated by the aryl hydrocarbon receptor (AHR), as well as cardiac toxicity and immune effects, substituted PAH structures are likely to also induce non-cancer toxic effects. A screen of OPAH toxicity in developing zebrafish demonstrated that OPAHs cause developmental effects at a wide range of waterborne concentrations, and different structural elements likely explain their differential induction of a variety of morphological abnormalities (Knecht et al. 2013). Like unsubstituted PAHs, OPAHs differentially induce expression of known targets of the aryl hydrocarbon receptor, such as *cyp1a*. They also induce expression of genes involved in redox homeostasis, suggesting that oxidative stress also plays a role in their toxicity. Because of their ubiquity, and potential greater prevalence in some environmental situations than parent PAHs, there is a need to understand

mechanisms by which OPAHs cause toxicity, and how these mechanisms are related to, or distinct from, those identified for parent PAHs.

Here we compare the transcriptional signatures and proposed toxicological mechanisms of two structurally-related OPAHs, 1,9 benz-10-anthrone (BEZO) and benz(a)anthracene-7,12-dione (7,12-B[a]AQ) during embryonic development. These 4-ring OPAHs, which are detected in environmental samples, differ in their ring arrangement and oxygenation pattern. BEZO is detected in air samples associated with high traffic emissions, but is also an important intermediate used in production of dyes (Nielsen et al. 1999; Wei et al. 2012). Exposure has been associated with hepatic and dermal lesions in workers, as well as ascorbic acid depletion in animal models (Singh et al. 2003). 7,12-B[a]AQ is also detected in air and at industrial waste sites, and was among the most abundant OPAHs detected in urban air in Beijing (Wang et al. 2011; Wei et al. 2012). 7,12-B[a]AQ can be formed from benz(a)anthracene (BAA), an EPA priority pollutant PAH which is mutagentic and induces developmental toxicity via the AHR (Incardona et al. 2006). Both compounds were identified in a zebrafish toxicity screen to disrupt normal development, while differentially inducing *cyp1a* expression, suggesting distinct modes of action. We investigated the role of Ahr2 in mediating developmental toxicity. We used whole genome mRNA sequencing to investigate global differences in transcription induced by these compounds. Despite their differential *cyp1a* activation and early morphology profiles, we found that developmental toxicity of both BEZO and 7,12-B[a]AQ was dependent on *ahr2*. Transcriptional profiling with RNA-seq showed largely similar gene expression between the compounds, with cellular redox homeostasis genes playing a large role in the toxicological response. The large difference in *cyp1a* expression, coupled with more subtle differences in the expression of interacting transcription factors, highlights the ability of the AHR to mediate toxicity via alternate pathways in a ligand-dependent manner. Understanding the multitude of AHR interactions is important for assessing and predicting health risk posed by OPAHs. Comparative transcriptional profiling additionally sheds light on conserved mechanisms and biomarkers that may be more appropriate than *cyp1a* for inferring exposure and AHR activation by this class of emerging contaminants.

Materials and Methods

Fish Husbandry

All experiments were conducted with wild-type 5D zebrafish. Adult zebrafish were maintained at the Sinnhuber Aquatic Research Laboratory on a recirculating system with a water temperature of 28 ± 1˚C, and a 14 hr light: 10 hr dark photoperiod. All experiments were conducted with embryos collected in the morning from multiple adult zebrafish set up for group spawning as described previously (Reimers et al. 2006). Adult care and reproductive techniques were conducted according to Institutional Animal Care and Use Committee protocols at Oregon State University.

Chemicals

Analytical grade (> 98% purity) 1,9-benz-10-anthrone (BEZO) was purchased from Fluka, and benz(a)anthracene-7,12-dione (7,12-B[a]AQ) was purchased from Sigma-Aldrich. Compounds were dissolved to 10 mM in dimethyl sulfoxide (DMSO). Stocks were sonicated in a bath sonicator for 15 min before each use. For embryo exposures, BEZO and 7,12-B[a]AQ stocks in 100% DMSO were dissolved in embryo media to a final concentration of 1% DMSO.

Developmental toxicity

Embryos were cleaned, developmentally staged and batch-exposed in glass vials at 6 hours post fertilization (hpf) to 5, 7.5 and 10 μM concentrations of OPAH or 1% DMSO vehicle control, 20 embryos per vial in 2 mL exposure solution (Kimmel et al. 1995). Vials were protected from light and incubated on a rocker at 28˚C for the duration of the exposure. Embryos were collected at 48 hpf for RNA and immunohistochemical analysis. For developmental toxicity evaluations, embryos remained in solution until 72 hpf, when they were rinsed 3 times, transferred to individual wells of a 96-well plate, and incubated in fresh embryo media until evaluation at 120 hpf. Embryos were then euthanized with MS-222 (tricaine methanesulfonate) and evaluated for yolk sac, axis, trunk, somite, fin, cardiac, eye, snout, jaw, otic vesicle, brain and pigment malformations. Mortality and the percentage of embryos with each malformation were calculated for each treatment group with the vial (20 embryos) as the experimental unit. Representative larvae were imaged at 48 and 120 hpf with a Nikon Coolpix 5000 digital camera. Experiments were repeated in triplicate, and percent incidence data across replicates was analyzed for significance using SigmaPlot

software. Data were analyzed for morpholino and treatment effects by two-way ANOVA with Tukey's all pairwise post hoc test for each endpoint.

Morpholino injection

Embryos were injected at the single cell stage with a fluorescein-tagged translation-blocking morpholinos targeting *ahr2* (*ahr2*-MO, 5' TGTACCGATACCCGCCGACATGGTT 3'), or a standard nonsense control (control-MO, 5' cctcttacctcagttacaatttata 3') purchased from Gene Tools (Philomath, OR) at a concentration of 0.75mM. Injection volume was ~2nL. Fertilized, normally developing embryos were screened for morpholino incorporation at 4 hpf by fluorescence microscropy. Three independent replicates were conducted.

RNA isolation

Groups of 20 embryos were homogenized at 48 hpf in RNAzol (Molecular Research Center, Cincinnati, OH) using a bullet blender with 0.5 mM zirconium oxide beads (Next Advance, Averill Park, NY). Samples were stored at -80˚C until RNA isolation via phenol guanidine extraction. RNA was quantified and quality assessed using a SynergyMx microplate reader with the Gen5 Take3 module to calculate O.D. 260/280 ratios. Quality of RNA samples for sequencing was additionally assessed with an Agilent Bioanalyzer 2100. cDNA was synthesized from 1 µg (confirmation with RNA from sequencing samples) or 2 µg of RNA using the ABI high capacity cDNA synthesis kit. cDNA was diluted and 50 ng equivalents of RNA were used for QPCR reactions. 3-4 biological replicates were collected per treatment group.

Quantitative RT-PCR

Gene-specific primers (MWG Operon) for qRT-PCR amplification are listed in Table S1. All qRT-PCR assays were performed in 20 µl reactions consisting of 10 µl Power SYBR Green PCR master mix (Applied Biosystems), 0.4 µl each primer, 9.2 µl H_2O and 50 ng equivalents of cDNA. Amplification (Step One Plus, Applied Biosystems) was performed with cycling parameters as follows: 95°C for 10 min; 40 cycles of 95°C for 15 s, 60°C for 1 min; 95°C for 15 sec and 60°C for 1 min. A melt curve was performed at 3° increments to assess for multiple products. Relative fold change values in PAH-treated samples compared to vehicle controls were calculated for genes of interest, normalized to β-actin, by the method

described by Pfaffl (Pfaffl 2001). Four biological replicates were assessed and statistically analyzed by Two-way ANOVA with Tukey's post-hoc test using Sigmaplot software.

Immunohistochemistry

Whole embryos were fixed at 48 hpf in 4% paraformaldehyde at 4˚C overnight. Mouse α fish CYP1A monoclonal (1:500 dilution, Biosense laboratories) primary antibody and Alexafluor® 594 goat α mouse IgG (H+L) (1:1000 dilution, Molecular Probes, Eugene, OR) secondary antibody were used for immunofluorescent labeling of Cyp1a as described by Svoboda et al (Svoboda et al. 2001). Briefly, embryos were washed in phosphate-buffered saline containing 0.1% Tween-20 (PBST), permeabilized by a 1 h incubation in distilled H2O followed by 20 min in cold acetone and 30 min incubation in 1 mg/ml collagenase. Embryos were blocked for 1h in PBST with 10% normal goat serum, incubated with 1˚ antibody in 10% NGS overnight at 4˚C, washed in PBST, and incubated in 2˚ antibody 4 h at RT. Embryos were imaged by epi-fluorescence microscopy using a Zeiss Axiovert 200M microscope with 5X objective. Merged images were created from z-stacks of 5 20uM slices captured under identical exposure conditions. Two overlapping frames were spliced to capture the entire length of each larva.

Paired-end mRNA sequencing

mRNA was isolated from total RNA samples, fractionated and libraries prepared with custom barcodes for sequencing at the University of Oregon Genomics Core Facility. 50 bp paired-end sequencing was conducted with an Illumina HiSeq 2000 sequencer; sequence (fastq) files were transferred to the Oregon State University Center for Genome Research and Biocomputing for analysis. Sequences were filtered based on Illumina quality scores and analyzed for quality using FastQC analytical software (Babraham Bioinformatics). Reads were trimmed to exclude low quality sequences at the end of reads.

Sequence mapping, transcriptome assembly and statistical analysis

Paired-end sequence reads were aligned with TopHat version 2.0.7 (Trapnell et al. 2009) to *Danio rerio* genome assembly Zv9.70 using the following parameters: 50 bp minimum intron length, 10000 bp maximum intron length, 200 mate pair inner distance, 150 bp mate pair inner distance stdev, --no-mixed option (only paired alignments). Transcripts were

assembled using Cufflinks, and merged with Cuffmerge using Zv9.70 as a guide to create a GTF file with all transcripts assembled from our data (Trapnell et al. 2012). Differentially expressed transcripts, first including novel transcripts from our dataset, were identified with Cuffdiff by comparing BEZO and 7,12 B[a]AQ exposed samples to the 1% DMSO control. Upper quartile normalization and bias correction using Zv9.70 were performed, and an FDR of 0.01 was applied. Differentially expressed genes with an adjusted p value of <0.05 were considered significant. We conducted a second statistical analysis in Cuffdiff, using only the Zv9.70 transcriptome (no novel transcripts), to identify significant gene lists with better annotation for analysis of biological functions for each BEZO and 7,12-B[a]AQ, using an FDR of 0.05. Log_2 fold change (log_2FC) values were calculated by comparing each sample FPKM value to the mean control FPKM value. For comparisons with microarray PAH data, log_2FC values based on fluorescence intensity values were used, microarray data is available in the NCBI GEO omnibus Accession: GSE44130 (Goodale et al. 2013). Hierarchical clustering and heatmap visualizations were conducted with Multiexperiment Viewer (MeV) (Saeed et al. 2003).

Pathway analysis

Go Rilla (Gene Ontology enRIchment anaLysis and visualization) tool was used to identify enriched gene ontology (GO) terms from clusters of genes in the BEZO-7,12-B[a]AQ heatmap (Eden et al. 2009). Official gene symbols were used, with the background of official gene symbols for all transcripts identified in the dataset. GO Rilla recognized 15,768 zebrafish gene symbols provided from our combined GTF file. GO terms with p values <0.001 were considered significant.

The Bioinformatics Resource Manager v 2.3 (BRM) was used to identify Entrez IDs and human homologs of significant genes identified in our statistical comparison using the Zv9 transcriptome (Tilton et al. 2012). We used Metacore GeneGo software to identify enriched biological processes, GO terms, and predicted transcription factors from the BEZO and 7,12-B[a]AQ significant gene lists (p <0.05). Statistical significance of over-connected interactions was calculated using a hypergeometric distribution, where the P value represents the probability of a particular mapping arising by chance for experimental data compared to the background (Nikolsky et al. 2009). Networks were constructed in

MetaCore for experimental data using an algorithm that identifies the shortest path to directly connect nodes in the dataset to transcription factors. Network visualizations were generated in Cytoscape (Shannon et al. 2003). MetaCore processes, GO terms, and predicted transcription factors were filtered to include only those associated with at least 10 genes in the dataset. For MetaCore processes, a statistical cutoff of $P < 0.005$ was used. GO terms with associated P values < 0.001 were considered significant, and the 20 most significant GO terms for each OPAH were reported. Transcription factors with statistical overconnectedness P values < 0.001 were considered significant.

Results

AHR2 morphants are resistant to BEZO and 7,12-B[a]AQ induced developmental toxicity

We investigated the role of AHR2 in developmental toxicity of 7,12-B[a]AQ and BEZO by exposing control and *ahr2* morpholino-injected (morphant) zebrafish embryos to 0, 5, 7.5 and 10 μM concentrations of OPAH and observing morphological changes at 120 hpf. Both 7,12-B[a]AQ and BEZO induced a concentration-dependent increase in the percentage of control -injected embryos with pericardial edema (Figure 2A and B, dark circles, F and G). Interestingly, neither 7,12-B[a]AQ nor BEZO caused a significant increase in pericardial edema in *ahr2* morphants (Figure 2A and B, light circles, I and J). BEZO and 7,12-B[a]AQ-exposed control morphants also had significant jaw, eye and axis malformations, which were not significantly increased in *ahr2* morphants (two way ANOVA, $P < 0.05$). While a slight increase in embryos exhibiting at least one malformation was observed in *ahr2* morphants exposed to 7,12-B[a]AQ or BEZO, the increases were not significant. As observed previously, 7,12-B[a]AQ and BEZO induced similar malformation profiles, which also included significant increases in jaw, yolk sac edema, and to a lesser extent curved body axis (Figure 2F, G). The compounds induced a similar concentration response, with the 7.5 μM exposure eliciting a response in nearly 100% of embryos.

BEZO and 7,12-B[a]AQ induce distinct malformations and cytochrome P450 phase 1 metabolizing enzyme expression at 48 hpf

To further compare the developmental toxicity of these 4-ring OPAHs, we focused on the earlier 48 hpf developmental time point, when robust Cyp1a expression can be visualized by immunohistochemistry following ligand exposure, but the liver and its metabolic activity

are not yet functional. We examined *cyp1a* and *cyp1b1* mRNA expression in control and *ahr2* morphant embryos exposed to 7.5 μM 7,12-B[a]AQ or BEZO, compared to vehicle control. As is expected for an Ahr2 agonist, 7,12 B[a]AQ exposure induced a robust, 197-fold average increase in *cyp1a* expression in control-injected embryos (Figure 3A). The *ahr2* morpholino caused a 64% reduction, with *cyp1a* induced 61-fold in *ahr2* morphants exposed to 7,12 B[a]AQ. *Cyp1b1* expression was also induced by 7,12-B[a]AQ exposure, with an average 17-fold change in control-injected embryos (Figure 3A). *cyp1b1* induction was 96% prevented with the *ahr2* morpholino; basal expression was significantly (albeit only 1.8 fold) higher in *ahr2* morphants compared to control morphants, and was not significantly induced by 7,12 B[a]AQ exposure. 7,12-B[a]AQ induced malformations, including pooling of blood ventral to the developing heart, slight curved body axis and reduced size, which were apparent by 48 hpf (Figure 3E, compared to 3C vehicle control). Cyp1a protein, visualized by whole-mount immunohistochemistry, was robustly expressed throughout the trunk and brain vasculature, as well as the developing heart (Figure 3K, compared to 3I control). The *ahr2* morpholino completely prevented all of the morphological effects observed (Figure 3F, compared to 3C,D controls). Cyp1a protein expression was largely prevented, with some remaining vascular expression in the eye, brain, and trunk (Figure 3L). This expression is in agreement with the 61-fold change in *cyp1a* mRNA in the *ahr2* morphants. The incomplete block of *cyp1a* expression by morpholino is in line with previous studies, and may be due to incomplete morpholino efficacy or induction via one of the other zebrafish AHR isoforms. It is important to note, however, that it does not correspond with any observable malformations at 48 or 120 hpf.

In contrast to the strong *cyp1a* induction observed with 7,12-B[a]AQ, exposure to 7.5 μM BEZO induced a 4.5-fold increase in *cyp1a* in control morphants (Figure 3B). While statistically significant, this *cyp1a* induction is only ~2% of the 7,12-B[a]AQ response. Knockdown of *ahr2* resulted in a 42% rescue of the cyp1a induction by BEZO. *Cyp1b1* was induced 1.8-fold by BEZO exposure. This expression level was similar to that in *ahr2* morphants, which showed no difference in cyp1b1 expression between treatment groups. While *cyp1a* and *cyp1b1* induction was minimal, BEZO induced distinct physiological abnormalities by 48hpf. Severe edema was consistently observed surrounding the developing heart (Figure 3G). Compared to the 120 hpf time point, the BEZO and 7,12

B[a]AQ phenotypes could be readily distinguished at 48 hpf, where BEZO-exposed embryos had more pronounced pericardial edema, but did not exhibit the axis and shorter trunk length induced by 7,12-B[a]AQ (Figure 3G compared to 3E). BEZO-induced malformations were not accompanied with any Cyp1a protein expression detectable by whole-mount immunohistochemistry (Figure 3M). This was consistent with the very low *cyp1a* mRNA induction. In agreement with the 120 hpf malformation data, however, knockdown of AHR2 completely rescued the BEZO-induced developmental abnormalities at 48 hpf (Figure 3H).

Comparison of mRNA expression profiles with mRNA-seq

The large difference in *cyp1a* mRNA expression at an EC100 concentration for malformations suggested differences in transcriptional regulation between BEZO and B[a]AQ. We used whole genome mRNA expression profiling (mRNA-seq) to identify global mRNA expression changes induced by these two OPAHs at 48 hpf. Paired-end 50bp RNA-seq was conducted on embryos exposed to 10 μM 7,12-B[a]AQ, 10 μM BEZO or 1% DMSO control from 6-48 hpf. Three biological replicates of pooled RNA from 20 embryos were sequenced for each exposure group. An average of 48.2 ± 7.9(stdev) million paired sequence reads passing Illumina QC were obtained per sample. Alignment to Sanger zebrafish genome build Zv9.70 resulted in 36.2 ± 6.1 million mapped reads per sample (75%). Of these, an average of 28.8 ± 5.3 million paired reads per sample (~80%) mapped uniquely to one location in the genome (Table S2). Transcripts were assembled with cufflinks and merged with the Zv9.70 transcriptome to obtain a combined transcript file with 32,432 Ensembl genes and 13,478 novel transcripts. Comparison of each treatment with the DMSO control using CuffDiff identified 964 differentially expressed transcripts in the 7,12-B[a]AQ group and 696 in the BEZO group ($p < 0.05$ with a FDR of 0.01). The union of significant transcripts was 1351.

Are transcripts misexpressed in embryos exposed to the parent PAH BAA similarly affected by OPAHs?

We previously identified transcripts misexpressed in response to three parent PAHs, dibenzothiophene (DBT), pyrene (PYR), and benz(a)anthracene (BAA) using the Agilent mRNA microarray platform (Goodale et al. 2013). BAA is a previously-identified AHR agonist, while the other two PAHs do not induce AHR-dependent toxicity. We hypothesized

that the genes significantly misexpressed by BAA would respond similarly to 7,12-B[a]AQ, because of their identical ring structures (Figure 1). We examined the expression of these genes across 7,12-B[a]AQ and BEZO, in comparison to the parent PAHs, in order to better refine a set of genes distinct to PAHs that induce toxicity via the AHR. We also sought to discern any differences between the three putative AHR ligands, and to identify misexpressed transcripts common to all PAH exposures. Ensembl IDs and corresponding transcripts were identified in the RNA-seq dataset for 57 transcripts that were significantly misexpressed by BAA at 48 hpf in the microarray study (Goodale et al. 2013). Of these transcripts, 32 were significant for 7,12-B[a]AQ and 17 for BEZO. Caution is necessary for comparison across studies on different platforms; the magnitude of fold change values detected by microarray vs. RNA-seq have been shown to be different, and the platforms can differ in their sensitivity (van Delft et al. 2012). Comparison of significant gene lists in particular is problematic because of the different background sets and statistical methods employed. We have found heatmaps useful for visualizing patterns in expression across PAHs and identifying clusters of similarly expressed transcripts for further analysis. A heatmap of the BAA significant transcripts was created using log2FC values for each sample compared to the average control (1% DMSO) value on its platform (Fluorescence intensity for microarray, FPKM value for RNA-seq). Bidirectional hierarchical clustering was conducted in the MultiExperiment Viewer to identify clusters of differentially expressed transcripts (Figure 4). Replicates of each PAH treatment group clustered together; no platform-based clustering was observed among the controls. 7,12-B[a]AQ samples were the most distant from controls, and clustered closely with BAA. As expected for compounds lacking AHR activity, DBT and PYR clustered most closely with the control samples. The BEZO cluster grouped between the BAA-7,12-B[a]AQ cluster and the other PAHs, with less robust expression than 7,12-B[a]AQ and BAA, but also notably different than PYR and DBT. Expression patterns on the whole were similar across BEZO, BAA and 7,12-B[a]AQ, but differed in magnitude, suggesting that differences between the PAHs for this set of transcripts lie in dose, potency, or platform, rather than mechanism. No directional differences were observed that would suggest distinct regulatory mechanisms between these PAHs. Several distinct clusters of genes were identified by the bidirectional hierarchical clustering. The genes in the top custer, *cyp1a*, *cyp1c1*, *wfikkn1*, *ahrrrb*, *cyp1c2*

and *cyp1b1*were the most highly induced by BAA and 7,12-B[a]AQ, with mean FC values reaching 230 for *cyp1a*. They were also induced by BEZO, but to a lower extent (4-fold or less), in agreement with our previous data for *cyp1a*. A large group of genes below the top cluster were only mildly induced by BAA, and were more variable for the rest of the PAHs. A small cluster of genes in the middle of the heatmap, *s100z*, *cxcr4a*, *slc1a4* and *psat1*, were consistently induced by all 5 PAH exposures. *Cxr4a* and *s100z* were previously identified in the microarray study among the most highly elevated transcripts across the three parent PAH structures (Goodale et al. 2013). The cluster below this consisted of transcripts that were robustly elevated by BEZO, B[a]AQ and BAA, such as *ctsl.1*, *sult6b1* and *gsr*, but were not increased in DBT and PYR samples. Finally, the bottom cluster of the heatmap consisted of transcripts generally repressed by all 5 PAHs. This transcript set had subtle differences in expression levels between the AHR-activating vs AHR-independent PAHs. In order to investigate whether log2FC values in the heatmap accurately portrayed expression trends across compounds that were tested on different platforms, we validated expression of genes from multiple clusters with qRT-PCR across all 5 PAHs (Table 1). Log_2FC values of *cyp1a*, *ctsl.1*, *sult6b1*, and *cxcr4a* were very consistent between q-RT-PCR and both genome-wide platforms. Expression patterns of *ctgfb* and *s100z*, which were not as highly induced by any of the PAHs, were consistent in the qRT-PCR, but statistical significance was more variable across platforms (Table 1). Statistical analysis methods employed (ANOVA across treatment groups vs. pairwise with control, multiple testing corrections) were different between qRT-PCR, microarray, and RNA-seq. Significant gene list comparisons may therefore result in erroneous conclusions with this data. Our qRT-PCR analysis supports the idea, however, that clustering based on gene expression values may be a useful way to identify meaningful differences between PAHs using data from these two platforms. Based on the clustering analysis presented here with the AHR-related transcript list, we might predict BEZO to be an AHR agonist with lower potency than BAA and 7,12-B[a]AQ.

Novel BEZO and 7,12-B[a]AQ-induced transcripts identified with RNA-seq

mRNA-seq data provides a rich resource to compare expression across the entire genome, including novel transcripts not yet annotated in the current assembly. In order to identify transcriptional differences between BEZO and 7,12-B[a]AQ that might explain morphological and apparent potency differences for inducing AHR-related targets, we used

Cuffdiff to compare each OPAH treatment group to the 1% DMSO control. In our primary analysis, we employed a transcript set that included all transcripts in the zebrafish genome (Zv9) as well as novel transcripts identified in our dataset.

Comparison of the significant gene lists ($p < 0.05$, 0.01 FDR) revealed 309 transcripts that were significantly misexpressed in response to both PAHs. 387 transcripts were unique to BEZO, and 655 were unique to BaAQ. 366 of the significant transcripts had a fold change > 2 (Figure 5A). Of these, 125 transcripts were unique to BEZO, while 171 were unique to 7,12-B[a]AQ. 70 transcripts were similarly expressed between the two OPAHs, and the numbers of over- vs under-expressed transcripts were nearly equivalent. 10 of the transcripts similarly expressed in the BEZO and 7,12-B[a]AQ groups were not annotated in Zv9 (novel to our dataset). BEZO and 7,12-B[a]AQ uniquely induced changes in 22 and 33 novel transcripts, respectively. Annotation of the zebrafish genome has undergone rapid improvement in recent years; experimentally identified transcripts from RNA-seq studies, however, have yet to be fully incorporated into the transcriptome. While "novel" transcripts may result from sequencing errors, repeats, or incorrect assembly, they may also represent as-of-yet unidentified genes that could be involved in mechanisms of toxicity, which are not yet fully elucidated for the AHR. We examined novel transcripts that had the largest fold changes between treatment groups using both Gbrowse, to examine coverage across our samples, as well as with the Ensembl genome browser to view predicted transcripts from publicly available RNA-seq data. Many of our "novel" transcripts corresponded with regions of high transcriptional coverage in other datasets, but were not annotated as transcripts in publicly available databases. Novel transcript XLOC_030523 was one of the most highly induced transcripts in the 7,12-B[a]AQ treatment groups (9.7 fold, $p < 0.05$). The genomic context of this predicted transcript (Chromosome 3: 63102008-63105155) is displayed in Figure 5B (top). The pooled RNA-seq alignment track shows a long region of moderate coverage upstream of *sox9b* on the -1 strand, and a predicted transcript based on this RNA-seq data (5 dpf exon track). There are no Ensembl/Havana annotated transcripts within this region. The Gbrowse view (Figure 5B, bottom) shows the predicted transcript from our dataset, along with neighboring transcripts that were likely predicted as separate because of the low coverage. The control samples all had very low coverage of this region, while the 7,12-B[a]AQ samples had much higher coverage. This supports the Cufflinks-Cuffdiff

identification of this as an overexpressed transcript in the 7,12-B[a]AQ -exposed embryos. Several of the neighboring predicted transcripts were also significantly increased by 7,12-B[a]AQ, but not by BEZO. By visualizing our data alongside a set of recently-identified long intergenic non-coding RNAs (lincRNAs) we discovered that several of our predicted "novel" transcripts are part of a leader-like lincRNA (Chew et al. 2013). Its location near *sox9b* is particularly intriguing, given the important role of *sox9b* in mediating AHR-dependent effects of TCDD on tissue regeneration, jaw and heart development (Andreasen et al. 2006; Xiong et al. 2008; Hofsteen et al. 2013). The mechanism by which AHR activation results in decreased *sox9b* expression is not known. In our dataset *sox9b* was decreased by 7,12-B[a]AQ, though not significantly, perhaps because RNA was from whole embryo rather than specific tissue types. Novel transcripts identified within this dataset will require additional validation. Examination of the genomic context, as demonstrated in Figure 5B, suggested that many are not sequencing artifacts but rather have not yet been annotated in Zv9, and should be included in our comparison of BEZO and 7,12-B[a]AQ.

We performed hierarchical clustering on the union of BEZO and 7,12-B[a]AQ significantly misexpressed transcripts with FC >2, and identified clusters of transcripts with different expression patterns (Figure 5C). Considering now the entire set of misexpressed transcripts, rather than the AHR agonist-associated transcripts discussed earlier, a set of genes underexpressed in BEZO and 7,12-B[a]AQ-exposed embryos was notable. Few genes were identified in the microarray as down-regulated by BAA. This could potentially reflect a difference in the platforms used; we nevertheless compared expression of the BEZO and 7,12-B[a]AQ significant transcripts from Figure 5C across all 5 PAHs (Figure S3). The genes down-regulated by the OPAHs were generally not affected by BAA. Many, however, were similarly misregulated by DBT and PYR. There was also a notable group of transcripts induced by the OPAHs, including *gstp1,* which were not strongly affected by the parent PAHs. Differences between the OPAHs remained subtle, however. No oppositely expressed gene clusters were apparent between BEZO and 7,12-B[a]AQ.

We used GO rilla Gene Ontology enrichment analysis to investigate biological processes associated with differentially expressed gene clusters (Figure 5C). Because significant transcripts were identified from our merged transcriptome, many did not have sufficient

annotation for ontology analysis (novel transcripts and transcripts lacking official gene names); biological processes were only identified for 5 of the clusters. The significant (P <0.001) GO process for cluster 12, which contained the previously-discussed *cyp1* genes, was Response to Chemical Stimulus (GO:0042221, Figure 5C bottom). The only significant term identified for the large group of transcripts overexpressed in both BEZO and 7,12-B[a]AQ was Oxidation-reduction Process (GO:0055114). Cluster 8, expressed more highly in BEZO-exposed embryos, was enriched for Cell-cycle Arrest (GO:0007050). Phototransduction (GO:0007602) was significant for the very small but prominent cluster 7, which was underexpressed in BEZO-exposed embryos and contained *opn1lw2*, *opn1sw1* (opsin isoforms) and *crygm2d11* (gamma D crystallin). Another group of genes in cluster 5, containing *rs1* (retinoschisin) and *arr3a* (retinal arrestin) were enriched for Visual Perception (GO:0007601). No significant biological processes were identified for the remaining clusters of misexpressed transcripts.

Ahr2 knockdown prevents induction of transcripts unique to BEZO and 7,12-B[a]AQ toxicity profiles

Knockdown of AHR2 prevented induction of morphological defects by BEZO and 7,12-B[a]AQ. AHR binding for these compounds, however, has not been determined. It is feasible that some transcriptional changes observed are not dependent on AHR2, particularly if AHR-dependent toxicity is mediated via an intermediate (endogenous ligand or metabolite) following an upstream interaction of the OPAH with another target. We selected a set of differentially expressed transcripts from the RNA-seq dataset and investigated their expression in *ahr2* morphants with qRT-PCR. We focused on lesser-known transcripts since AHR2-dependence of known AHR targets (Cyp1 enzymes) was previously established. *wfikkn1*, a top BAA target identified in the microarray analysis, was induced 5.5 fold by 7,12-B[a]AQ (Figure 6A), but not by BEZO. No significant induction was observed in the *ahr2* morphants. *Glutathione S-transferase pi 2* (*gstp2*), by contrast, is a redox responsive gene and was significantly induced by both 7,12-B[a]AQ (4.8 fold) and BEZO (3.1 fold). Again no significant induction was observed in *ahr2* morphants (Figure 6B). *Arginase 2* (*arg2*) was one of the few genes significantly induced by BEZO but unchanged by 7,12-B[a]AQ (Figure 5C cluster 8). This differential expression was confirmed by qRT-PCR in the control morphants, and AHR2 knockdown prevented *arg2* induction by BEZO (Figure 6C).

We also confirmed induction of *insulin-like growth factor binding protein 1a* (*igfbp1a*), one of the transcripts most highly induced by BEZO, and *plac-8 onzin related like 4* (*ponzr4*), one of the transcripts most reduced by OPAH exposure in the RNA-seq dataset (Figure 6D,E). Comparative expression levels detected by qRT-PCR between BEZO and 7,12-B[a]AQ were consistent with those observed with RNA-seq, and AHR2 knockdown prevented misexpression of both of these genes (Figure 6D,E striped bars). Finally, we investigated *p53* expression, as a master regulator that was induced by BEZO in the RNA-seq dataset. A very slight (1.2 fold) induction was detected by qRT-PCR, with no significant difference between 7,12-B[a]AQ and BEZO. *ahr2* morphants had slightly higher *p53* expression than control morphants, which was not affected by OPAH exposure (Figure 6F).

Predicted transcription factors and biological processes affected by BEZO and 7,12-B[a]AQ

To better understand biological processes affected by exposure to BEZO and 7,12-B[a]AQ during development, we analyzed the entire sets of genes significantly affected by each OPAH for statistically enriched biological processes. For this analysis, we used significant genes identified by CuffDiff analysis across transcripts annotated in the zebrafish (Zv9) transcriptome (novel transcripts were not included). Significant transcripts for 7,12-B[a]AQ and BEZO exposures are listed in Tables S3 and S4, respectively. When compared to the previous analysis, which included novel transcripts, we observed good overlap in the significant gene lists; 337(93%) and 317(83%) of genes significant for 7,12-BaAQ and BEZO, respectively, were previously identified as significant. Entrez IDs (human homolog preferred) were identified for 587 of the 600 significant genes. Metacore GeneGo software was used to identify significant biological processes, gene ontology terms, and transcriptional regulators associated with the significant gene lists. The most significant process invoked by both OPAHs was Hypoxia and Oxidative Stress Response (Figure 7A). As suggested by the heatmap clustering (Figure 5C), Visual Perception was highly affected by BEZO, but not 7,12-B[a]AQ. Immune-Related Processes Involving the Complement and Kallikrein-Kinin Systems, as well as Inflammation (Phagocytosis) and Cell Cycle were affected by 7,12-B[a]AQ. The significant gene ontology terms agreed with these processes, and provide more insight into affected molecular mechanisms. Metabolic processes and response to chemical stimulus were the most significantly enriched GO terms, and involved over 150 genes (Table 2). The majority of the most enriched GO terms for each OPAH were

significant for both ($p < 0.001$). Notably, Tissue Development and Oxidation-reduction Processes were less significant in BEZO, while Visual Perception and Sensory Perception of Light Stimulus were not significant for 7,12-B[a]AQ. The decreased expression of eye-related genes (opsins, crystallins, *visual system homeobox 1*, *retina and anterior neural fold homeobox 1*) was also reflected in the transcription factors (TFs) predicted to be important in the BEZO response. NR2E3 nuclear receptor subfamily 2, group E, member 3 (also known as PNR, photoreceptor-specific nuclear receptor) and Maf (v-maf avian musculaponeurotic fibrosarcoma oncogene homolog) were among the most significant, and are both important in eye development. Other top predicted TFs for BEZO were C/EBPbeta and SP1. Among the most significant TFs predicted for 7,12-B[a]AQ were C/EBPbeta, ATF-2, RELA and c-Jun (Table 2). Both OPAHs were predicted to regulate networks involving HIF1A, C/EBPalpha, c-Myc and NRF2. Many of these TFs interact, and tightly coordinate responses to oxidative stress/redox homeostasis as well as many other cellular functions. The enriched biological processes and transcription factors highlight the prominent role of oxidative stress and hypoxia-related signaling for both of these oxygenated PAHs, while other processes, such as visual perception and AHR-mediated metabolic/chemical response processes, represent potential structure-dependent differences in toxicological mechanisms.

While AHR was significant ($P < 4.26E-04$ and $2.49E-03$ for 7,12-B[a]AQ and BEZO, respectively), it was not as significant as other TFs. The number of genes predicted with high confidence to interact with AHR was also much lower. Because we experimentally determined that both BEZO and 7,12-B[a]AQ-induced developmental toxicity depended on AHR2, we were interested to identify known interactors of AHR that were misexpressed in BEZO and 7,12-B[a]AQ-exposed embryos. Significantly misexpressed genes that have been shown to interact with the AHR (including both high and low confidence interactions) are displayed in Figure 6B. BEZO (blue) and 7,12-B[a]AQ (yellow) significant genes had some overlap, which included overexpressed TFs NFE2L1 (homolog of antioxidant regulator NRF, which shares highest similarity with *nrf2b* in zebrafish) and CITED2 (Cbp/p300-interacting transactivator), among others (Timme-Laragy et al. 2012). Though a large group of AHR-interacting genes were unique to 7,12-B[a]AQ (Figure 7B, yellow), the only TF was FOXQ1 (Forkead box Q1) which mediates TCDD-induced jaw malformations (Planchart and Mattingly 2010). AHR-interacting genes unique to BEZO included TFs P53, C/EBPbeta, and

CXCR4. While these were induced by both OPAHs, they were induced more strongly (and significantly) by BEZO, and could potentially contribute to the severe developmental toxicity of BEZO despite its weak induction of canonical AHR target genes.

Discussion

We found that BEZO and 7,12-B[a]AQ caused morphological abnormalities in developing zebrafish, including disrupted heart development, craniofacial abnormalities, eye defects and edema, at similar waterborne exposure concentrations. Their disparate induction of *cyp1a* mRNA and protein expression, which was identified previously and confirmed in this study, led us to predict differential involvement of the AHR in the toxicity mechanisms of these environmentally relevant OPAHs (Knecht et al. 2013). Despite the practically negligible induction of *cyp1a* and lack of *cyp1b1* induction by BEZO, the BEZO-induced toxicological endpoints evaluated in this study were dependent on AHR2. Cyp1a expression and metabolic activity are both widely used as biomarkers of AHR activation, particularly for environmental monitoring studies. For dioxin-like compounds, Cyp1a has been demonstrated to correlate well with ligand affinity for the receptor as well as AHR-associated toxicological effects in a plethora of organisms (Safe 1998; Billiard et al. 2002). AHR activation and Cyp1a expression are also associated with toxicity of many PAHs, such as benzo(a)pyrene. However, the structural diversity of the parent compounds, not to mention their substituted derivatives and propensity for metabolism, complicates prediction and interpretation of AHR interactions for this chemical class. This has been previously shown in zebrafish, where 4- and 5-ring PAH structures differentially interact with AHRs, resulting in tissue-specific Cyp1a expression patterns as well as a range of morphological effects (Incardona et al. 2006; Incardona et al. 2011; Knecht et al. 2013). Fluoranthene and dibenzothiophene inhibit Cyp1a activity, though the mechanism by which these PAHs act as inhibitors remains unknown (Willett et al. 2001; Wassenberg et al. 2005). Studies with AHR agonists and alternative AHR ligands, both in cell culture and animal models, have recently highlighted how ligands can differentially mediate a multitude of AHR-dependent biological processes (Patel et al. 2009; Murray et al. 2011; Narayanan et al. 2012). Adding to the complexity are reports of AHR-mediated responses in the absence of a xenobiotic ligand, including both *cyp1a* induction in hyperoxia, as well as decreased *cyp1a* expression resulting from oxidative stress (Barker et al. 1994; Couroucli et al. 2002) In this

context, an environmentally-relevant OPAH that induces AHR-dependent developmental toxicity in the absence of strong Cyp1a expression should perhaps not be entirely unexpected.

Because of its use in dye manufacture and associated reports of dermal lesions and decreased liver function in exposed workers, a number of studies have investigated BEZO toxicity in rodents. Exposure to high concentrations of BEZO causes decreased ascorbic acid associated with liver, kidney, and testis histopathological changes in guinea pigs, which could be attenuated with ascorbic acid supplementation (Das et al. 1994). In agreement with our study, Singh et al. observed decreased cytochrome P-450 phase 1 enzymes and ethoxyresorufin-O-deethylase (EROD) activity in guinea pigs exposed to BEZO (2003). Both in that study and others, BEZO caused a decrease in glutathione (GSH), and an increase in cytochrome P-450 phase 2 enzymes including glutathione peroxidase and glutathione reductase (Dwivedi et al. 2001). To our knowledge, involvement of the AHR in these effects awaits investigation.

Using mRNA sequencing, we compared the global transcriptional profile of BEZO with that of Cyp1a-inducer 7,12-B[a]AQ, as well as parent PAHs previously investigated via microarray. Because of the 60-fold difference in *cyp1a* expression at concentrations that induce comparable toxicity, we anticipated that other genes associated with AHR activation would be differentially regulated by BEZO and 7,12-B[a]AQ, helping to elucidate alternate toxicity mechanisms. We previously identified a set of transcripts differentially expressed in zebrafish embryos exposed to BAA, the unsubstituted parent PAH of 7,12-B[a]AQ, which induces AHR2-dependent morphological abnormalities in zebrafish, and toxicity in a variety of model systems (Incardona et al. 2006; Jennings 2012). Few of the BAA-induced transcripts were similarly regulated by other parent PAHs that exert AHR-independent toxic effects. We identified differential regulation of this specific set of AHR-interacting transcripts by examining their fold change values in BEZO and 7,12-B[a]AQ exposed embryos (compared to controls). In addition to *cyp1a*, *cyp1c1*, *cyp1c2*, *cyp1b1*, *ahrrb* and *wfikkn1* were more highly expressed in 7,12-B[a]AQ than BEZO. With the exception of *wfikkn1*, these transcripts are all known targets of the AHR (Baba et al. 2001; Jonsson et al. 2007). Other transcripts associated with AHR activation, such as oxidative stress and phase

2 metabolizing enzymes *gsr*, *prdx1*, and *sult6b1*, were induced at comparable levels by BEZO, 7,12-B[a]AQ and BAA. None of the BAA transcripts were oppositely expressed in response to BEZO vs. 7,12-B[a]AQ and BAA. While expression of the phase 1 metabolizing enzymes implies a potency, dose, or metabolism difference between these 4-ring PAHs, the consistency of expression for the rest of the transcripts across all three compounds perhaps suggests that the majority of transcriptional responses are not directly mediated by AHR, but rather by the network of transcription factors, such as NRF2, NFkB subunit RELA, CEBPB that are known to interact with AHR (Tian et al. 1999; Vogel et al. 2004; Timme-Laragy et al. 2009).

Examination of all identified transcripts that were significantly misexpressed in response to BEZO or 7,12-B[a]AQ supported the notion that these and multiple other TFs mediate the web of transcriptional changes that result from exposure to OPAHs. Many interacting and tightly regulated mechanisms coordinate to respond to stimuli such as xenobiotic exposure, hypoxia, UV irradiation and endogenous (hormone) signaling. Crosstalk between the AHR and other transcription factors has been widely reported. As a member of the PAS family of transcription factors, the AHR interacts with other PAS proteins and shares the requirement of ARNT for dimerization and transcriptional activation with HIF1A (Gu et al. 2000). AHR crosstalk via other binding partners and coactivators, including *p300* (CREB binding protein), HSP90, and the AHR repressor (AHRR) have been reported (Beischlag et al. 2008; Evans et al. 2008). We identified 366 transcripts that were significantly differentially expressed 2 fold or greater in response to OPAH exposure. Despite the fact that only 19% of these were significant in both OPAH exposure groups, we did not identify any clusters of transcripts with strong evidence of opposite (down vs. up) regulation between BEZO and 7,12-B[a]AQ. Rather, we observed clusters with more subtle differences in the degree of regulation. Phase 1 enzymes, as discussed previously, were more robustly induced by 7,12-B[a]AQ. BEZO, on the other hand, induced striking decreased expression of a cluster of genes involved in photoreception, which were relatively unaffected by 7,12-B[a]AQ. The largest clusters of significant transcripts in the BEZO/7,12-B[a]AQ comparison (Figure 5C) had similar levels of expression, however. The observation that relatively few of these transcripts were similarly disrupted in embryos exposed to the parent PAH BAA (Figure S1) suggests that perhaps the bulk of the transcriptional response to OPAHs is not mediated by

direct ligand activation of the AHR. These more reactive PAHs likely interact with cells in a multitude of other ways, causing oxidative stress, DNA and/or protein damage. It is important to keep in mind that a global transcriptional analysis does not discern between adaptive and harmful responses; indeed many of the pathways induced by these PAHs, such as NRF2-mediated antioxidant activity, likely protect the embryo from damage rather than mediate toxic effects (Van Tiem and Di Giulio 2011; Garner and Di Giulio 2012).

By comparing the BEZO and 7,12-B[a]AQ-induced transcripts across all PAHs, we identified clusters of transcripts that are differentially expressed in response to diverse PAH structures. We found that many of the transcripts misexpressed in response to the OPAHs followed similar expression patterns in DBT and PYR-exposed embryos. Inflammatory signaling via NFkB and CEBPB was significant for both OPAHs as well as DBT and PYR in the microarray analysis (Goodale et al. 2013); these pathways may be similarly involved in responding to PAH exposure via a mechanism that can be activated by a broad range of structures. We compared transcript expression across all 5 PAHs at concentrations that induced malformations, and were able to identify genes more consistently expressed than the *cyp1* transcripts, which may be useful to predict AHR-independent biological effects for mixtures containing multiple PAHs. Of all PAHs investigated, BAA induced expression of the smallest number of significant genes, a pattern which is supported in the heatmap (Figure S1). We attributed some of this difference to uptake, which was much lower for BAA than the other two parent PAHs. The toxicity of BAA demonstrated, for the parent PAHs, that a PAH with affinity for the AHR can induce toxicity at a much lower body burden than PAHs that do not induce AHR activity. Without body burden data we cannot definitively discern uptake vs. mechanistic differences between the OPAHs. Based on the similar log Kows of BEZO and 7,12-B[a]AQ (4.81 and 4.4, respectively), however, we would not predict large uptake differences (Meylan and Howard 1995) The similarity of expression among transcripts (in exception of the canonical AHR targets), as well as transcriptional clusters that were more strongly misexpressed by BEZO than 7,12-B[a]AQ, additionally suggests that the difference between these compounds is more complex than uptake. Further exploration of differential AHR-mediated pathways will be necessary to identify true mechanistic differences.

The relative insignificance of AHR in the predicted biological functions and transcription factors for BEZO and 7,12-B[a]AQ raises the question of why AHR2 knockdown offers pronounced protection against these compounds. For 7,12-B[a]AQ and parent PAH BAA, we might predict that following the initial binding to the AHR, a cascade of effects mediated by both the AHR response complex and metabolites leads to the wide array of transcriptional changes. For BEZO, however, the mechanism is less clear; is BEZO binding the AHR directly? If so, why isn't Cyp1a robustly expressed? We investigated *ahr2* dependence of transcripts that were highly misexpressed by BEZO, but are not known to be associated with AHR. If BEZO was affecting other pathways, and perhaps activating the AHR via a crosstalk mechanism, we would expect some transcripts to be induced in the absence of AHR2. In the small set of transcripts investigated here, however, we saw no evidence of AHR2-independent regulation. Rather, we identified *arg2*, and *igfbp1a*, AHR2-dependent transcripts induced more strongly by BEZO than 7,12-B[a]AQ.

The subtle differences in gene expression that correspond with distinct morphologies at 48 hpf in our study suggest differential interactions of the AHR with other TFs or co-activators. Regulation of TFs often occurs post-transcriptionally, so we would not necessarily expect large changes in TF transcripts themselves. A predicted network from the distinct cluster of genes associated with visual perception in BEZO-exposed embryos predicted the involvement of p53, which has been previously demonstrated to be involved in disrupted eye development in zebrafish (Kim et al. 2013). P53 was significantly induced 1.5 fold by BEZO, but only 1.1 fold (not significantly) by 7,12-B[a]AQ in the RNA-seq analysis. We saw no difference, however, between BEZO and 7,12-B[a]AQ with qRT-PCR, and expression of p53 was actually increased slightly in *ahr2* mutants. Interestingly, *arg2*, which is induced by hypoxia and involved in endothelial dysfunction/reduced NO signaling, has also recently been implicated in retinopathy (Durante 2013). *Arg2* deficient mice are resistant to retinal degeneration induced in oxygen induced retinopathy (Narayanan et al. 2011). While the mechanism of AHR2-dependent *arg2* induction remains unknown, it may be a mediator of eye-specific effects observed with BEZO. Another transcription factor, CCAAT/enhancer binding protein beta (CEBPB), was also significantly induced by BEZO 1.7 fold, but not by 7,12-B[a]AQ. CEBPB is a mediator of the acute phase response, and AHR activation by TCDD

was shown to decrease C/EBPB presence on the promoter of acute phase gene *Saa3* (Patel et al. 2009).

Further studies are needed to elucidate the involvement of other predicted transcription factors and interacting proteins, such as ARNT and CEBPB, in the toxicological effects mediated by these OPAHs. Several selective AHR modulators (SAhRMs), which bind to the AHR and repress inflammatory signaling via a mechanism that does not involve binding to AHR response elements, have been identified in cell culture (Patel et al. 2009). SAhRMs do not induce canonical AHR signaling such as CYP1A expression, and it is hypothesized that repressive activity occurs via AHR interaction with coactivators /repressors (Narayanan et al. 2012). We observed reductions in acute phase response genes (*c3*, *c4*, *cfdl*) in response to OPAH exposure, which suggests that these alternate mechanisms could also explain some of the *ahr2*-dependent gene regulation observed in our study. There is evidence of cell-type specific activities of the AHR,however. Interactions observed in a particular cell line may therefore not translate to the same transcriptional relationships in the whole animal context.Further investigation will be necessary to elucidate non-canonical AHR2 signaling pathways in the developing embryo. Comparative transcriptional profiling of PAHs demonstrates that PAHs likely mediate toxicity via a suite of mechanisms, including both canonical and non-canonical AHR signaling, depending on structure. We have identified clusters of transcripts associated with structures that induced AHR-dependent and AHR-independent toxicity; further elucidation of the complex web of AHR interactions will catalyze predictive capability for this diverse group of environmentally pertinent chemicals.

Acknowledgements

The authors would like to thank Cari Buchner, Carrie Barton and the staff at the Sinnhuber Aquatic Research Laboratory for their excellent fish husbandry and expertise. Jason Carriere processed the RNA samples and conducted mRNA sequencing at the University of Oregon Genomics Core Facility, Eugene, OR. We would also like to thank Tanguay laboratory members, in particular Lisa Truong, Derik Haggard and Jill Franzosa for their advice with RNA-seq analysis and critical review of the manuscript. We are grateful to OSU Superfund Research Program Core D/Anderson Laboratory for their analytical support, advice, and providing OPAHs. This work was supported by the NIEHS Superfund Research Program P42 ES016465, Core Center Grant P30 ES000210 and the NIEHS Training Grant T32 ES007060.

Pacific Northwest National Laboratory is a multi-program national laboratory operated by Battelle Memorial Institute for the DOE under contract number DE-AC05-76RLO1830.

References

Andreasen, E. A., M. E. Hahn, et al. (2002). "The zebrafish (Danio rerio) aryl hydrocarbon receptor type 1 is a novel vertebrate receptor." Mol Pharmacol **62**(2): 234-249.

Andreasen, E. A., L. K. Mathew, et al. (2006). "Regenerative growth is impacted by TCDD: gene expression analysis reveals extracellular matrix modulation." Toxicol Sci **92**(1): 254-269.

Baba, T., J. Mimura, et al. (2001). "Structure and expression of the Ah receptor repressor gene." J Biol Chem **276**(35): 33101-33110.

Barker, C. W., J. B. Fagan, et al. (1994). "Down-regulation of P4501A1 and P4501A2 mRNA expression in isolated hepatocytes by oxidative stress." J Biol Chem **269**(6): 3985-3990.

Beischlag, T. V., J. Luis Morales, et al. (2008). "The aryl hydrocarbon receptor complex and the control of gene expression." Crit Rev Eukaryot Gene Expr **18**(3): 207-250.

Billiard, S. M., M. E. Hahn, et al. (2002). "Binding of polycyclic aromatic hydrocarbons (PAHs) to teleost aryl hydrocarbon receptors (AHRs)." Comp Biochem Physiol B Biochem Mol Biol **133**(1): 55-68.

Bostrom, C. E., P. Gerde, et al. (2002). "Cancer risk assessment, indicators, and guidelines for polycyclic aromatic hydrocarbons in the ambient air." Environ Health Perspect **110 Suppl 3**: 451-488.

Chew, G. L., A. Pauli, et al. (2013). "Ribosome profiling reveals resemblance between long non-coding RNAs and 5' leaders of coding RNAs." Development **140**(13): 2828-2834.

Couroucli, X. I., S. E. Welty, et al. (2002). "Regulation of pulmonary and hepatic cytochrome P4501A expression in the rat by hyperoxia: implications for hyperoxic lung injury." Mol Pharmacol **61**(3): 507-515.

Das, M., K. Garg, et al. (1994). "Attenuation of benzanthrone toxicity by ascorbic acid in guinea pigs." Fundam Appl Toxicol **22**(3): 447-456.

Durant, J. L., W. F. Busby, Jr., et al. (1996). "Human cell mutagenicity of oxygenated, nitrated and unsubstituted polycyclic aromatic hydrocarbons associated with urban aerosols." Mutat Res **371**(3-4): 123-157.

Durante, W. (2013). "Role of arginase in vessel wall remodeling." Front Immunol **4**: 111.

Dwivedi, N., M. Das, et al. (2001). "Role of biological antioxidants in benzanthrone toxicity." Archives of Toxicology **75**(4): 221-226.

Eden, E., R. Navon, et al. (2009). "GOrilla: a tool for discovery and visualization of enriched GO terms in ranked gene lists." BMC Bioinformatics **10**: 48.

EPA, U. (2012). "Integrated Risk Information System." from http://www.epa.gov/iris/.

Evans, B. R., S. I. Karchner, et al. (2008). "Repression of aryl hydrocarbon receptor (AHR) signaling by AHR repressor: role of DNA binding and competition for AHR nuclear translocator." Mol Pharmacol **73**(2): 387-398.

Garner, L. V. and R. T. Di Giulio (2012). "Glutathione transferase pi class 2 (GSTp2) protects against the cardiac deformities caused by exposure to PAHs but not PCB-126 in zebrafish embryos." Comp Biochem Physiol C Toxicol Pharmacol **155**(4): 573-579.

Goodale, B. C., S. C. Tilton, et al. (2013). "Structurally distinct polycyclic aromatic hydrocarbons induce differential transcriptional responses in developing zebrafish." Toxicol Appl Pharmacol.

Gu, Y. Z., J. B. Hogenesch, et al. (2000). "The PAS superfamily: sensors of environmental and developmental signals." Annu Rev Pharmacol Toxicol **40**: 519-561.

Gurbani, D., S. K. Bharti, et al. (2013). "Polycyclic aromatic hydrocarbons and their quinones modulate the metabolic profile and induce DNA damage in human alveolar and bronchiolar cells." Int J Hyg Environ Health.

Hahn, M. E. (1998). "The aryl hydrocarbon receptor: a comparative perspective." Comp Biochem Physiol C Pharmacol Toxicol Endocrinol **121**(1-3): 23-53.

Hofsteen, P., J. Plavicki, et al. (2013). "Sox9b is Required for Epicardium Formation and Plays a Role in TCDD-induced Heart Malformation in Zebrafish." Mol Pharmacol.

Incardona, J. P., H. L. Day, et al. (2006). "Developmental toxicity of 4-ring polycyclic aromatic hydrocarbons in zebrafish is differentially dependent on AH receptor isoforms and hepatic cytochrome P4501A metabolism." Toxicol Appl Pharmacol **217**(3): 308-321.

Incardona, J. P., T. L. Linbo, et al. (2011). "Cardiac toxicity of 5-ring polycyclic aromatic hydrocarbons is differentially dependent on the aryl hydrocarbon receptor 2 isoform during zebrafish development." Toxicol Appl Pharmacol **257**(2): 242-249.

Jennings, A. A. (2012). "Worldwide regulatory guidance values for surface soil exposure to carcinogenic or mutagenic polycyclic aromatic hydrocarbons." J Environ Manage **110**: 82-102.

Jonsson, M. E., M. J. Jenny, et al. (2007). "Role of AHR2 in the expression of novel cytochrome P450 1 family genes, cell cycle genes, and morphological defects in developing zebra fish exposed to 3,3',4,4',5-pentachlorobiphenyl or 2,3,7,8-tetrachlorodibenzo-p-dioxin." Toxicol Sci **100**(1): 180-193.

Karchner, S. I., D. G. Franks, et al. (2005). "AHR1B, a new functional aryl hydrocarbon receptor in zebrafish: tandem arrangement of ahr1b and ahr2 genes." Biochem J **392**(Pt 1): 153-161.

Kim, K. T., T. Zaikova, et al. (2013). "Gold nanoparticles disrupt zebrafish eye development and pigmentation." Toxicol Sci **133**(2): 275-288.

Kimmel, C. B., W. W. Ballard, et al. (1995). "Stages of embryonic development of the zebrafish." Dev Dyn **203**(3): 253-310.

Knecht, A. L., B. C. Goodale, et al. (2013). "Comparative developmental toxicity of environmentally relevant oxygenated PAHs." Toxicol Appl Pharmacol.

Layshock, J. A., G. Wilson, et al. (2010). "Ketone and Quinone-Substituted Polycyclic Aromatic Hydrocarbons in Mussel Tissue, Sediment, Urban Dust, and Diesel Particulate Matrices." Environmental Toxicology and Chemistry **29**(11): 2450-2460.

Lundstedt, S., Y. Persson, et al. (2006). Transformation of PAHs during ethanol-Fenton treatment of an aged gasworks' soil. Chemosphere. England. **65:** 1288-1294.

Lundstedt, S., P. A. White, et al. (2007). "Sources, fate, and toxic hazards of oxygenated polycyclic aromatic hydrocarbons (PAHs) at PAH-contaminated sites." Ambio **36**(6): 475-485.

Meylan, W. M. and P. H. Howard (1995). "Atom Fragment Contribution Method for Estimating Octanol-Water Partition-Coefficients." Journal of Pharmaceutical Sciences **84**(1): 83-92.

Murray, I. A., C. A. Flaveny, et al. (2011). "Suppression of cytokine-mediated complement factor gene expression through selective activation of the Ah receptor with 3',4'-dimethoxy-alpha-naphthoflavone." Mol Pharmacol **79**(3): 508-519.

Narayanan, G. A., I. A. Murray, et al. (2012). "Selective aryl hydrocarbon receptor modulator-mediated repression of CD55 expression induced by cytokine exposure." J Pharmacol Exp Ther **342**(2): 345-355.

Narayanan, S. P., J. Suwanpradid, et al. (2011). "Arginase 2 deletion reduces neuro-glial injury and improves retinal function in a model of retinopathy of prematurity." PLoS One **6**(7): e22460.

Nielsen, T., A. Feilberg, et al. (1999). "The variation of street air levels of PAH and other mutagenic PAC in relation to regulations of traffic emissions and the impact of atmospheric processes." Environmental Science and Pollution Research **6**(3): 133-137.

Nikolsky, Y., E. Kirillov, et al. (2009). "Functional analysis of OMICs data and small molecule compounds in an integrated "knowledge-based" platform." Methods Mol Biol **563**: 177-196.

Patel, R. D., I. A. Murray, et al. (2009). "Ah receptor represses acute-phase response gene expression without binding to its cognate response element." Lab Invest **89**(6): 695-707.

Pfaffl, M. W. (2001). "A new mathematical model for relative quantification in real-time RT-PCR." Nucleic Acids Res **29**(9): e45.

Planchart, A. and C. J. Mattingly (2010). "2,3,7,8-Tetrachlorodibenzo-p-dioxin upregulates FoxQ1b in zebrafish jaw primordium." Chem Res Toxicol **23**(3): 480-487.

Ramirez, N., A. Cuadras, et al. (2011). "Risk assessment related to atmospheric polycyclic aromatic hydrocarbons in gas and particle phases near industrial sites." Environ Health Perspect **119**(8): 1110-1116.

Reimers, M. J., J. K. La Du, et al. (2006). "Ethanol-dependent toxicity in zebrafish is partially attenuated by antioxidants." Neurotoxicol Teratol **28**(4): 497-508.

Saeed, A. I., V. Sharov, et al. (2003). "TM4: a free, open-source system for microarray data management and analysis." Biotechniques **34**(2): 374-378.

Safe, S. H. (1998). "Development validation and problems with the toxic equivalency factor approach for risk assessment of dioxins and related compounds." J Anim Sci **76**(1): 134-141.

Shannon, P., A. Markiel, et al. (2003). "Cytoscape: a software environment for integrated models of biomolecular interaction networks." Genome Res **13**(11): 2498-2504.

Singh, R. P., R. Khanna, et al. (2003). "Comparative effect of benzanthrone and 3-bromobenzanthrone on hepatic xenobiotic metabolism and anti-oxidative defense system in guinea pigs." Archives of Toxicology **77**(2): 94-99.

Svoboda, K. R., A. E. Linares, et al. (2001). "Activity regulates programmed cell death of zebrafish Rohon-Beard neurons." Development **128**(18): 3511-3520.

Tian, Y., S. Ke, et al. (1999). "Ah receptor and NF-kappaB interactions, a potential mechanism for dioxin toxicity." J Biol Chem **274**(1): 510-515.

Tilton, S. C., T. L. Tal, et al. (2012). "Bioinformatics resource manager v2.3: an integrated software environment for systems biology with microRNA and cross-species analysis tools." BMC Bioinformatics **13**(1): 311.

Timme-Laragy, A. R., S. I. Karchner, et al. (2012). "Nrf2b, novel zebrafish paralog of oxidant-responsive transcription factor NF-E2-related factor 2 (NRF2)." J Biol Chem **287**(7): 4609-4627.

Timme-Laragy, A. R., L. A. Van Tiem, et al. (2009). "Antioxidant responses and NRF2 in synergistic developmental toxicity of PAHs in zebrafish." Toxicol Sci.

Trapnell, C., L. Pachter, et al. (2009). "TopHat: discovering splice junctions with RNA-Seq." Bioinformatics **25**(9): 1105-1111.

Trapnell, C., A. Roberts, et al. (2012). "Differential gene and transcript expression analysis of RNA-seq experiments with TopHat and Cufflinks." Nat Protoc **7**(3): 562-578.

van Delft, J., S. Gaj, et al. (2012). "RNA-Seq provides new insights in the transcriptome responses induced by the carcinogen benzo[a]pyrene." Toxicol Sci **130**(2): 427-439.

Van Tiem, L. A. and R. T. Di Giulio (2011). "AHR2 knockdown prevents PAH-mediated cardiac toxicity and XRE- and ARE-associated gene induction in zebrafish (Danio rerio)." Toxicol Appl Pharmacol **254**(3): 280-287.

Vogel, C. F., E. Sciullo, et al. (2004). "Dioxin increases C/EBPbeta transcription by activating cAMP/protein kinase A." J Biol Chem **279**(10): 8886-8894.

Walgraeve, C., K. Demeestere, et al. (2010). "Oxygenated polycyclic aromatic hydrocarbons in atmospheric particulate matter: Molecular characterization and occurrence." Atmospheric Environment **44**(15): 1831-1846.

Wang, W., N. Jariyasopit, et al. (2011). "Concentration and photochemistry of PAHs, NPAHs, and OPAHs and toxicity of PM2.5 during the Beijing Olympic Games." Environ Sci Technol **45**(16): 6887-6895.

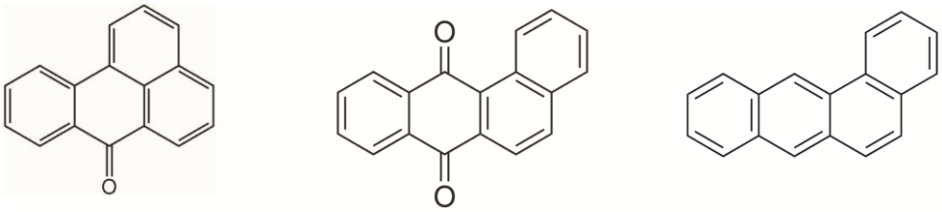
Wassenberg, D. M., A. L. Nerlinger, et al. (2005). "Effects of the polycyclic aromatic hydrocarbon heterocycles, carbazole and dibenzothiophene, on in vivo and in vitro CYP1A activity and polycyclic aromatic hydrocarbon-derived embryonic deformities." Environ Toxicol Chem **24**(10): 2526-2532.

Wei, S. L., B. Huang, et al. (2012). "Characterization of PM2.5-bound nitrated and oxygenated PAHs in two industrial sites of South China." Atmospheric Research **109**: 76-83.

Willett, K. L., D. Wassenberg, et al. (2001). "In vivo and in vitro inhibition of CYP1A-dependent activity in Fundulus heteroclitus by the polynuclear aromatic hydrocarbon fluoranthene." Toxicol Appl Pharmacol **177**(3): 264-271.

Xiong, K. M., R. E. Peterson, et al. (2008). "Aryl hydrocarbon receptor-mediated down-regulation of sox9b causes jaw malformation in zebrafish embryos." Mol Pharmacol **74**(6): 1544-1553.

Yu, H. (2002). "Environmental carcinogenic polycyclic aromatic hydrocarbons: photochemistry and phototoxicity." J Environ Sci Health C Environ Carcinog Ecotoxicol Rev **20**(2): 149-183.



1,9-benz-10-anthrone
BEZO
CAS# 82-05-3

benz(a)anthracene-7,12-dione
7,12-B[a]AQ
CAS# 2498-66-0

benz(a)anthracene
BAA
CAS# 56-55-3

Figure 4-1 Structures of BEZO, 7,12-B[a]AQ and BAA

BEZO and 7,12-B[a]AQ, both oxygenated 4-ring PAHs, are compared with 4-ring parent PAH BAA.

Figure 4-2 Developmental toxicity of 7,12-B[a]AQ and BEZO at 120 hpf

(**A**) Exposure from 6-72 hpf to 5, 7.5 and 10 μM 7,12-B[a]AQ caused concentration-dependent increases in the percent of control morpholino(MO)-injected larvae with pericardial edema (dark circles). 7,12-B[a]AQ exposure did not cause significant pericardial edema above control levels in *ahr2*-MO injected embryos (light circles) (**B**) BEZO exposure induced pericardial edema at 5, 7.5 and 10 uM in control-MO injected embryos (dark circles), ahr2 morphants showed no significant increase (light circles). The percentage of embryos with at least 1 malformation was significantly increased by both 7,12-B[a]AQ(**C**) and BEZO (**D**) in control-MO injected embryos (dark circles), while ahr2 morphants had no significant increase in the percentage of malformed embryos (light circles). Data represent 3 independent replicates analyzed by one-way ANOVA with Tukey's pairwise posthoc test. [a] significantly different than control [b]significantly different than control and 5 uM, $p < 0.05$. (**E**, **F**, **G**) Representative images of control-MO injected embryos exposed to 1% DMSO vehicle control, 10 μM 7,12-B[a]AQ, and 10 μM BEZO, respectively. Severe malformations including pericardial edema, yolk sac edema, craniofacial malformations and eye defects can be observed in F and G. (**H, I, J**) Representative images of *ahr2*-MO injected embryos exposed to 1% DMSO vehicle control, 10 μM 7,12-B[a]AQ, and 10 μM BEZO, respectively show rescue of morphological abnormalities.

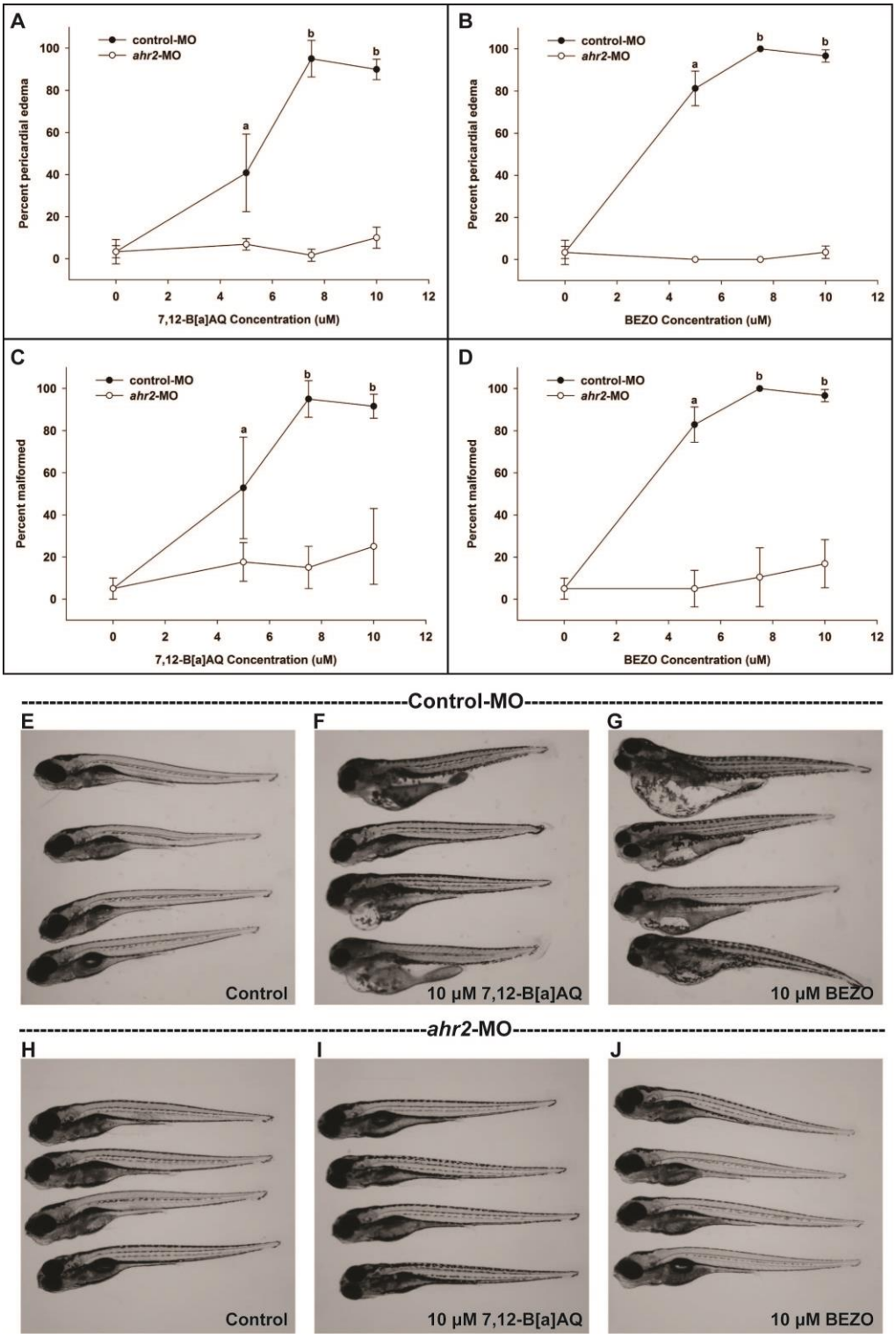

A
control-MO
ahr2-MO
Percent pericardial edema
7,12-B[a]AQ Concentration (uM)
B
BEZO Concentration (uM)
C
Percent malformed
D
Control-MO
E
F
G
Control
10 μM 7,12-B[a]AQ
10 μM BEZO
ahr2-MO
H
I
J

Figure 4-2

Figure 4-3 Cyp1 expression and morphology in OPAH-exposed embryos at 48 hpf

A) Exposure to 7.5 μM 7,12-B[a]AQ induced differential expression of cyp1a and cyp1b1 detected by qRT-PCR in control morpholino injected (c-MO) and ahr2-MO injected embryos compared to DMSO controls. B) Exposure to 7.5 μM BEZO induced differential expression of cyp1a and cyp1b1 in c-MO and ahr2-MO injected embryos compared to DMSO controls, but with much smaller fold change values than 7,12-B[a]AQ. Note different scale compare to Figure A. Data represent 4 biological replicates analyzed by two-way ANOVA with Tukey's posthoc test. Treatment groups not sharing a letter are statistically different ($p < 0.05$).
C, D) Representative light microscope images of 48 hpf control-MO and ahr2-MO injected embryos exposed to 1% DMSO vehicle control. E, F) 48 hpf control-MO and ahr2-MO injected embryos exposed to 7.5 μM 7,12-B[a]AQ. Pooling of blood is apparent in E (arrow). G, H) 48 hpf control-MO and ahr2-MO injected embryos exposed to 7.5 μM BEZO. Pericardial edema is apparent in G (arrow). I, J) Immofluorescent labeling of Cyp1a protein in 48 hpf control-MO and ahr2-MO injected embryos exposed to 1% DMSO vehicle control. K, L) Cyp1a protein expression in 48 hpf control-MO and ahr2-MO injected embryos exposed to 7.5 μM 7,12-B[a]AQ, c-MO injected embryos showed strong fluorescence throughout the vasculature (K). BEZO exposed embryos showed no Cyp1a protein expression (M, N).

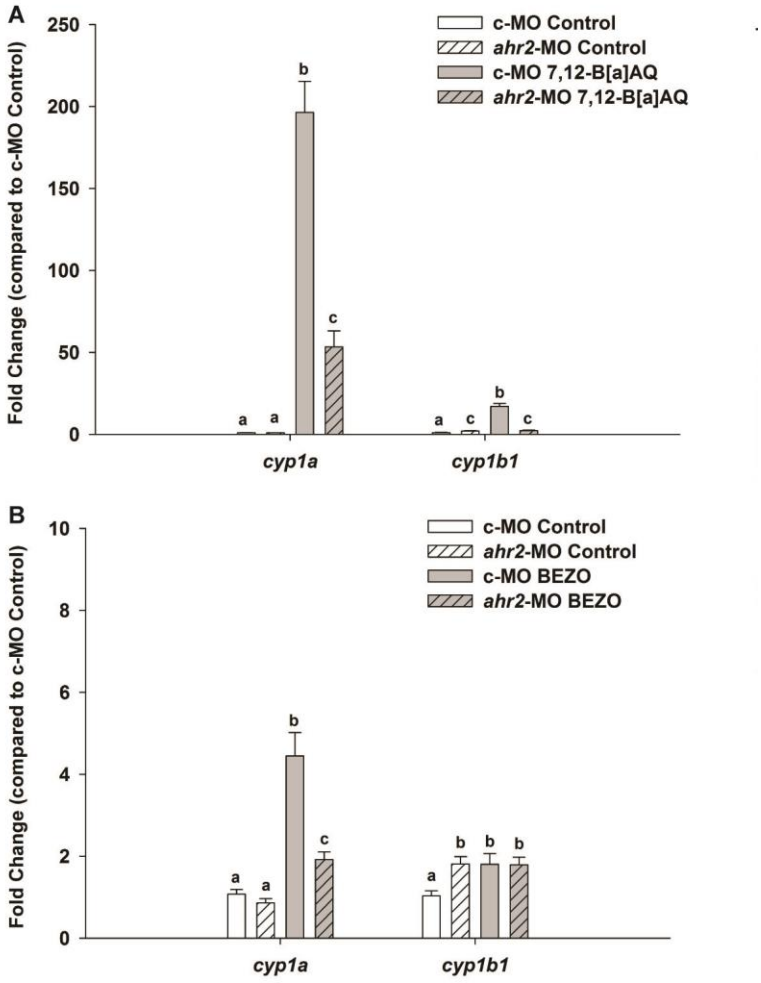

A
Fold Change (compared to c-MO Control)
250
200
150
100
50
0
c-MO Control
ahr2-MO Control
c-MO 7,12-B[a]AQ
ahr2-MO 7,12-B[a]AQ
a a b c
a c b c
cyp1a
cyp1b1
B
Fold Change (compared to c-MO Control)
10
8
6
4
2
0
c-MO Control
ahr2-MO Control
c-MO BEZO
ahr2-MO BEZO
a a b c
a b b b
cyp1a
cyp1b1

Figure 4-3

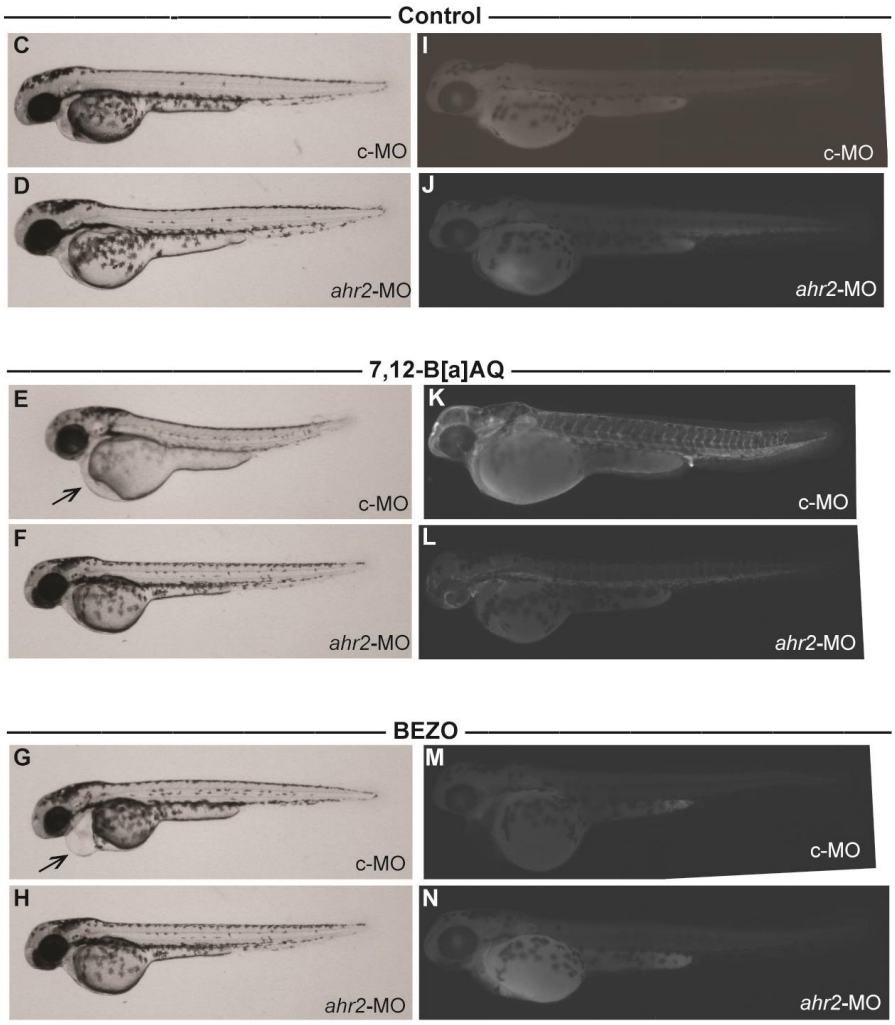


Figure 4-3 (Continued)

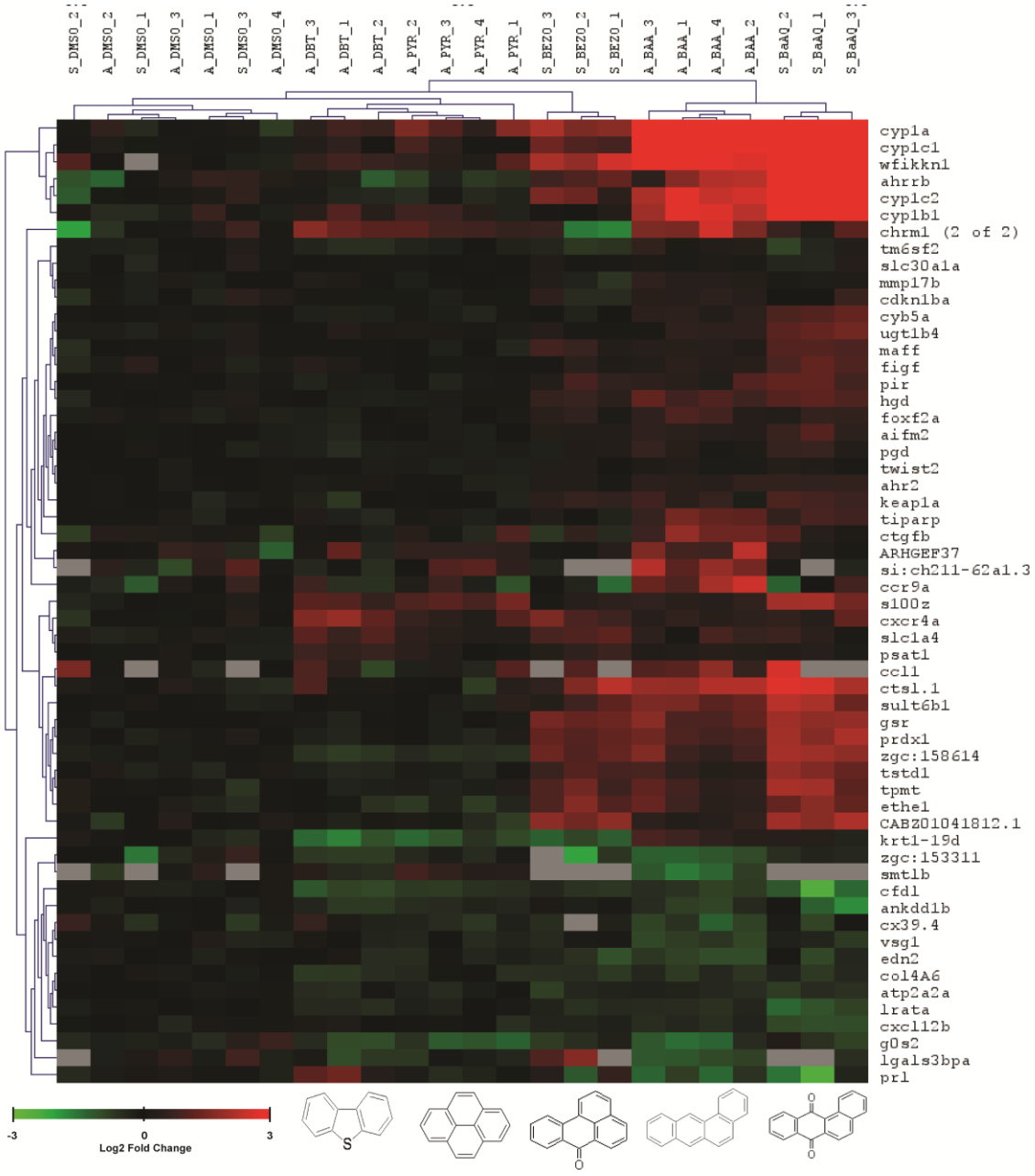


Figure 4-4 Heatmap of transcripts significantly induced by BAA exposure at 48 hpf.

Log2FC values were cluster using birectional hierarchical clustering. Individual samples are normalized to respective platform mean control value. A= array, fluorescence intensity values. S=mRNA-seq FPKM values). Insufficient data is represented in gray.

Figure 4-5 Comparison of transcripts induced by BEZO and 7,12-B[a]AQ exposure

A) Venn comparison of significant gene lists identified in mRNA-seq analysis of BEZO and 7,12-B[a]AQ with fold change >2 compared to control. **B**) novel transcript underexpressed in both BEZO and 7,12-B[a]AQ exposed samples occurs in a region with high transcriptional coverage but no annotation in Zv9 (top). Visualization of individual sample transcript reads across the predicted transcripts shows consistency within treatments (bottom). **C**) Bidirectional hierarchical clustering of all 366 transcripts significantly induced > 2 fold by BEZO or 7,12-B[a]AQ. Biological functions that were significantly enriched in clusters of transcripts are noted.

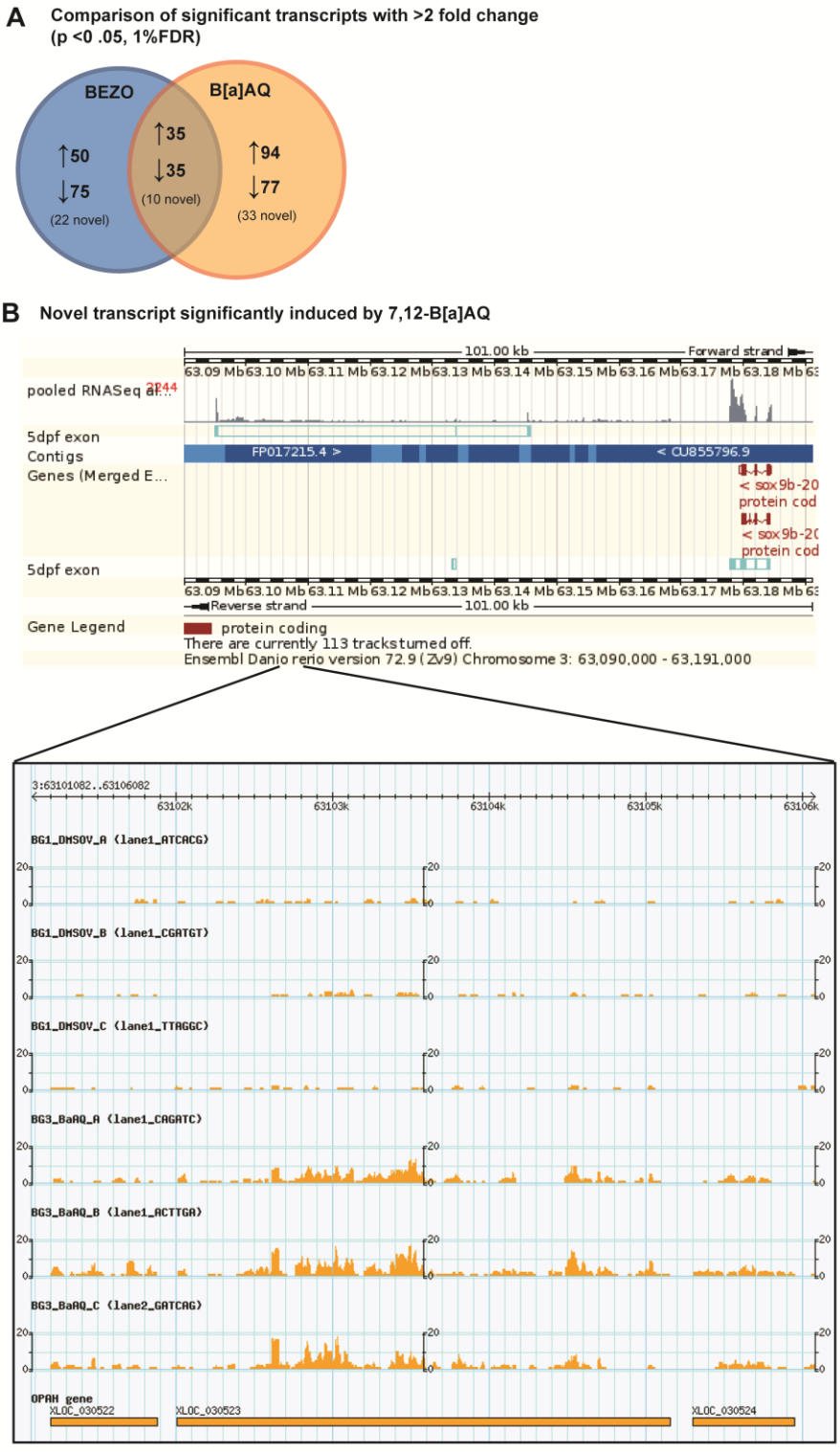

A
Comparison of significant transcripts with >2 fold change
(p <0 .05, 1%FDR)
BEZO
B[a]AQ
↑50
↓75
(22 novel)
↑35
↓35
(10 novel)
↑94
↓77
(33 novel)
B
Novel transcript significantly induced by 7,12-B[a]AQ
101.00 kb
Forward strand
63.09 Mb 63.10 Mb 63.11 Mb 63.12 Mb 63.13 Mb 63.14 Mb 63.15 Mb 63.16 Mb 63.17 Mb 63.18 Mb
pooled RNASeq al...
5dpf exon
Contigs
Genes (Merged E...
FP017215.4 >
< CU855796.9
< sox9b-20
protein cod
5dpf exon
Reverse strand
Gene Legend
protein coding
There are currently 113 tracks turned off.
Ensembl Danio rerio version 72.9 (Zv9) Chromosome 3: 63,090,000 - 63,191,000
3:63101082..63106082
63102k
63103k
63104k
63105k
63106k
BG1_DMSOV_A (lane1_ATCACG)
BG1_DMSOV_B (lane1_CGATGT)
BG1_DMSOV_C (lane1_TTAGGC)
BG3_BaAQ_A (lane1_CAGATC)
BG3_BaAQ_B (lane1_ACTTGA)
BG3_BaAQ_C (lane2_GATCAG)
OPAH gene
XLOC_030522
XLOC_030523
XLOC_030524

Figure 4-5

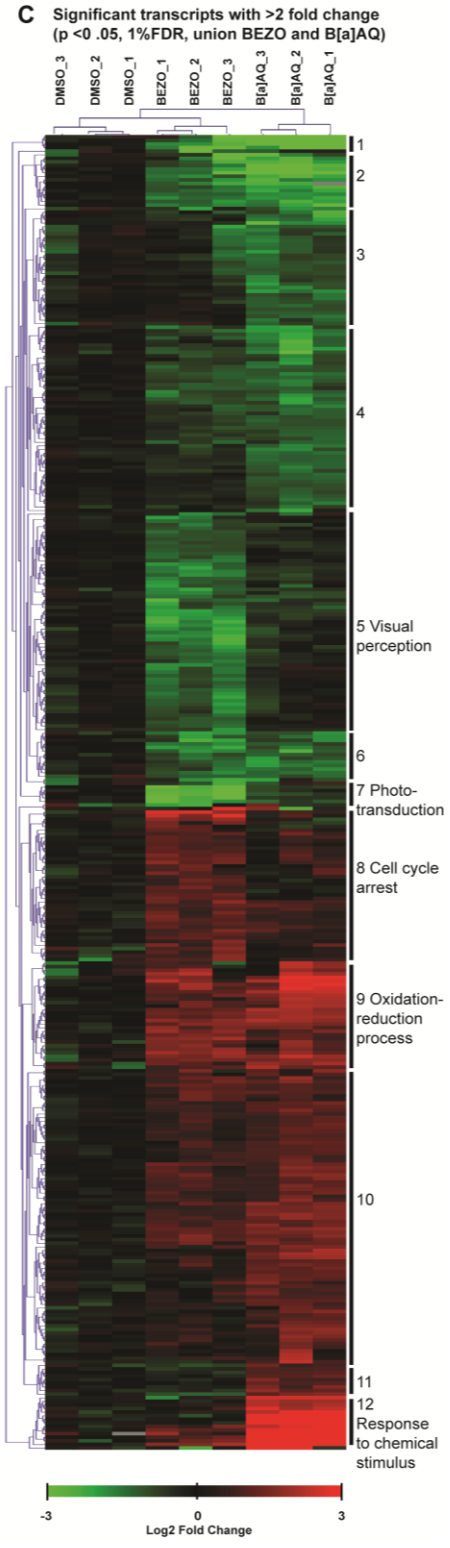


Figure 4-5 (Continued)

Figure 4-6 qRT-PCR analysis of OPAH targets in control and AHR2 morphants

Exposure to 7.5 µM 7,12-B[a]AQ or BEZO induced differential expression of *wfikkn1* (**A**), *gstp2* (**B**), *arg2* (**C**), *igfbp1a* (**D**), *ponzr4* (**E**) and *p53* (**F**) detected by qRT-PCR in c-MO and *ahr2*-MO injected embryos compared to DMSO controls. Data represent 4 biological replicates analyzed by two-way ANOVA with Tukey's posthoc test. Significantly different induction is designated with different letters ($p < 0.05$).

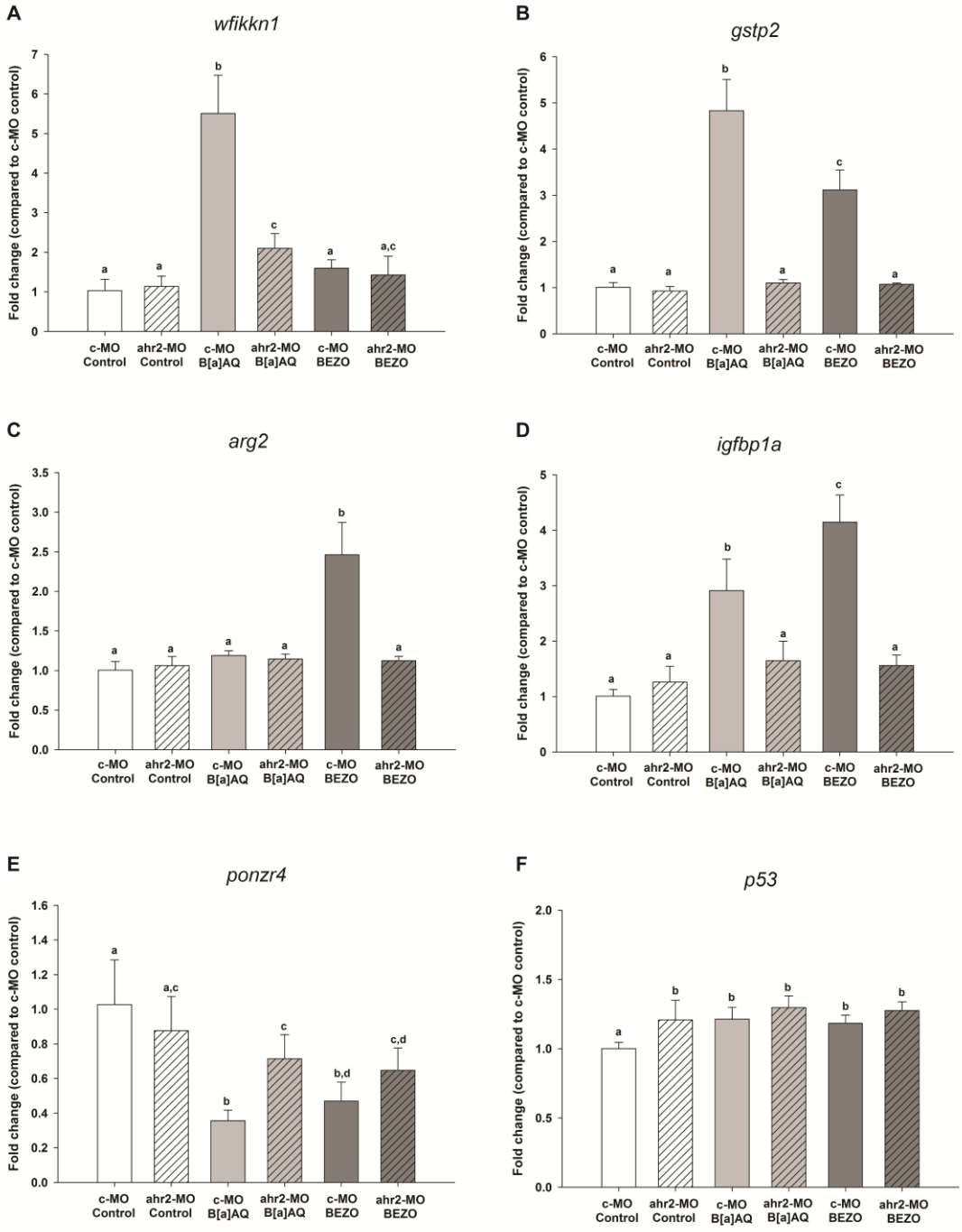

A
wfikkn1
Fold change (compared to c-MO control)
c-MO Control
ahr2-MO Control
c-MO B[a]AQ
ahr2-MO B[a]AQ
c-MO BEZO
ahr2-MO BEZO
B
gstp2
C
arg2
D
igfbp1a
E
ponzr4
F
p53

Figure 4-6

Figure 4-7 Biological processes affected by BEZO and 7,12-B[a]AQ exposure

A)MetaCore processes significantly over-represented among transcripts misexpressed by BEZO and 7,12-B[a]AQ. **B**) Predicted interactions between the AHR and transcripts differentially expressed in BEZO (blue) and 7,12-B[a]AQ (yellow) exposed embryos. Interactions were predicted using the Metacore statistical interactome tool. Color of circles represents expression of transcript compared to control (red = increased, blue = decreased). Symbols designating network object classes

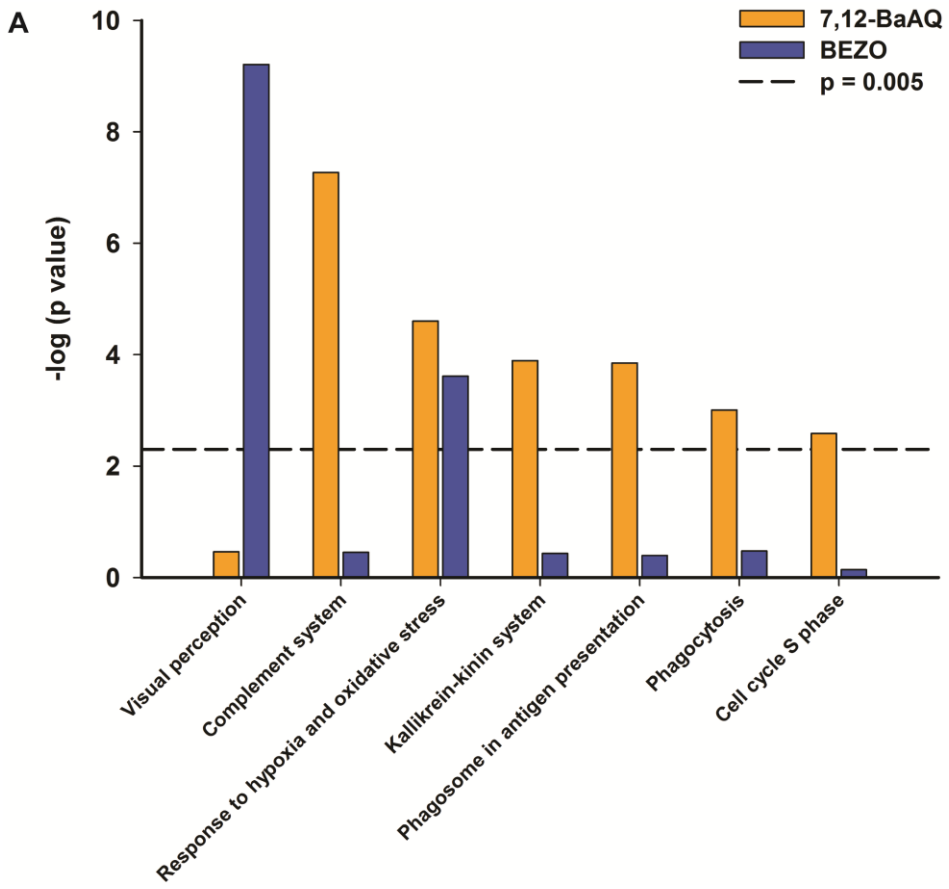

A
10
8
6
4
2
0
-log (p value)
7,12-BaAQ
BEZO
p = 0.005
Visual perception
Complement system
Response to hypoxia and oxidative stress
Kallikrein-kinin system
Phagosome in antigen presentation
Phagocytosis
Cell cycle S phase

Figure 4-7

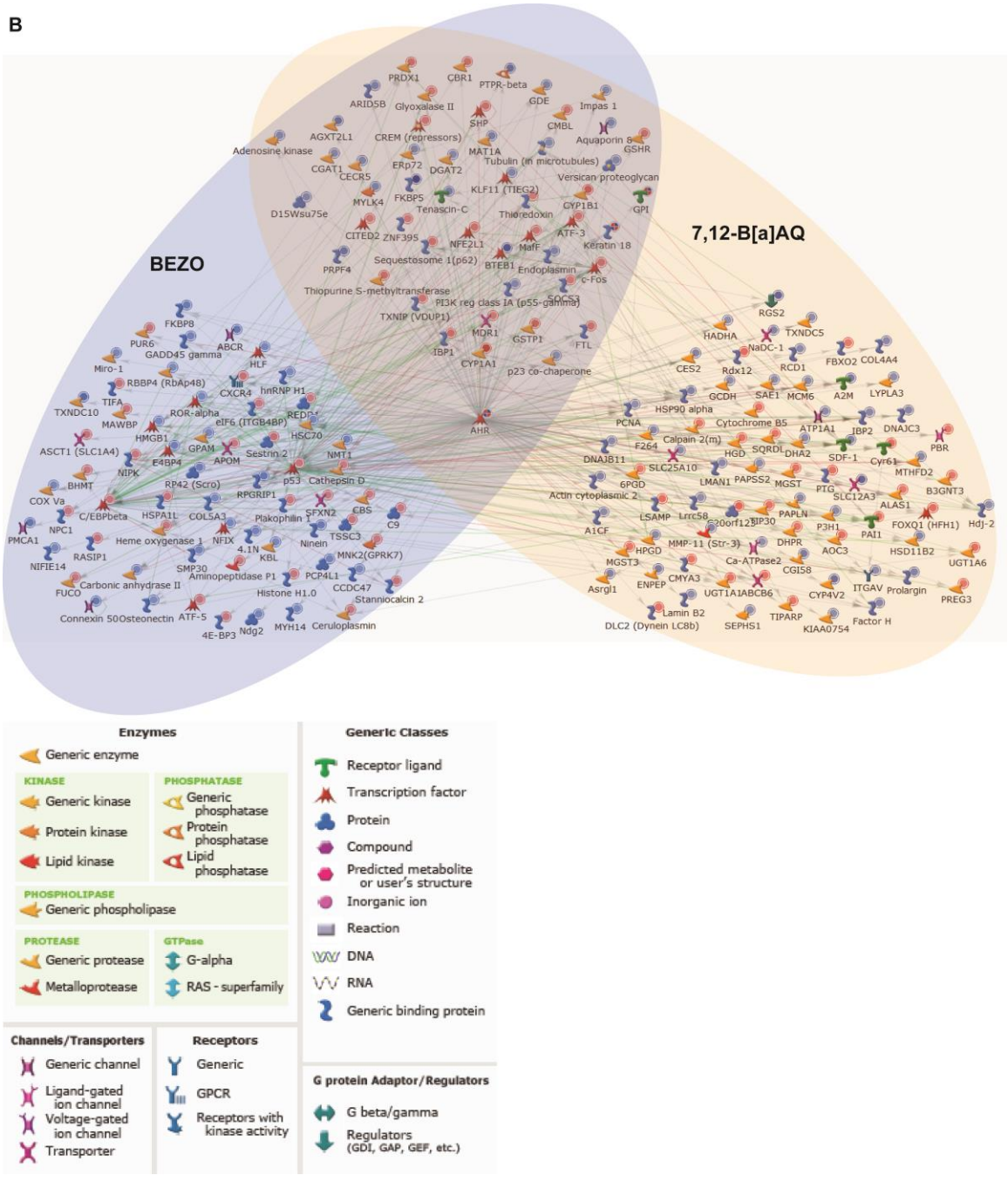


Figure 4-7 (Continued)

Table 4-1 Cross-platform comparison of expression data for genes differentially induced by PAHs

Log2 fold change values of PAH-treated samples compared to 1% DMSO control detected by microarray fluorescence intensity (DBT, PYR and BAA, previous study) or RNA-seq FPKM values (BEZO and 7,12-B[a]AQ, this study) compared to qRT-PCR log2FC values. *Significantly different than control, one-way ANOVA with Tukey's all pairwise posthoc test, $p < 0.05$ † At least one probe significantly different than control, one-way ANOVA with Tukey's all pairwise posthoc test, 0.05 FDR, adjusted p value < 0.05. ‡ Significantly different than control, Cuffdiff pairwise comparison, 0.01 FDR, adjusted p value < 0.05)

	DBT		PYR		BEZO		BAA		7,12-B[a]AQ	
Gene	Microarray	QPCR	Microarray	QPCR	RNA-seq	QPCR	Microarray	QPCR	RNA-seq	QPCR
cyp1a	0.7 ± 0.21	0.81 ± 0.23	1.24 ± 0.54‡	1.43 ± 0.47*	1.84 ± 0.29†	1.61 ± 0.15*	4.56 ± 0.14‡	6.63 ± 0.32*	7.85 ± 0.25†	7.24 ± 0.09*
ctsl.1	0.46 ± 0.5	-0.17 ± 0.35	-0.08 ± 0.49	-0.39 ± 0.09	1.53 ± 0.88†	1.63 ± 0.16*	2.08 ± 0.2‡	1.68 ± 0.16*	2.51 ± 0.31†	2.32 ± 0.23*
sult6b1	0.03 ± 0.1	-0.27 ± 0.26	0.04 ± 0.33	-0.28 ± 0.18	0.8 ± 0.42†	0.87 ± 0.33*	1.42 ± 0.2‡	1.03 ± 0.24*	1.94 ± 0.5†	2.42 ± 0.26*
cxcr4a	1.61 ± 0.33‡	1.19 ± 0.13*	0.63 ± 0.36‡	0.28 ± 0.33	1.14 ± 0.42†	0.83 ± 0.22*	0.5 ± 0.11‡	0.21 ± 0.38	0.77 ± 0.46†	0.74 ± 0.09*
ctgfb	-0.05 ± 0.13	-0.73 ± 0.23	0.25 ± 0.61	-0.56 ± 0.21	-0.13 ± 0.67	-0.02 ± 0.06	1.37 ± 0.46‡	1.23 ± 0.17*	0.56 ± 0.48	1.25 ± 0.07*
s100z	0.96 ± 0.27‡	0.33 ± 0.17	1.17 ± 0.25‡	0.51 ± 0.16	0.34 ± 0.22	0.91 ± 0.08*	0.52 ± 0.12‡	0.06 ± 0.25	1.89 ± 0.36†	2 ± 0.12*

Table 4-2 Biological processes misregulated by OPAHs

Gene ontology terms over-represented in BEZO and 7,12-B[a]AQ significant gene lists. The 20 most significant GO terms for each OPAH are listed (Metacore GeneGO, $p < 0.001$, shading according to degree of significance).

GO term	BEZO	7,12-BaAQ	# of genes/total in process
single-organism metabolic process	6.22E-13	1.76E-20	184/2713
small molecule metabolic process	1.40E-13	3.96E-16	156/2169
response to chemical stimulus	5.70E-09	6.98E-16	164/2599
response to organic substance	6.54E-09	4.83E-14	129/1889
response to lipid	5.69E-08	2.08E-13	67/718
organ development	1.24E-12	2.40E-11	155/2420
organic acid metabolic process	1.25E-06	1.70E-12	75/839
response to hormone stimulus	6.90E-08	1.78E-12	73/840
response to organic cyclic compound	8.57E-09	4.81E-12	68/754
developmental process	9.87E-10	9.06E-12	219/4138
tissue development	4.37E-04	3.26E-11	81/1261
metabolic process	7.02E-06	4.38E-11	332/7643
response to endogenous stimulus	1.34E-08	6.01E-11	89/1187
system development	8.77E-11	2.04E-10	189/3339
oxidation-reduction process	4.98E-05	8.80E-11	67/846
response to oxygen-containing compound	4.57E-09	1.17E-10	73/881
visual perception	1.18E-10	7.67E-02	26/203
sensory perception of light stimulus	1.30E-10	7.84E-02	26/204
multicellular organismal development	5.17E-10	5.07E-10	205/3781
anatomical structure development	1.14E-09	1.13E-09	199/3744

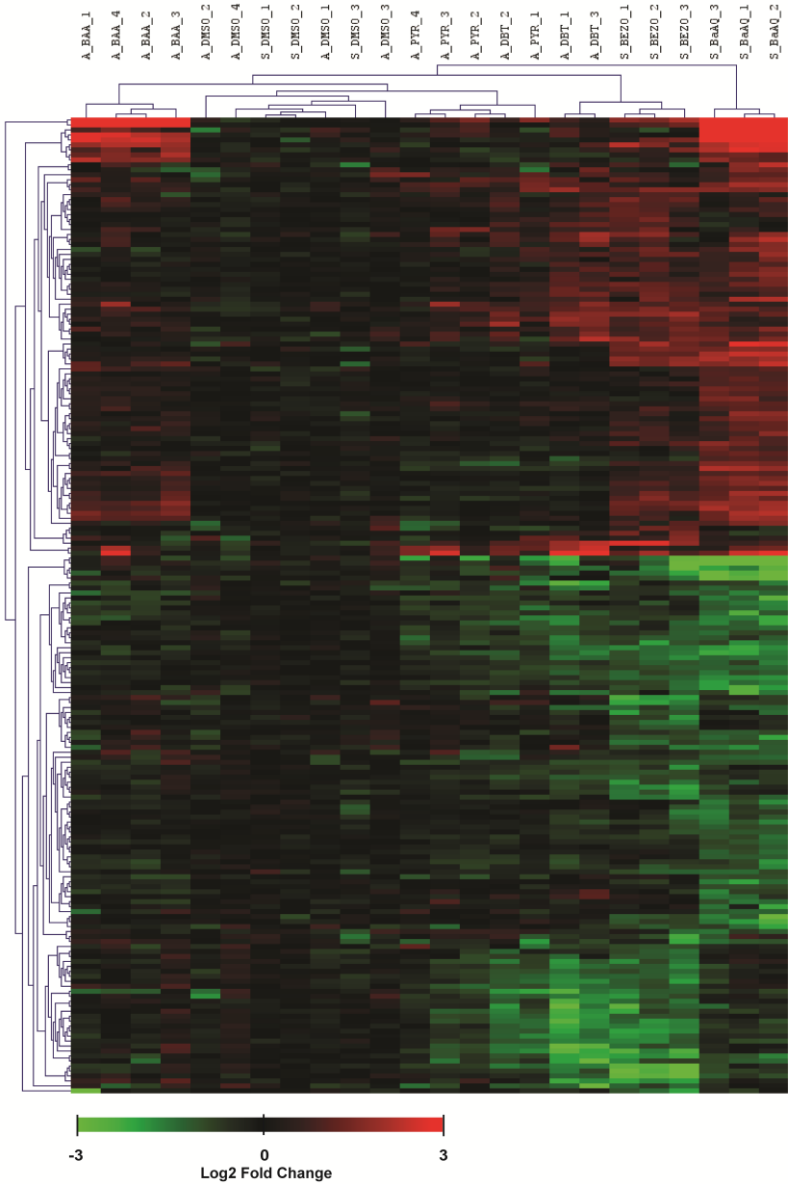


Figure 4-S1 Comparative expression across 5 PAH structures

Bidirectional hierarchically clustered heatmap of log2FC values of transcripts significantly induced by BEZO or 7,12-B[a]AQ exposure, for which probes were identified on the Agilent zebrafish microarray platform. Individual samples are normalized to respective platform mean control value. A= array, fluorescence intensity values. S=mRNA-seq FPKM values.

Table 4-S1 Primers used for qRT-PCR analysis

Gene	Ensembl ID	Forward Primer (5'- 3')	Reverse Primer (5'- 3')
arg2	ENSDARG00000039269	AACGGCGGACTGACCTAC	CCAGAGCGGATGCAACTA
β-actin	ENSDARG00000037746	AAGCAGGAGTACGATGAGTC	TGGAGTCCTCAGATGCATTG
cyp1a	ENSDARG00000026039	TGCCGATTTCATCCCTTTCC	AGAGCCGTGCTGATAGTGTC
cyp1b1	ENSDARG00000068934	CTGCATTGATTTCCGAGACGTG	CACACTCCGTGTTGACAGC
ctgfb	ENSDARG00000070586	TGTAACCAATGACAATGAGC	CATCCAGACAACTCGAAACG
ctsl.1	ENSDARG00000003902	GGACTCCTACCCCTATGAAG	ATAACCAACAGCCAGAACAC
cxcr4a	ENSDARG00000057633	ACACGGTAAACTTGTACAGC	ATGTGACAAACGAGTCCTG
gstp2	ENSDARG00000057338	TCTGGACTCTTTCCCGTCTCTCAA	ATTCACTGTTTGCCGTTGCCGT
igfbpa	ENSDARG00000014947	AAGCGGTGTGCACCGAGAGC	CCCGGTCACGAACACGGTGG
ponzr4	ENSDARG00000087440	GCTGTATTCCTCCAATCACG	CCTTGCCTTCATCTCTCGTC
p53	ENSDARG00000035559	CTCTCCCACCAACATCCACT	ACGTCCACCACCATTTGAAC
s100z	ENSDARG00000038729	GATCACCGTCTTCCACAAC	GTCCTTCTGAGACATGAGG
wfikkn1	ENSDARG00000044671	TATGCACACACAGTCAACAC	GGACTCATTTACCTGTCGAG

Table 4-S2 Sequencing mapping

Summary of total counts and mapping statistics of reads that passed Illumina CASAVA QC for RNA-seq samples in this study. Reads were mapped to genome assembly Zv9.

Sample Name	Total paired reads (Illumina QC ok)	Reads mapped to genome (Zv9)	% reads mapped	Reads mapped uniquely	% reads uniquely mapped	Reads mapped to two locations	Reads mapped to 3 or more locations	Total hits (only paired accepted)
DMSO_A	41,403,566	31,136,272	75.2	25,206,504	81	1,681,278	4,248,490	40,196,648
DMSO_B	59,745,304	45,224,497	75.7	37,320,636	82.5	2,876,464	5,027,397	59,347,598
DMSO_C	59,035,420	44,516,465	75.4	36,303,716	81.6	2,424,866	5,787,883	59,634,276
BEZO_A	51,837,910	38,800,129	74.8	31,274,174	80.6	2,056,482	5,469,473	51,499,242
BEZO_B	46,499,422	35,095,720	75.5	25,391,954	72.4	1,827,628	7,876,138	46,369,830
BEZO_C	41,818,250	31,305,119	74.9	25,532,988	81.6	1,664,374	4,107,757	39,621,128
BaAQ_A	49,416,290	37,181,558	75.2	29,973,554	80.6	1,953,912	5,254,092	50,418,194
BaAQ_B	36,241,038	26,888,497	74.2	21,517,188	80	1,372,508	3,998,801	35,143,112
BaAQ_C	47,733,496	35,477,524	74.3	27,090,316	76.4	1,971,892	6,415,316	51,354,196

Table 4-S3 7,12-B[a]AQ significantly misexpressed transcripts

Zebrafish genome assembly Zv9 transcripts identified by Cuffdiff as significantly differentially expressed in 7,12-B[a]AQ-exposed embryos compared to control (1% FDR, $q < 0.05$).). Log_2 fold change values represent mean BEZO-exposed compared to control.

Gene ID	Gene	Log2(fold_change)	q_value
ENSDARG00000026039	cyp1a	7.81	0.00E+00
ENSDARG00000058980	cyp1c1	4.81	0.00E+00
ENSDARG00000018298	cyp1c2	4.41	0.00E+00
ENSDARG00000059387	fgf7	3.82	4.35E-03
ENSDARG00000068934	cyp1b1	3.57	0.00E+00
ENSDARG00000076534	si:ch211-14a17.10	3.11	1.29E-02
ENSDARG00000057338	gstp2	2.69	1.32E-02
ENSDARG00000003902	ctsl.1	2.44	1.22E-05
ENSDARG00000015355	fosl1a	2.31	4.96E-03
ENSDARG00000005039	gstp1	2.27	0.00E+00
ENSDARG00000007344	tcap	2.25	0.00E+00
ENSDARG00000022139	ocstamp	2.06	7.57E-03
ENSDARG00000074971	DHRS13 (2 of 5)	2.00	1.93E-07
ENSDARG00000061481	CABZ01041812.1	1.96	0.00E+00
ENSDARG00000058734	prdx1	1.92	0.00E+00
ENSDARG00000086826	sult6b1	1.92	0.00E+00
ENSDARG00000061634	zgc:158614	1.90	1.87E-13
ENSDARG00000055974	TPMT (1 of 2)	1.86	2.88E-08
ENSDARG00000038729	s100z	1.84	5.98E-03
ENSDARG00000016713	dhrs13l1	1.83	5.80E-06
ENSDARG00000031683	fos	1.80	2.23E-10
ENSDARG00000086047	CABZ01067657.1	1.75	0.00E+00
ENSDARG00000019236	gsr	1.74	8.45E-11
ENSDARG00000075014	sqstm1	1.73	2.90E-02
ENSDARG00000073695	mamdc2b	1.70	1.40E-03
ENSDARG00000021149	cbr1l	1.69	0.00E+00
ENSDARG00000056638	pir	1.68	1.85E-11
ENSDARG00000044685	nr0b2a	1.59	9.65E-12
ENSDARG00000091715	CR926130.2	1.58	1.61E-12
ENSDARG00000071567	TSTD1	1.58	3.77E-08
ENSDARG00000067652	im:7150988	1.57	0.00E+00
ENSDARG00000089936	sepw2b	1.53	3.96E-08
ENSDARG00000089697	si:dkey-3d18.4	1.52	3.59E-03
ENSDARG00000035422	cyr61l1	1.48	1.71E-02
ENSDARG00000033285	gsto2	1.47	1.14E-07
ENSDARG00000002204	hspb11	1.46	2.23E-10
ENSDARG00000030896	foxq1a	1.44	3.00E-02
ENSDARG00000005713	ethe1	1.41	3.30E-07
ENSDARG00000034852	nt5c2l1	1.39	8.31E-04
ENSDARG00000089507	ugt1b4	1.34	2.43E-11
ENSDARG00000089586	AL844567.2	1.33	2.45E-03
ENSDARG00000063223	arl14	1.32	4.60E-02
ENSDARG00000044935	hpdb	1.29	7.05E-07
ENSDARG00000075524	CYLC2	1.29	5.53E-11
ENSDARG00000056795	serpine1	1.28	6.52E-03

Gene ID	Gene	Log2(fold_change)	q_value
ENSDARG00000014646	AOC2	1.25	1.93E-07
ENSDARG00000090375	ALPK3 (2 of 2)	1.24	1.22E-03
ENSDARG00000002405	si:ch211-225b11.1	1.22	4.78E-04
ENSDARG00000053136	b2m	1.22	1.68E-02
ENSDARG00000070000	txnipb	1.18	1.49E-08
ENSDARG00000043442	MAL (2 of 3)	1.18	5.20E-06
ENSDARG00000007823	atf3	1.16	5.80E-06
ENSDARG00000079938	zgc:173594	1.16	4.00E-03
ENSDARG00000061081	arpp21	1.15	1.04E-04
ENSDARG00000025122	abhd4	1.15	2.02E-02
ENSDARG00000006220	ugt1ab	1.14	3.76E-02
ENSDARG00000073786	cmbl	1.13	3.07E-05
ENSDARG00000094719	si:dkeyp-1h4.9	1.12	2.89E-04
ENSDARG00000058005	hgd	1.09	9.84E-04
ENSDARG00000018566	CU302436.1	1.09	5.51E-11
ENSDARG00000022165	MGST1 (1 of 2)	1.08	4.73E-02
ENSDARG00000055643	cyb5a	1.08	9.57E-07
ENSDARG00000067701	myoz3a	1.07	1.54E-03
ENSDARG00000071005	ppp1r3ca	1.06	2.34E-05
ENSDARG00000014947	igfbp1a	1.06	8.85E-03
ENSDARG00000078674	hspb9	1.05	2.07E-08
ENSDARG00000023217	CREM	1.04	4.35E-02
ENSDARG00000028386	htatip2	1.02	8.05E-03
ENSDARG00000091116	pkhd1l1	1.01	8.62E-07
ENSDARG00000032496	zgc:91887	1.00	2.93E-04
ENSDARG00000063297	abcb6	0.99	1.24E-02
ENSDARG00000039232	DUSP8 (2 of 2)	0.94	1.84E-03
ENSDARG00000031776	zgc:92066	0.94	2.09E-05
ENSDARG00000061120	slc43a2b	0.93	4.75E-04
ENSDARG00000092379	si:dkeyp-51b9.3	0.92	2.81E-02
ENSDARG00000093494	si:ch211-217k17.9	0.92	3.25E-06
ENSDARG00000093584	zgc:193505	0.91	3.97E-05
ENSDARG00000028957	maff	0.90	1.53E-05
ENSDARG00000070020	cyp2aa9	0.90	9.84E-04
ENSDARG00000026611	socs3b	0.86	1.54E-03
ENSDARG00000055510	ypel3	0.86	3.59E-03
ENSDARG00000016132	keap1a	0.85	3.98E-03
ENSDARG00000090014	CAPN2 (1 of 4)	0.85	6.87E-03
ENSDARG00000061841	tiparp	0.84	2.07E-02
ENSDARG00000017034	sqrdl	0.84	7.79E-03
ENSDARG00000074634	keap1b	0.82	5.94E-03
ENSDARG00000093521	B3GNT3 (2 of 4)	0.82	3.77E-02
ENSDARG00000087093	si:ch211-157c3.4	0.82	4.33E-02
ENSDARG00000060315	si:dkey-193b15.6	0.81	5.36E-03
ENSDARG00000095434	si:ch211-217k17.8	0.79	7.76E-03
ENSDARG00000033364	zgc:158387	0.79	8.80E-06
ENSDARG00000089046	-	0.79	2.93E-02
ENSDARG00000055045	casp3b	0.77	3.57E-02
ENSDARG00000036457	cacng6a	0.76	1.33E-02

Gene ID	Gene	Log2(fold_change)	q_value
ENSDARG00000060113	znf395a	0.75	1.99E-03
ENSDARG00000042310	blvrb	0.75	5.20E-03
ENSDARG00000094300	si:ch211-160e1.5	0.75	4.91E-04
ENSDARG00000030722	xirp1	0.75	2.05E-03
ENSDARG00000026655	tspo	0.74	6.60E-03
ENSDARG00000006982	msxd	0.73	2.91E-02
ENSDARG00000077549	AIFM2	0.73	5.72E-03
ENSDARG00000089920	MLIP	0.73	7.09E-05
ENSDARG00000040190	qdpra	0.73	2.90E-02
ENSDARG00000030905	cited2	0.72	3.67E-03
ENSDARG00000091996	si:ch211-117m20.5	0.72	3.46E-04
ENSDARG00000094557	nupr1	0.70	1.19E-02
ENSDARG00000059914	SDR42E2	0.70	3.45E-02
ENSDARG00000069559	si:ch211-239f4.7	0.69	1.64E-03
ENSDARG00000021787	abcb5	0.69	9.22E-04
ENSDARG00000028106	glrx	0.69	2.61E-02
ENSDARG00000038147	hbbe3	0.67	5.46E-03
ENSDARG00000079634	FILIP1 (1 of 2)	0.66	3.56E-02
ENSDARG00000019646	twist3	0.64	4.35E-02
ENSDARG00000035810	rgcc	0.64	5.36E-03
ENSDARG00000079119	CR848759.2	0.64	4.17E-02
ENSDARG00000013968	psap	0.59	4.32E-03
ENSDARG00000021059	alas1	0.59	5.20E-03
ENSDARG00000044212	CR385063.1	0.59	5.87E-03
ENSDARG00000089358	CABZ01035189.1	0.59	1.63E-02
ENSDARG00000011245	esrp1	0.58	1.06E-02
ENSDARG00000021833	ahr2	0.57	4.57E-03
ENSDARG00000021366	fbp1a	0.57	1.66E-02
ENSDARG00000070355	FBXO2	0.56	6.89E-03
ENSDARG00000025338	hagh	0.56	4.13E-02
ENSDARG00000070047	rgs4	0.54	2.46E-02
ENSDARG00000071601	pvalb9	0.54	2.19E-02
ENSDARG00000036107	txnipa	0.54	1.16E-02
ENSDARG00000015343	pgd	0.53	2.22E-02
ENSDARG00000038643	alas2	0.53	2.46E-02
ENSDARG00000044125	txn	0.52	3.05E-02
ENSDARG00000035519	histh1l	0.51	2.46E-02
ENSDARG00000007216	abce1	-0.46	4.73E-02
ENSDARG00000006008	dct	-0.46	4.73E-02
ENSDARG00000039578	pa2g4a	-0.48	3.13E-02
ENSDARG00000005122	atp2a2b	-0.49	3.29E-02
ENSDARG00000014179	pfkma	-0.50	2.91E-02
ENSDARG00000030913	zgc:152873	-0.51	4.67E-02
ENSDARG00000061375	sgpl1	-0.51	3.55E-02
ENSDARG00000000212	KRT23 (1 of 15)	-0.51	1.89E-02
ENSDARG00000060594	hadhab	-0.51	2.44E-02
ENSDARG00000068076	psmd7	-0.51	2.02E-02
ENSDARG00000007697	fabp7a	-0.51	2.64E-02
ENSDARG00000030972	dnaja1l	-0.52	4.73E-02

Gene ID	Gene	Log2(fold_change)	q_value
ENSDARG00000010571	ezh2	-0.53	4.03E-02
ENSDARG00000017568	HNRNPAB (2 of 2)	-0.53	3.55E-02
ENSDARG00000056248	wu:fb15e04	-0.53	1.39E-02
ENSDARG00000012234	psme3	-0.53	4.33E-02
ENSDARG00000054155	pcna	-0.53	1.10E-02
ENSDARG00000056314	a2ml	-0.53	1.16E-02
ENSDARG00000037284	ptges3a	-0.54	3.73E-02
ENSDARG00000059357	SARNP	-0.54	1.80E-02
ENSDARG00000063631	ch1073-291c23.1	-0.54	4.91E-02
ENSDARG00000036371	acta1a	-0.54	2.19E-02
ENSDARG00000063509	lrrc58b	-0.55	4.76E-02
ENSDARG00000095268	si:dkey-261h17.1	-0.55	1.21E-02
ENSDARG00000033760	pmelb	-0.55	1.44E-02
ENSDARG00000037057	gcdh	-0.55	2.90E-02
ENSDARG00000037116	cxcl12a	-0.55	1.70E-02
ENSDARG00000071353	AL929007.1	-0.55	7.82E-03
ENSDARG00000007354	pdia3	-0.55	1.63E-02
ENSDARG00000076790	si:ch211-55g3.6	-0.56	2.91E-02
ENSDARG00000006314	itgav	-0.56	1.15E-02
ENSDARG00000090495	pla2g15	-0.56	3.25E-02
ENSDARG00000026829	cotl1	-0.57	5.84E-03
ENSDARG00000012729	hcls1	-0.57	1.73E-02
ENSDARG00000054259	nat10	-0.57	4.02E-02
ENSDARG00000058292	sephs1	-0.57	4.59E-02
ENSDARG00000012694	c3a	-0.57	2.01E-02
ENSDARG00000060041	LIG1	-0.57	6.44E-03
ENSDARG00000037997	tubb5	-0.57	2.64E-02
ENSDARG00000016235	rbp1a	-0.57	1.50E-02
ENSDARG00000012066	dcn	-0.57	2.01E-02
ENSDARG00000070651	PRKCD (1 of 2)	-0.57	3.36E-02
ENSDARG00000017261	gdpd1	-0.58	3.48E-02
ENSDARG00000056600	papss2b	-0.58	1.10E-02
ENSDARG00000009342	txndc5	-0.58	3.98E-03
ENSDARG00000045367	tuba1	-0.58	1.74E-02
ENSDARG00000070050	sfrp2	-0.58	3.41E-02
ENSDARG00000037845	col9a3	-0.58	4.13E-03
ENSDARG00000021720	COL4A6	-0.59	8.75E-03
ENSDARG00000001057	bysl	-0.59	3.12E-02
ENSDARG00000008732	zgc:66479	-0.59	3.59E-03
ENSDARG00000045297	phb2	-0.60	1.80E-02
ENSDARG00000088514	and1	-0.60	2.79E-03
ENSDARG00000061124	srpr	-0.60	7.79E-03
ENSDARG00000091792	BX088649.1	-0.60	3.45E-02
ENSDARG00000040041	mcm4	-0.60	5.44E-03
ENSDARG00000087055	BX842614.2	-0.61	1.10E-02
ENSDARG00000036700	si:ch211-114n24.6	-0.62	1.14E-03
ENSDARG00000053493	aldh1a2	-0.63	5.44E-03
ENSDARG00000054807	sec13	-0.63	8.60E-03
ENSDARG00000038056	FGFBP2 (1 of 2)	-0.63	1.87E-03

Gene ID	Gene	Log2(fold_change)	q_value
ENSDARG00000011404	fen1	-0.63	1.01E-02
ENSDARG00000039345	drg1	-0.63	7.20E-03
ENSDARG00000043276	calr	-0.63	1.42E-03
ENSDARG00000086370	apoea	-0.63	2.27E-03
ENSDARG00000020956	pck2	-0.63	2.04E-03
ENSDARG00000009001	pdip5	-0.64	9.24E-04
ENSDARG00000071212	lepre1	-0.64	2.85E-03
ENSDARG00000056767	itgb3a	-0.65	3.01E-03
ENSDARG00000009401	vcanb	-0.65	2.81E-03
ENSDARG00000014091	osr1	-0.66	3.62E-02
ENSDARG00000005023	fkbp9	-0.66	2.87E-02
ENSDARG00000010962	fkbp7	-0.66	4.09E-02
ENSDARG00000042021	mapk12a	-0.66	2.19E-02
ENSDARG00000038768	mrpl12	-0.66	6.56E-03
ENSDARG00000010487	sae1	-0.66	5.24E-03
ENSDARG00000092467	si:ch73-46j18.5	-0.66	2.88E-03
ENSDARG00000057683	mcm6	-0.67	6.51E-04
ENSDARG00000018846	dgat2	-0.67	2.42E-02
ENSDARG00000037158	rcc1	-0.67	3.94E-02
ENSDARG00000001993	myhb	-0.67	3.74E-02
ENSDARG00000015088	dnajb11	-0.68	2.19E-02
ENSDARG00000055945	asph	-0.68	2.19E-02
ENSDARG00000037846	hm13	-0.68	6.32E-04
ENSDARG00000005161	gpib	-0.68	2.79E-03
ENSDARG00000020103	calrl	-0.68	1.96E-04
ENSDARG00000033855	rqcd1	-0.69	1.54E-02
ENSDARG00000015911	mcm2	-0.69	1.45E-03
ENSDARG00000021004	c5	-0.69	6.70E-03
ENSDARG00000019507	mcm5	-0.69	1.12E-03
ENSDARG00000000796	nr4a1	-0.69	1.32E-02
ENSDARG00000037961	rcn3	-0.70	1.14E-03
ENSDARG00000074908	col6a1	-0.70	1.67E-04
ENSDARG00000027495	elovl4b	-0.70	2.19E-02
ENSDARG00000039131	atp1a1a.3	-0.71	1.68E-02
ENSDARG00000010640	SLC12A2 (6 of 6)	-0.71	4.65E-03
ENSDARG00000039041	sfrp5	-0.71	4.00E-02
ENSDARG00000044975	KRT23 (9 of 15)	-0.72	2.88E-03
ENSDARG00000044261	si:ch211-243g18.2	-0.72	2.51E-02
ENSDARG00000040535	CSGALNACT1 (1 of 2)	-0.73	3.93E-02
ENSDARG00000079233	E2F2	-0.73	2.67E-02
ENSDARG00000002968	a1cf	-0.73	3.38E-02
ENSDARG00000075016	APOB (3 of 3)	-0.73	5.84E-03
ENSDARG00000012016	HPGD	-0.73	2.91E-02
ENSDARG00000069116	timm10	-0.73	1.16E-02
ENSDARG00000092155	apoc2	-0.73	3.84E-04
ENSDARG00000060797	pfkmb	-0.74	8.38E-04
ENSDARG00000002071	adss	-0.74	6.29E-04
ENSDARG00000062688	gpnmb	-0.74	3.49E-03
ENSDARG00000069415	col17a1a	-0.75	9.61E-05

Gene ID	Gene	Log2(fold_change)	q_value
ENSDARG00000055493	hic1	-0.75	4.05E-02
ENSDARG00000069980	lman1	-0.75	5.98E-03
ENSDARG00000015495	klf3	-0.76	2.61E-04
ENSDARG00000002831	col4a4	-0.77	4.57E-02
ENSDARG00000071219	pik3r3a	-0.77	3.11E-03
ENSDARG00000038153	lgals2b	-0.77	4.62E-02
ENSDARG00000014594	anxa1b	-0.77	6.94E-04
ENSDARG00000069823	PROCA1	-0.78	1.16E-02
ENSDARG00000057738	hells	-0.78	6.94E-04
ENSDARG00000024928	ITIH4	-0.78	9.58E-03
ENSDARG00000035652	sat1a	-0.78	1.37E-02
ENSDARG00000007221	pbk	-0.79	4.10E-02
ENSDARG00000063177	manf	-0.79	3.48E-03
ENSDARG00000056778	cfhl2	-0.79	2.78E-02
ENSDARG00000069048	serpinf1	-0.79	1.53E-04
ENSDARG00000012366	fbp2	-0.79	4.34E-03
ENSDARG00000079370	utp18	-0.79	3.40E-02
ENSDARG00000052039	caspb	-0.80	2.38E-03
ENSDARG00000069261	metap2a	-0.80	4.35E-02
ENSDARG00000055278	cfb	-0.80	1.93E-05
ENSDARG00000069706	prmt6	-0.81	4.73E-02
ENSDARG00000091397	-	-0.82	1.99E-02
ENSDARG00000018258	ADK (2 of 2)	-0.82	9.14E-03
ENSDARG00000076624	ptprb	-0.82	2.12E-02
ENSDARG00000027867	PAPLN (1 of 2)	-0.82	8.10E-03
ENSDARG00000007377	odc1	-0.83	1.24E-03
ENSDARG00000018266	mthfd1a	-0.83	5.67E-03
ENSDARG00000052470	igfbp2a	-0.83	3.72E-04
ENSDARG00000090468	PPP1R3A (2 of 2)	-0.83	4.34E-03
ENSDARG00000090802	MCM3 (2 of 2)	-0.83	1.77E-02
ENSDARG00000033140	desi1a	-0.84	6.28E-04
ENSDARG00000042641	cyp51	-0.84	1.19E-02
ENSDARG00000004665	hspa5	-0.84	1.86E-06
ENSDARG00000045141	aqp8a.1	-0.84	4.61E-02
ENSDARG00000029075	pfkfb4l	-0.84	1.87E-04
ENSDARG00000056938	kera	-0.85	5.80E-06
ENSDARG00000045843	apex1	-0.85	1.59E-03
ENSDARG00000073928	mrc1a	-0.85	1.48E-04
ENSDARG00000009782	myh11a	-0.85	3.55E-02
ENSDARG00000035891	acana	-0.85	3.45E-02
ENSDARG00000041110	dnajc3	-0.87	3.51E-02
ENSDARG00000004282	zgc:77375	-0.88	2.07E-02
ENSDARG00000029204	TYRP1 (2 of 2)	-0.88	1.87E-05
ENSDARG00000052738	hmgcs1	-0.89	3.84E-04
ENSDARG00000018491	pdia4	-0.90	8.75E-08
ENSDARG00000001975	hsd11b2	-0.90	3.62E-04
ENSDARG00000003820	nr1d2a	-0.90	3.49E-02
ENSDARG00000057575	pnp4a	-0.90	2.28E-04
ENSDARG00000003570	hsp90b1	-0.91	2.70E-07

Gene ID	Gene	Log2(fold_change)	q_value
ENSDARG00000014488	ca2	-0.92	2.62E-08
ENSDARG00000090467	CABZ01074130.1	-0.92	2.53E-08
ENSDARG00000016491	aglb	-0.93	2.23E-06
ENSDARG00000016718	mmp11b	-0.94	4.33E-02
ENSDARG00000073699	-	-0.94	2.44E-08
ENSDARG00000079111	zgc:86725	-0.95	4.68E-04
ENSDARG00000013670	hyou1	-0.96	1.50E-02
ENSDARG00000060345	apod	-0.96	1.78E-04
ENSDARG00000069827	crygm2d11	-0.97	1.88E-04
ENSDARG00000041595	ces3	-0.98	2.37E-05
ENSDARG00000070597	prelp	-0.98	7.57E-03
ENSDARG00000018351	hpda	-0.98	1.79E-02
ENSDARG00000055172	BX470254.2	-0.98	1.04E-07
ENSDARG00000055100	cxcl12b	-0.99	1.30E-05
ENSDARG00000053853	slc13a2	-1.00	2.37E-05
ENSDARG00000076357	CABZ01102039.1	-1.01	3.40E-02
ENSDARG00000010478	hsp90aa1.1	-1.03	2.90E-10
ENSDARG00000090268	KRT23 (12 of 15)	-1.03	4.81E-08
ENSDARG00000039462	CABZ01076094.1	-1.03	2.73E-08
ENSDARG00000042780	APOB (1 of 3)	-1.03	8.62E-07
ENSDARG00000026771	tmem41ab	-1.04	3.45E-02
ENSDARG00000052917	im:7154842	-1.04	3.51E-09
ENSDARG00000091260	MYLK4 (1 of 2)	-1.04	1.63E-04
ENSDARG00000035914	tmem167a	-1.05	3.05E-06
ENSDARG00000038785	abcf2a	-1.06	8.08E-10
ENSDARG00000090623	CR392352.1	-1.07	5.51E-03
ENSDARG00000007480	rpe65a	-1.07	2.48E-05
ENSDARG00000036840	krt15	-1.09	1.68E-05
ENSDARG00000069988	ARID5A (2 of 2)	-1.09	4.51E-02
ENSDARG00000043806	postna	-1.10	6.50E-04
ENSDARG00000045180	acta2	-1.11	3.40E-02
ENSDARG00000039605	mat1a	-1.11	2.39E-12
ENSDARG00000037278	lrata	-1.11	2.81E-02
ENSDARG00000075161	defbl1	-1.13	4.37E-08
ENSDARG00000093774	rbp2b	-1.14	3.05E-06
ENSDARG00000030215	matn1	-1.14	1.05E-04
ENSDARG00000045808	rlbp1b	-1.16	2.91E-02
ENSDARG00000054753	col10a1	-1.17	2.44E-11
ENSDARG00000057064	enpep	-1.20	2.46E-02
ENSDARG00000023324	rab11bb	-1.23	7.79E-03
ENSDARG00000001760	tnxb	-1.23	2.55E-09
ENSDARG00000095321	si:dkey-9l20.3	-1.26	0.00E+00
ENSDARG00000038424	C4A	-1.26	1.62E-07
ENSDARG00000035309	entpd3	-1.28	2.33E-09
ENSDARG00000055118	mylipb	-1.32	1.63E-02
ENSDARG00000077872	CR626907.1	-1.33	2.88E-03
ENSDARG00000095239	si:dkeyp-106c3.1	-1.35	2.28E-02
ENSDARG00000009018	rhbg	-1.36	0.00E+00
ENSDARG00000076874	abhd5	-1.37	1.33E-02

Gene ID	Gene	Log2(fold_change)	q_value
ENSDARG00000016412	agt	-1.37	2.44E-08
ENSDARG00000079302	and2	-1.40	0.00E+00
ENSDARG00000076192	ankrd1b	-1.43	2.23E-10
ENSDARG00000041685	BX663520.1	-1.47	1.07E-03
ENSDARG00000087345	CABZ01059415.2	-1.57	7.28E-07
ENSDARG00000088636	wu:fa03e10	-1.64	0.00E+00
ENSDARG00000052631	thbs4a	-1.69	1.61E-07
ENSDARG00000030604	phkg1a	-1.70	2.90E-03
ENSDARG00000062132	cyp4v8	-1.76	2.64E-02
ENSDARG00000052207	c3c	-1.76	0.00E+00
ENSDARG00000067639	prpf4	-1.78	7.68E-04
ENSDARG00000002311	fabp11b	-1.81	0.00E+00
ENSDARG00000079933	SLC46A3 (1 of 2)	-1.84	6.91E-05
ENSDARG00000031952	mb	-1.84	0.00E+00
ENSDARG00000035544	agxt2l1	-1.91	1.42E-02
ENSDARG00000042379	zgc:103681	-2.02	4.35E-02
ENSDARG00000056875	rgs2	-2.19	1.28E-02
ENSDARG00000071173	slc12a10.2	-2.22	1.48E-04
ENSDARG00000087359	c3b	-2.26	1.58E-04
ENSDARG00000056587	cyp2r1	-2.29	2.15E-03
ENSDARG00000007024	uox	-2.94	4.67E-02
ENSDARG00000068194	klf9	-3.13	4.33E-06
ENSDARG00000088589	ponzr3	-3.41	7.93E-10
ENSDARG00000028396	fkbp5	-4.23	0.00E+00
ENSDARG00000087440	ponzr4	-4.42	3.80E-09

Table 4-S4 BEZO significantly misexpressed transcripts

Zebrafish genome assembly Zv9 transcripts identified by Cuffdiff as significantly differentially expressed in BEZO-exposed embryos compared to control (1% FDR, $q < 0.05$). Log_2 fold change values represent mean BEZO-exposed compared to control.

Gene ID	Gene	Log2(fold_change)	q_value
ENSDARG00000069375	zgc:162608	1.94	9.06E-09
ENSDARG00000014947	igfbp1a	1.91	0.00E+00
ENSDARG00000026039	cyp1a	1.83	1.27E-12
ENSDARG00000086047	CABZ01067657.1	1.72	0.00E+00
ENSDARG00000061481	CABZ01041812.1	1.72	0.00E+00
ENSDARG00000074971	DHRS13 (2 of 5)	1.68	1.43E-05
ENSDARG00000003902	ctsl.1	1.63	2.46E-02
ENSDARG00000044685	nr0b2a	1.53	1.36E-13
ENSDARG00000038025	cbx7a	1.49	1.21E-04
ENSDARG00000076221	zgc:198419	1.43	1.09E-02
ENSDARG00000007344	tcap	1.42	2.93E-12
ENSDARG00000091047	CABZ01045617.2	1.39	6.79E-08
ENSDARG00000070012	sesn2	1.36	5.73E-08
ENSDARG00000075524	CYLC2	1.35	0.00E+00
ENSDARG00000019236	gsr	1.33	1.45E-07
ENSDARG00000005713	ethe1	1.29	2.50E-07
ENSDARG00000037121	mat2ab	1.26	6.22E-06
ENSDARG00000023217	CREM	1.25	4.36E-04
ENSDARG00000005039	gstp1	1.25	0.00E+00
ENSDARG00000007823	atf3	1.23	6.38E-09
ENSDARG00000061120	slc43a2b	1.23	0.00E+00
ENSDARG00000061634	zgc:158614	1.22	2.65E-06
ENSDARG00000021149	cbr1l	1.21	0.00E+00
ENSDARG00000058734	prdx1	1.21	0.00E+00
ENSDARG00000089697	si:dkey-3d18.4	1.18	4.96E-02
ENSDARG00000094719	si:dkeyp-1h4.9	1.18	2.65E-06
ENSDARG00000057633	cxcr4a	1.18	2.51E-03
ENSDARG00000030872	cetp	1.17	2.12E-05
ENSDARG00000059914	SDR42E2	1.15	6.40E-09
ENSDARG00000078878	METTL21C (1 of 2)	1.14	2.06E-04
ENSDARG00000058980	cyp1c1	1.11	7.89E-11
ENSDARG00000051956	isca1	1.11	5.54E-10
ENSDARG00000060189	ESM1	1.09	4.13E-02
ENSDARG00000055974	TPMT (1 of 2)	1.07	1.12E-02
ENSDARG00000067857	pmt	1.04	5.14E-04
ENSDARG00000056638	pir	1.03	1.15E-04
ENSDARG00000000551	slc1a4	1.03	4.71E-12
ENSDARG00000091871	si:ch211-13o20.3	1.00	2.35E-06
ENSDARG00000039269	arg2	0.99	5.73E-08
ENSDARG00000031683	fos	0.98	4.03E-03
ENSDARG00000045708	adm2a	0.98	8.88E-03
ENSDARG00000002405	si:ch211-225b11.1	0.97	7.03E-04
ENSDARG00000056680	stc2a	0.96	8.50E-08
ENSDARG00000058476	stc1l	0.94	2.70E-02
ENSDARG00000052694	micall2a	0.94	1.55E-03

Gene ID	Gene	Log2(fold_change)	q_value
ENSDARG00000016457	irf9	0.93	2.62E-03
ENSDARG00000071567	TSTD1	0.91	9.14E-03
ENSDARG00000055723	hsp70l	0.90	4.12E-04
ENSDARG00000078882	CR407583.2	0.89	9.16E-06
ENSDARG00000077559	NCOA7 (2 of 2)	0.89	4.17E-03
ENSDARG00000061081	arpp21	0.88	2.90E-03
ENSDARG00000021242	mvp	0.88	6.92E-08
ENSDARG00000079938	zgc:173594	0.88	4.80E-02
ENSDARG00000076386	epdl1	0.88	3.93E-03
ENSDARG00000070020	cyp2aa9	0.88	7.92E-05
ENSDARG00000031776	zgc:92066	0.85	1.49E-06
ENSDARG00000020645	slc7a3a	0.85	5.98E-08
ENSDARG00000086826	sult6b1	0.83	1.04E-07
ENSDARG00000074829	RASIP1	0.83	4.21E-02
ENSDARG00000091111	TIFA	0.83	4.69E-04
ENSDARG00000033539	paics	0.83	7.89E-11
ENSDARG00000077785	ATF5 (2 of 2)	0.81	1.59E-05
ENSDARG00000075014	sqstm1	0.80	2.15E-04
ENSDARG00000073786	cmbl	0.80	4.80E-03
ENSDARG00000052148	ptgs1	0.80	1.64E-02
ENSDARG00000057121	C7 (1 of 2)	0.79	2.19E-02
ENSDARG00000019274	rasd1	0.79	8.98E-05
ENSDARG00000054058	h1fx	0.79	1.22E-11
ENSDARG00000042725	cebpb	0.78	9.16E-06
ENSDARG00000035602	dao.1	0.78	2.70E-03
ENSDARG00000025338	hagh	0.77	1.26E-05
ENSDARG00000093156	si:ch73-21g5.7	0.76	8.39E-03
ENSDARG00000040907	gcgb	0.76	1.85E-02
ENSDARG00000030905	cited2	0.73	1.05E-04
ENSDARG00000092337	gas5	0.73	1.41E-05
ENSDARG00000094557	nupr1	0.72	4.72E-04
ENSDARG00000026359	PBLD (2 of 2)	0.70	1.40E-02
ENSDARG00000091350	MYO18B	0.69	3.57E-02
ENSDARG00000045568	bcat1	0.69	3.77E-02
ENSDARG00000045976	sidt2	0.68	3.41E-04
ENSDARG00000027529	hmox1	0.68	3.18E-04
ENSDARG00000032496	zgc:91887	0.68	2.40E-02
ENSDARG00000070000	txnipb	0.68	1.89E-03
ENSDARG00000060113	znf395a	0.66	1.40E-04
ENSDARG00000001873	phgdh	0.65	1.93E-06
ENSDARG00000056379	si:ch211-154o6.6	0.65	1.85E-02
ENSDARG00000060315	si:dkey-193b15.6	0.65	1.86E-02
ENSDARG00000035559	tp53	0.64	2.83E-02
ENSDARG00000026611	socs3b	0.64	1.81E-02
ENSDARG00000038559	h1f0	0.63	7.48E-08
ENSDARG00000068096	ATF5 (1 of 2)	0.63	1.47E-03
ENSDARG00000035519	histh1l	0.63	9.62E-08
ENSDARG00000090552	wu:fb12e11	0.62	9.72E-03
ENSDARG00000059202	TSPAN2 (2 of 2)	0.62	1.53E-02
ENSDARG00000020742	NAGA	0.61	2.59E-02

Gene ID	Gene	Log2(fold_change)	q_value
ENSDARG00000021787	abcb5	0.61	7.61E-05
ENSDARG00000088885	PHGR1	0.61	1.02E-02
ENSDARG00000006019	tkt	0.60	5.66E-04
ENSDARG00000042874	phlda2	0.59	2.59E-02
ENSDARG00000070426	chac1	0.59	1.14E-06
ENSDARG00000016733	psat1	0.58	7.23E-06
ENSDARG00000035890	fuca1	0.58	1.17E-04
ENSDARG00000076838	APOM	0.57	6.17E-03
ENSDARG00000095147	KRT23 (2 of 15)	0.57	4.93E-02
ENSDARG00000033666	pi4k2a	0.56	1.03E-02
ENSDARG00000038147	hbbe3	0.56	3.51E-03
ENSDARG00000017388	gstt1b	0.56	1.82E-02
ENSDARG00000091816	CABZ01088039.1	0.56	3.58E-03
ENSDARG00000029011	xpnpep1	0.55	1.56E-02
ENSDARG00000052705	pkp1	0.55	3.21E-02
ENSDARG00000067652	im:7150988	0.54	1.92E-03
ENSDARG00000028618	KRT18 (2 of 3)	0.54	1.54E-04
ENSDARG00000041607	eif4ebp3l	0.53	1.26E-02
ENSDARG00000020232	eif6	0.53	4.29E-02
ENSDARG00000070047	rgs4	0.53	1.22E-03
ENSDARG00000018566	CU302436.1	0.53	1.78E-04
ENSDARG00000012987	gpia	0.53	9.23E-04
ENSDARG00000015164	mknk2b	0.52	3.54E-04
ENSDARG00000041394	dnajb1b	0.51	2.59E-02
ENSDARG00000007955	iars	0.51	5.19E-04
ENSDARG00000010312	cp	0.50	2.37E-04
ENSDARG00000016319	c9	0.50	1.02E-02
ENSDARG00000013968	psap	0.49	1.05E-04
ENSDARG00000036107	txnipa	0.49	1.49E-04
ENSDARG00000028957	maff	0.49	3.41E-02
ENSDARG00000094300	si:ch211-160e1.5	0.49	1.92E-02
ENSDARG00000016200	trib3	0.48	2.52E-02
ENSDARG00000056395	onecut3	0.48	2.13E-02
ENSDARG00000013430	bhmt	0.48	3.62E-03
ENSDARG00000089920	MLIP	0.48	1.22E-03
ENSDARG00000076667	ccng1	0.47	1.24E-03
ENSDARG00000017960	sfxn2	0.46	1.96E-02
ENSDARG00000054030	hoxb5b	0.46	2.17E-02
ENSDARG00000010946	cbsb	0.45	2.66E-02
ENSDARG00000069142	aars	0.45	3.18E-03
ENSDARG00000037910	FILIP1L (2 of 2)	0.44	3.98E-02
ENSDARG00000033609	map1lc3a	0.44	3.75E-02
ENSDARG00000071082	p4ha1b	0.44	1.13E-02
ENSDARG00000091996	si:ch211-117m20.5	0.43	2.01E-03
ENSDARG00000017180	npc1	0.42	3.47E-03
ENSDARG00000042934	ctgfa	0.41	1.49E-02
ENSDARG00000037618	ddit4	0.41	4.21E-02
ENSDARG00000076241	txlnbb	0.41	2.27E-02
ENSDARG00000044125	txn	0.38	4.93E-02
ENSDARG00000061100	nars	0.36	4.21E-02

Gene ID	Gene	Log2(fold_change)	q_value
ENSDARG00000057698	ctsd	0.36	3.48E-02
ENSDARG00000058656	desma	0.35	4.67E-02
ENSDARG00000079745	si:ch211-166a6.5	0.34	3.72E-02
ENSDARG00000041811	rps25	0.34	4.95E-02
ENSDARG00000026726	anxa1a	0.33	4.81E-02
ENSDARG00000042245	MYL4	-0.34	4.67E-02
ENSDARG00000057052	DSCAML1	-0.34	4.29E-02
ENSDARG00000018259	atp1a3a	-0.34	4.83E-02
ENSDARG00000019566	neurod	-0.35	3.21E-02
ENSDARG00000055216	tuba1l	-0.35	2.82E-02
ENSDARG00000009001	pdip5	-0.35	4.21E-02
ENSDARG00000074908	col6a1	-0.35	3.82E-02
ENSDARG00000017568	HNRNPAB (2 of 2)	-0.36	4.37E-02
ENSDARG00000056151	tyrp1b	-0.36	2.55E-02
ENSDARG00000073732	myh14	-0.36	4.58E-02
ENSDARG00000029058	rbb4	-0.36	3.10E-02
ENSDARG00000005551	hnrnph1l	-0.37	2.96E-02
ENSDARG00000056725	hmgb3a	-0.37	3.37E-02
ENSDARG00000019353	sparc	-0.37	4.57E-02
ENSDARG00000037846	hm13	-0.37	4.64E-02
ENSDARG00000079772	hmgb1a	-0.38	1.49E-02
ENSDARG00000090268	KRT23 (12 of 15)	-0.38	3.91E-02
ENSDARG00000068507	crybb1	-0.39	4.82E-02
ENSDARG00000004665	hspa5	-0.40	7.57E-03
ENSDARG00000071353	AL929007.1	-0.40	1.04E-02
ENSDARG00000028524	col5a3b	-0.40	5.37E-03
ENSDARG00000086222	NAT16	-0.40	1.81E-02
ENSDARG00000020103	calrl	-0.40	5.12E-03
ENSDARG00000027355	slc25a4	-0.40	5.12E-03
ENSDARG00000013963	mipb	-0.40	6.08E-03
ENSDARG00000016235	rbp1a	-0.40	4.21E-02
ENSDARG00000003570	hsp90b1	-0.41	6.65E-03
ENSDARG00000088514	and1	-0.42	2.84E-03
ENSDARG00000054362	ccdc47	-0.42	4.46E-03
ENSDARG00000069737	pou4f2	-0.42	1.47E-02
ENSDARG00000031100	ivns1abpa	-0.43	1.53E-02
ENSDARG00000063914	mt-nd3	-0.43	2.18E-03
ENSDARG00000008732	zgc:66479	-0.43	4.85E-03
ENSDARG00000058117	snap25b	-0.43	3.78E-02
ENSDARG00000061124	srpr	-0.43	1.60E-02
ENSDARG00000056292	vsx1	-0.44	1.45E-02
ENSDARG00000037284	ptges3a	-0.44	1.45E-02
ENSDARG00000062688	gpnmb	-0.44	4.90E-02
ENSDARG00000054807	sec13	-0.44	2.75E-02
ENSDARG00000076768	REPS2	-0.45	3.42E-02
ENSDARG00000018491	pdia4	-0.45	1.40E-03
ENSDARG00000011125	snrpb	-0.45	3.08E-02
ENSDARG00000014179	pfkma	-0.46	1.77E-03
ENSDARG00000037285	mipa	-0.46	1.78E-03
ENSDARG00000039913	tmem147	-0.46	4.67E-02

Gene ID	Gene	Log2(fold_change)	q_value
ENSDARG00000074169	GPAM	-0.47	1.28E-02
ENSDARG00000030411	crygn2	-0.47	3.72E-04
ENSDARG00000090467	CABZ01074130.1	-0.48	2.73E-04
ENSDARG00000001889	tuba1l2	-0.48	2.55E-04
ENSDARG00000045843	apex1	-0.48	4.29E-02
ENSDARG00000092467	si:ch73-46j18.5	-0.48	7.23E-03
ENSDARG00000036840	krt15	-0.48	4.83E-02
ENSDARG00000012381	zgc:63663	-0.49	1.01E-03
ENSDARG00000018130	rhot1a	-0.49	4.61E-02
ENSDARG00000056248	wu:fb15e04	-0.50	1.14E-04
ENSDARG00000045143	hbbe2	-0.50	4.25E-03
ENSDARG00000043257	ckbb	-0.50	1.26E-02
ENSDARG00000073699	-	-0.51	2.07E-04
ENSDARG00000071219	pik3r3a	-0.51	3.64E-02
ENSDARG00000057738	hells	-0.51	1.08E-02
ENSDARG00000002071	adss	-0.51	3.61E-03
ENSDARG00000007576	crybb1l1	-0.51	1.03E-04
ENSDARG00000038056	FGFBP2 (1 of 2)	-0.51	3.55E-04
ENSDARG00000042780	APOB (1 of 3)	-0.51	2.40E-03
ENSDARG00000005161	gpib	-0.52	4.36E-03
ENSDARG00000074752	hlfa	-0.52	1.92E-03
ENSDARG00000063631	ch1073-291c23.1	-0.52	5.49E-03
ENSDARG00000053875	cryba1b	-0.52	1.49E-04
ENSDARG00000001910	rorab	-0.53	4.63E-04
ENSDARG00000042021	mapk12a	-0.53	3.35E-02
ENSDARG00000018119	cox5ab	-0.53	1.30E-02
ENSDARG00000053502	cryaa	-0.53	4.55E-03
ENSDARG00000059357	SARNP	-0.53	2.78E-04
ENSDARG00000044562	cycsb	-0.54	2.37E-05
ENSDARG00000005643	gcat	-0.54	4.60E-02
ENSDARG00000087765	si:ch211-212n6.17	-0.54	2.44E-04
ENSDARG00000057575	pnp4a	-0.55	1.76E-02
ENSDARG00000031316	six6b	-0.55	6.48E-03
ENSDARG00000086030	PCBP3 (2 of 2)	-0.55	4.67E-02
ENSDARG00000038785	abcf2a	-0.55	8.56E-05
ENSDARG00000090468	PPP1R3A (2 of 2)	-0.56	4.64E-02
ENSDARG00000015495	klf3	-0.56	8.15E-04
ENSDARG00000037997	tubb5	-0.57	3.80E-05
ENSDARG00000036344	calb2b	-0.57	2.31E-04
ENSDARG00000024548	cryba4	-0.58	2.77E-05
ENSDARG00000070386	KRTCAP2	-0.58	2.52E-02
ENSDARG00000061836	nfixb	-0.59	2.33E-03
ENSDARG00000037588	bhlhe23	-0.59	4.51E-02
ENSDARG00000007697	fabp7a	-0.60	1.38E-06
ENSDARG00000054804	anp32e	-0.60	4.49E-04
ENSDARG00000044975	KRT23 (9 of 15)	-0.61	1.30E-03
ENSDARG00000077341	PPP1R14C (1 of 2)	-0.61	3.55E-02
ENSDARG00000007715	lgsn	-0.61	2.13E-04
ENSDARG00000009401	vcanb	-0.63	1.46E-05
ENSDARG00000036058	gnao1b	-0.63	5.90E-05

Gene ID	Gene	Log2(fold_change)	q_value
ENSDARG00000010717	chchd10	-0.63	1.66E-02
ENSDARG00000032929	cryba1l	-0.64	7.06E-08
ENSDARG00000021720	COL4A6	-0.65	1.11E-05
ENSDARG00000039605	mat1a	-0.65	4.51E-08
ENSDARG00000056938	kera	-0.65	8.98E-05
ENSDARG00000093774	rbp2b	-0.66	1.97E-03
ENSDARG00000033760	pmelb	-0.66	3.90E-06
ENSDARG00000011166	cahz	-0.66	4.72E-04
ENSDARG00000087324	crygm2d1	-0.66	1.72E-04
ENSDARG00000030349	cryba2a	-0.66	5.73E-08
ENSDARG00000018846	dgat2	-0.67	6.40E-04
ENSDARG00000069415	col17a1a	-0.67	1.21E-07
ENSDARG00000075161	defbl1	-0.67	1.12E-04
ENSDARG00000029689	TKT	-0.67	1.05E-05
ENSDARG00000069823	PROCA1	-0.68	7.87E-03
ENSDARG00000032200	rgn	-0.68	4.67E-02
ENSDARG00000095863	zgc:161979	-0.68	1.24E-08
ENSDARG00000014488	ca2	-0.69	7.78E-09
ENSDARG00000023181	pcp4l1	-0.69	1.96E-02
ENSDARG00000029019	epb41b	-0.69	4.43E-03
ENSDARG00000086917	si:ch211-212n6.18	-0.71	1.14E-02
ENSDARG00000094760	si:dkey-125i10.3	-0.72	6.00E-03
ENSDARG00000004282	zgc:77375	-0.72	3.64E-02
ENSDARG00000076693	si:ch211-212n6.8	-0.74	5.29E-05
ENSDARG00000018258	ADK (2 of 2)	-0.74	3.67E-03
ENSDARG00000012366	fbp2	-0.75	5.15E-04
ENSDARG00000014594	anxa1b	-0.76	5.45E-06
ENSDARG00000011989	crx	-0.76	4.71E-11
ENSDARG00000086658	si:ch211-212n6.16	-0.76	9.16E-06
ENSDARG00000079302	and2	-0.76	2.12E-11
ENSDARG00000040535	CSGALNACT1 (1 of 2)	-0.77	3.95E-03
ENSDARG00000003820	nr1d2a	-0.77	2.40E-03
ENSDARG00000052700	si:dkey-162b23.4	-0.77	2.33E-03
ENSDARG00000091260	MYLK4 (1 of 2)	-0.78	1.15E-03
ENSDARG00000086281	zgc:112992	-0.78	1.37E-03
ENSDARG00000052039	caspb	-0.78	1.56E-04
ENSDARG00000002193	rho	-0.78	8.72E-04
ENSDARG00000074001	crygmxl2	-0.78	5.07E-10
ENSDARG00000045685	cntn1b	-0.78	4.64E-02
ENSDARG00000002311	fabp11b	-0.78	9.38E-05
ENSDARG00000019417	gadd45g	-0.78	6.00E-03
ENSDARG00000090689	si:busm1-118j2.5	-0.79	2.41E-07
ENSDARG00000042641	cyp51	-0.79	3.32E-03
ENSDARG00000075270	CU896655.3	-0.80	3.42E-02
ENSDARG00000033140	desi1a	-0.80	2.48E-05
ENSDARG00000060345	apod	-0.82	9.09E-05
ENSDARG00000057206	nmt1b	-0.82	3.28E-02
ENSDARG00000038643	alas2	-0.83	1.61E-08
ENSDARG00000016491	aglb	-0.84	3.05E-08
ENSDARG00000087164	crygm2d4	-0.85	1.93E-06

Gene ID	Gene	Log2(fold_change)	q_value
ENSDARG00000071488	AGL (3 of 3)	-0.86	2.38E-02
ENSDARG00000052917	im:7154842	-0.86	5.59E-11
ENSDARG00000087390	hbbe1.1	-0.87	0.00E+00
ENSDARG00000015076	cx44.1	-0.87	2.78E-04
ENSDARG00000089963	hbbe1.1	-0.87	2.54E-13
ENSDARG00000043961	BX957322.1	-0.88	1.62E-04
ENSDARG00000087188	nfil3-6	-0.90	1.14E-03
ENSDARG00000088823	crygm2d3	-0.91	2.16E-05
ENSDARG00000045141	aqp8a.1	-0.91	6.26E-03
ENSDARG00000079305	hbae3	-0.94	2.54E-13
ENSDARG00000037371	dcun1d1	-0.94	3.88E-02
ENSDARG00000023537	ahr1b	-0.97	5.06E-08
ENSDARG00000057460	crygm2d13	-0.98	1.22E-11
ENSDARG00000069792	crygm2d5	-0.98	1.92E-05
ENSDARG00000052631	thbs4a	-0.98	3.93E-04
ENSDARG00000035309	entpd3	-0.98	1.27E-07
ENSDARG00000007655	crybb1l3	-0.98	2.41E-02
ENSDARG00000023082	krt1-19d	-0.99	5.84E-03
ENSDARG00000069801	crygm2d12	-1.01	0.00E+00
ENSDARG00000030215	matn1	-1.03	7.50E-04
ENSDARG00000011640	syt5b	-1.03	1.10E-10
ENSDARG00000067639	prpf4	-1.04	4.67E-02
ENSDARG00000001760	tnxb	-1.04	2.09E-08
ENSDARG00000037921	gng13b	-1.04	0.00E+00
ENSDARG00000045808	rlbp1b	-1.06	2.41E-02
ENSDARG00000069988	ARID5A (2 of 2)	-1.07	3.41E-02
ENSDARG00000078440	CCDC88A	-1.08	1.70E-08
ENSDARG00000040321	rx2	-1.09	1.98E-03
ENSDARG00000092945	si:ch211-250g4.3	-1.11	0.00E+00
ENSDARG00000001976	si:ch211-13k12.1	-1.11	6.26E-03
ENSDARG00000037337	cnrip1b	-1.12	4.05E-02
ENSDARG00000044212	CR385063.1	-1.12	0.00E+00
ENSDARG00000023324	rab11bb	-1.13	8.54E-03
ENSDARG00000091148	zgc:162402	-1.13	7.16E-09
ENSDARG00000004358	gnb3a	-1.14	0.00E+00
ENSDARG00000041382	si:dkey-283b15.2	-1.15	4.35E-03
ENSDARG00000088330	si:ch211-5k11.2	-1.16	2.09E-06
ENSDARG00000055118	mylipb	-1.16	2.55E-02
ENSDARG00000068194	klf9	-1.16	1.05E-02
ENSDARG00000003991	fhl2b	-1.18	3.42E-02
ENSDARG00000069451	cx50.5	-1.20	1.98E-03
ENSDARG00000087345	CABZ01059415.2	-1.20	9.59E-06
ENSDARG00000051981	STX3 (2 of 2)	-1.20	8.23E-03
ENSDARG00000088636	wu:fa03e10	-1.21	0.00E+00
ENSDARG00000057629	slc30a8	-1.22	9.96E-07
ENSDARG00000093318	CRYGB	-1.24	8.68E-05
ENSDARG00000031952	mb	-1.25	0.00E+00
ENSDARG00000012504	rlbp1a	-1.26	8.73E-10
ENSDARG00000076572	crygm2d7	-1.27	1.07E-12
ENSDARG00000033382	grifin	-1.29	2.03E-02

Gene ID	Gene	Log2(fold_change)	q_value
ENSDARG00000007788	atp2b1b	-1.29	1.60E-02
ENSDARG00000062661	ABCA4 (1 of 2)	-1.29	2.84E-05
ENSDARG00000058556	CR854881.1	-1.31	2.68E-09
ENSDARG00000073874	crygm2d6	-1.32	0.00E+00
ENSDARG00000086912	zgc:86723	-1.33	0.00E+00
ENSDARG00000076055	RPGRIP1	-1.33	1.26E-02
ENSDARG00000086360	RP1 (2 of 2)	-1.33	1.94E-02
ENSDARG00000076192	ankrd1b	-1.40	0.00E+00
ENSDARG00000035544	agxt2l1	-1.40	3.88E-02
ENSDARG00000069817	crygm2d8	-1.42	0.00E+00
ENSDARG00000027495	elovl4b	-1.46	1.60E-11
ENSDARG00000028396	fkbp5	-1.50	0.00E+00
ENSDARG00000073750	crygm2d9	-1.50	9.85E-13
ENSDARG00000037656	C17H2orf71	-1.55	2.29E-02
ENSDARG00000007480	rpe65a	-1.58	1.36E-13
ENSDARG00000019902	rcv1	-1.58	0.00E+00
ENSDARG00000069826	crygm2d15	-1.62	0.00E+00
ENSDARG00000012126	zgc:109965	-1.62	9.69E-12
ENSDARG00000038634	CCK (1 of 2)	-1.68	1.58E-03
ENSDARG00000056511	arr3a	-1.71	7.92E-08
ENSDARG00000069615	CKMT2 (1 of 2)	-1.75	8.26E-05
ENSDARG00000075295	-	-1.76	7.06E-06
ENSDARG00000088589	ponzr3	-1.78	1.83E-06
ENSDARG00000094990	si:dkey-91f15.1	-1.87	2.33E-02
ENSDARG00000052223	rcvrna	-1.88	1.03E-07
ENSDARG00000038894	tmx3	-1.90	1.82E-07
ENSDARG00000087301	crygm2d14	-1.90	0.00E+00
ENSDARG00000076624	ptprb	-1.93	1.22E-11
ENSDARG00000076790	si:ch211-55g3.6	-2.07	0.00E+00
ENSDARG00000088687	zgc:165347	-2.11	0.00E+00
ENSDARG00000027236	rs1	-2.11	4.49E-06
ENSDARG00000057427	SV2B (3 of 3)	-2.21	7.83E-05
ENSDARG00000087440	ponzr4	-2.27	7.35E-10
ENSDARG00000045677	opn1sw1	-2.82	8.92E-03
ENSDARG00000094310	si:ch211-255g12.6	-2.86	0.00E+00
ENSDARG00000069827	crygm2d11	-3.05	5.59E-11
ENSDARG00000026855	cacna2d4a	-3.10	3.22E-03
ENSDARG00000044861	opn1lw2	-3.43	2.06E-04

Chapter 5 - Discussion

Individual PAHs interact with biological systems in numerous ways, resulting in complex responses in many organs and tissue types. To respond to exposures, organisms at all levels of biological complexity have the ability to detect, metabolize and excrete these multi-ringed structures that are ubiquitous in the environment. In vertebrates, the most well-known mediator of xenobiotic response is arguably the AHR, which can bind some PAH structures with high affinity. Metabolism and receptor affinity play large roles in the toxicity of AHR agonists. TCDD, one of the most toxic halogenated hydrocarbons, binds the AHR with high affinity but is not readily metabolized. Compounds naturally present in plants, such as indole 3-carbinol, are also AHR agonists but are more commonly thought of as beneficial components of the diet (Denison and Nagy 2003). Research on PAHs both in aquatic systems and mammals has highlighted a need to better understand both the AHR-dependent and -independent mechanisms by which PAH structures exert toxicological effects, and the concentrations at which these biological mechanisms become concerns for human and wildlife health.

Many studies have screened PAHs for their mutagenic activity, and PAHs are classified as carcinogenic or non-carcinogenic for human health risk assessment purposes. Because little is known about other toxicity mechanisms, it is not currently possible to classify PAHs as "inflammatory" or "ion channel disrupters", which would be necessary for predicting risk of these effects from exposure to complex PAH mixtures. The involvement of metabolism and receptor binding in the toxicological mechanisms causes small difference in PAH structure to result in very different biological effects. The presence of a "bay" or "fjord" region in higher molecular weight PAHs affects DNA damage potential (Mattsson et al. 2009). A recent study demonstrated that this is also true for non-genotoxic effects of methylanthracenes; 1-methylanthracene (1-MeA), which contains a bay-like region, was compared with 2-methylanthracene (2-MeA) for inflammatory related effects(Osgood et al. 2013) In an alveolar cell line, 1-MeA induced inflammatory signaling associated with tumor promotion, including MAP Kinase signaling, p38 phosphorylation, and inhibited gap junctional intercellular communication, while 2-MeA did not (Osgood et al. 2013). Recognizing these differences is particularly important as we begin to assess the toxicity of

substituted PAHs. Associating molecular mechanisms with structural differences would greatly increase our predictive power for toxicological effects of this large family of compounds.

The objective of the research presented here was to create profiles of biological activities of diverse PAH structures during embryonic development. The zebrafish model can rapidly provide a rich *in vivo* dataset from which to tease apart different biological mechanisms. Morphology analysis can be used to assess toxicity potential with high sensitivity (Truong et al. 2011). Whole genome analysis of mRNA expression via microarrays and RNA-seq provides a snapshot of the transcriptional effects of exposure at a point in time. By comparing these snapshots between compounds, we identified common effects, biomarker genes, and proposed mechanisms for further investigation of PAH-mediated toxixity.

In chapter 2, we compared microarray mRNA profiles of three parent PAHs, dibenzothiophene (DBT), pyrene (PYR), and benz(a)anthracene (BAA). By analyzing early transcriptional changes at concentrations that eventually elicited biological effects, we identified genes that were differentially involved in toxicological effects of these PAHs. We also measured body burdens of PAHs at the exposure concentrations and time points employed in the microarray. This information was essential for discerning potential mechanism from body burden differences. PAHs are hydrophobic; they are readily absorbed by embryos but also adhere to plastic, complicating exposures. While the studies presented here were all conducted in glass vials, solubility likely played a substantial role in the biological effects observed for the parent PAHs. Precipitation of PAH occurs for both PYR and BAA at the 25 μM concentration. PYR is slightly more soluble than BAA, and we expected some differences in body burden, but the extent to which this varied between compounds was surprising. The large difference in body burden between DBT and the 4-ring parent PAHs was important for interpreting the expression data. By comparing $\log_2$ FC values across all transcripts that were significantly induced by DBT or PYR, we found very similar patterns of expression. We found little evidence for distinct biological mechanisms between DBT and PYR, while BAA induced a very different profile of genes. BAA- induced genes were examined in further chapters. DBT and PYR, however, induced a large suite of inflammatory-related genes, which has been previously observed for PAHs in other model

systems. The exact mechanism of inflammation is not clear, and I observed no sign of a specific inflammatory site, using transgenic zebrafish with mpx-driven expression of GFP (neutrophils). I also observed a hyperactive phenotype elicited by PYR exposure. DBT also induced hyperactivity, but it decreased, while PYR-exposed embryos remained hyperactive for 5 hours (Appendix 2). Hyperactivity was observed at concentrations as low as 2.5 μM; future studies will investigate the mechanism that results in this behavioral phenotype.

The role of the AHR in mediating PAH-induced developmental toxicity is not yet well-defined. In Chapter 3, we characterized an *ahr2* mutant zebrafish, which will be an important tool for defining the role of the AHR in both PAH-mediated toxicity as well as normal development. We determined that the TILLING-identified *ahr2* mutant was indeed a functional AHR2 knockout, and was completely resistant to TCDD-induced toxicity. We additionally investigated the effects of an alternative ligand, leflunomide, in the *ahr2*^{hu3335} line. Interestingly, we found that leflunomide induced liver-specific Cyp1a in the mutant line, and found that the AHR isoform AHR1A was responsible for mediating this induction. While this study showed that AHR1A is a functional receptor, future work is needed to characterize the functions of this receptor and potential roles in mediating toxicity.

Building off of a screen of substituted PAHs, we investigated the role of AHR2 in mediating the developmental toxicity of two similarly-structured OPAHs, BEZO and 7,12-B[a]AQ in Chapter 4. Intriguingly, we found that despite different CYP1A induction profiles, developmental effects of both BEZO and 7,12-B[a]AQ were mediated by AHR2. We conducted paired-end 50 bp RNA-seq to compare mRNA expression profiles of embryos exposed to the two compounds from 6-48 hpf. Using the Tuxedo suite of software, we identified novel transcripts that are promising targets for future investigation of AHR-dependent toxicological pathways. By comparing BEZO and 7,12-B[a]AQ transcriptional profiles, we found differences in groups of transcripts, such as eye-related genes, that suggest the AHR interacts with other transcription factors or coactivators to mediate the differential toxicological effects observed for these two compounds. Future studies using morpholino knockdown of other predicted interactors would be a promising way to determine their roles in the toxicity of BEZO and 7,12-B[a]AQ, as well as discover new AHR toxicity mechanisms.

We identified transcripts with common identifiers that could be compared across the microarray and RNA-seq platforms. By clustering transcripts by expression changes observed with all 5 PAHs, we identified genes that could be potential biomarkers of PAH-related toxicity. *Ctsl.1*, for example, was induced by BAA, BEZO, and 7,12-B[a]AQ. Cathepsin L1 is a protease that influences blood pressure, and a recent study identified a polymorphic locus in its promoter, which was associated with high blood pressure in a human population (Mbewe-Campbell et al. 2012). By examining the locus, the authors found it was located in an XRE, and determined that CTSL.1 is AHR-responsive. To our knowledge, the effects of xenobiotic exposure on CTSL.1 and blood pressure regulation have not yet been investigated. Chemokine receptor 4a (*cxcr4a)* was induced mildly, but consistently, by all PAHs in these studies and would also be an interesting target for future investigation.

In the work presented here, we investigated global transcriptional responses to diverse PAH structures and began to identify expression patterns associated with PAH exposure. Investigating PAH toxicity in AHR2 deficient zebrafish added additional insight into potential toxicity mechanisms. This work, along with other recent studies of both the AHR and PAHs, demonstrates that the AHR has functions well beyond xenobiotic-activated binding to the canonical AHR-responsive genes. Unraveling the pathways by which PAHs differentially interact with the receptor will be an engaging direction for future research.

References

Denison, M. S. and S. R. Nagy (2003). "Activation of the aryl hydrocarbon receptor by structurally diverse exogenous and endogenous chemicals." Annu Rev Pharmacol Toxicol **43**: 309-334.

Jonsson, M. E., D. G. Franks, et al. (2009). "The tryptophan photoproduct 6-formylindolo[3,2-b]carbazole (FICZ) binds multiple AHRs and induces multiple CYP1 genes via AHR2 in zebrafish." Chem Biol Interact.

Mattsson, A., B. Jernstrom, et al. (2009). "H2AX phosphorylation in A549 cells induced by the bulky and stable DNA adducts of benzo[a]pyrene and dibenzo[a,l]pyrene diol epoxides." Chem Biol Interact **177**(1): 40-47.

Mbewe-Campbell, N., Z. Wei, et al. (2012). "Genes and environment: novel, functional polymorphism in the human cathepsin L (CTSL1) promoter disrupts a xenobiotic response element (XRE) to alter transcription and blood pressure." J Hypertens.

Osgood, R. S., B. L. Upham, et al. (2013). "Polycyclic aromatic hydrocarbon-induced signaling events relevant to inflammation and tumorigenesis in lung cells are dependent on molecular structure." PLoS One **8**(6): e65150.

Truong, L., S. L. Harper, et al. (2011). "Evaluation of embryotoxicity using the zebrafish model." Methods Mol Biol **691**: 271-279.

Chapter 6 - Future Directions

In the studies present in this dissertation, we employed a comparative transcriptomics approach to identify global changes in mRNA expression that lead to developmental effects of PAH exposure in developing embryos. We identified clusters of genes and associated biological processes that are misexpressed in response to different PAH exposures, and further investigated the role of AHR2 in mediating responses. The rich datasets presented in this dissertation could lead to many future research directions. The ion channel disruption/inflammatory/NfKB-related processes disrupted by DBT and PYR exposure are complex, and attempted to characterize endpoints more specifically related to these processes in Appendix 2. Further studies of the PYR behavioral response, in comparison with other PAHs and in combination with potential antagonists are in progress in the laboratory.

Involvement of the AHR in toxicity pathways and normal development remains an exciting and complex area for research. With three AHR isoforms, zebrafish provide an opportunity to unravel differential functions that may be partitioned between the isoforms. We showed the AHR1A is a functional receptor, and can mediate CYP1A expression. Beyond ability to induce that downstream target, little is known about function of AHR1A or AHR1B. Characterization of where they are expressed over the course of development, and their interactions with a variety of ligands could provide valuable information about different functions of the AHR. These studies will be easier to carry out in the *ahr2*^{hu3335} line.

The need to outcross the *ahr2*^{hu3335} line has put many exciting studies of endogenous AHR2 functions on hold, but there is promise to further investigate how loss of AHR2 affects developmental processes with them in the future. Beyond this, determining global transcriptional responses to PAH exposure in the AHR2 mutant line would be an exciting direction for research. In Chapter 4, we investigated the dependence of select transcriptional changes on AHR2. This could be continued with additional genes, but provides only a piece-wise picture, and we have continually found the AHR to be involved in mechanisms in ways we have not predicted. Analysis across the genome would provide information about the entirety of processes the AHR is affecting.

From the RNA-seq analysis, we were able to identify misexpressed transcripts, and compare across PAHs. This dataset however, could be analyzed in many more interesting ways. As annotation of the zebrafish genome improves, promoter analysis and/or prediction of non-coding RNA targeting may be able to better predict expression regulation than our current analysis based on known interactions in mammals. Some novel transcripts were identified and discussed in this dissertation; many are present within the dataset that would be interesting to pursue for interaction with the AHR and involvement in zebrafish development processes. Annotation of the zebrafish genome has improved much over the past 5 years, but there are many transcripts that remain un-annotated and could be important mediators of toxicity.

Comparison of all 5 PAH structures in these studies shed light on interesting patterns in expression. Better statistical comparisons across multiple treatment groups may help to identify promising transcriptional differences between BEZO and 7,12-B[a]AQ for further analysis. Comparing additional PAH structures, will of course add power to identify which structural differences may be responsible for mediating groups of transcripts involved in different biological processes. Not discussed here are other samples collected in parallel with our BEZO and 7,12-B[a]AQ samples, which included phenanthrene-quinone and environmental mixture exposed samples. Adding these to the analysis, and comparing patterns across the larger dataset will be an interesting future direction.

Finally, comparing the genes and responses identified here across model systems will be a valuable direction for future studies. Homologues of many genes identified here have similar functions in other species, and eventually could be developed into biomarkers of PAH effects relevant to human or wildlife populations. Determining first whether PAH exposure affects their expression in rodents or human cell lines remains to be determined, and an interesting area of future work.

Bibliography

(2010). R: A language and environment for statistical computing. R. D. C. Team. Vienna, Austria.

Abbott, B. D., J. E. Schmid, et al. (1999). "Adverse reproductive outcomes in the transgenic Ah receptor-deficient mouse." Toxicol Appl Pharmacol **155**(1): 62-70.

Alexeyenko, A., D. M. Wassenberg, et al. (2010). "Dynamic zebrafish interactome reveals transcriptional mechanisms of dioxin toxicity." PLoS One **5**(5): e10465.

Andreasen, E. A., M. E. Hahn, et al. (2002). "The zebrafish (Danio rerio) aryl hydrocarbon receptor type 1 is a novel vertebrate receptor." Mol Pharmacol **62**(2): 234-249.

Andreasen, E. A., L. K. Mathew, et al. (2006). "Regenerative growth is impacted by TCDD: gene expression analysis reveals extracellular matrix modulation." Toxicol Sci **92**(1): 254-269.

Andreasen, E. A., J. M. Spitsbergen, et al. (2002). "Tissue-specific expression of AHR2, ARNT2, and CYP1A in zebrafish embryos and larvae: effects of developmental stage and 2,3,7,8-tetrachlorodibenzo-p-dioxin exposure." Toxicol Sci **68**(2): 403-419.

Antkiewicz, D. S., R. E. Peterson, et al. (2006). "Blocking expression of AHR2 and ARNT1 in zebrafish larvae protects against cardiac toxicity of 2,3,7,8-tetrachlorodibenzo-p-dioxin." Toxicol Sci **94**(1): 175-182.

Archuleta, M. M., G. L. Schieven, et al. (1993). "7,12-Dimethylbenz[a]anthracene activates protein-tyrosine kinases Fyn and Lck in the HPB-ALL human T-cell line and increases tyrosine phosphorylation of phospholipase C-gamma 1, formation of inositol 1,4,5-trisphosphate, and mobilization of intracellular calcium." Proc Natl Acad Sci U S A **90**(13): 6105-6109.

Baba, T., J. Mimura, et al. (2001). "Structure and expression of the Ah receptor repressor gene." J Biol Chem **276**(35): 33101-33110.

Baird, W. M., L. A. Hooven, et al. (2005). "Carcinogenic polycyclic aromatic hydrocarbon-DNA adducts and mechanism of action." Environ Mol Mutagen **45**(2-3): 106-114.

Barker, C. W., J. B. Fagan, et al. (1994). "Down-regulation of P4501A1 and P4501A2 mRNA expression in isolated hepatocytes by oxidative stress." J Biol Chem **269**(6): 3985-3990.

Barron, M. G., M. G. Carls, et al. (2004). "Evaluation of fish early life-stage toxicity models of chronic embryonic exposures to complex polycyclic aromatic hydrocarbon mixtures." Toxicol Sci **78**(1): 60-67.

Barron, M. G., R. Heintz, et al. (2004). "Relative potency of PAHs and heterocycles as aryl hydrocarbon receptor agonists in fish." Mar Environ Res **58**(2-5): 95-100.

Beischlag, T. V., J. Luis Morales, et al. (2008). "The aryl hydrocarbon receptor complex and the control of gene expression." Crit Rev Eukaryot Gene Expr **18**(3): 207-250.

Benjamini, Y. and Y. Hochberg (1995). "Controlling the False Discovery Rate - a Practical and Powerful Approach to Multiple Testing." Journal of the Royal Statistical Society Series B-Methodological **57**(1): 289-300.

Billiard, S. M., M. E. Hahn, et al. (2002). "Binding of polycyclic aromatic hydrocarbons (PAHs) to teleost aryl hydrocarbon receptors (AHRs)." Comp Biochem Physiol B Biochem Mol Biol **133**(1): 55-68.

Billiard, S. M., A. R. Timme-Laragy, et al. (2006). "The role of the aryl hydrocarbon receptor pathway in mediating synergistic developmental toxicity of polycyclic aromatic hydrocarbons to zebrafish." Toxicol Sci **92**(2): 526-536.

Binder, R. L. and J. J. Stegeman (1984). "Microsomal electron transport and xenobiotic monooxygenase activities during the embryonic period of development in the killifish, Fundulus heteroclitus." Toxicol Appl Pharmacol **73**(3): 432-443.

Bisson, W. H., D. C. Koch, et al. (2009). "Modeling of the aryl hydrocarbon receptor (AhR) ligand binding domain and its utility in virtual ligand screening to predict new AhR ligands." J Med Chem **52**(18): 5635-5641.

Bock, K. W. (2012). "Ah receptor- and Nrf2-gene battery members: Modulators of quinone-mediated oxidative and endoplasmic reticulum stress." Biochem Pharmacol **83**(7): 833-838.

Bostrom, C. E., P. Gerde, et al. (2002). "Cancer risk assessment, indicators, and guidelines for polycyclic aromatic hydrocarbons in the ambient air." Environ Health Perspect **110 Suppl 3**: 451-488.

Brasier, A. R., M. Jamaluddin, et al. (2000). "Angiotensin II induces gene transcription through cell-type-dependent effects on the nuclear factor-kappaB (NF-kappaB) transcription factor." Mol Cell Biochem **212**(1-2): 155-169.

Brasier, A. R. and J. Li (1996). "Mechanisms for inducible control of angiotensinogen gene transcription." Hypertension **27**(3 Pt 2): 465-475.

Bugel, S. M., L. A. White, et al. (2010). "Impaired reproductive health of killifish (Fundulus heteroclitus) inhabiting Newark Bay, NJ, a chronically contaminated estuary." Aquat Toxicol **96**(3): 182-193.

Burstyn, I., H. Kromhout, et al. (2005). "Polycyclic aromatic hydrocarbons and fatal ischemic heart disease." Epidemiology **16**(6): 744-750.

Bussmann, J., S. A. Wolfe, et al. (2011). "Arterial-venous network formation during brain vascularization involves hemodynamic regulation of chemokine signaling." Development **138**(9): 1717-1726.

Carney, S. A., J. Chen, et al. (2006). "Aryl hydrocarbon receptor activation produces heart-specific transcriptional and toxic responses in developing zebrafish." Mol Pharmacol **70**(2): 549-561.

Carney, S. A., R. E. Peterson, et al. (2004). "2,3,7,8-Tetrachlorodibenzo-p-dioxin activation of the aryl hydrocarbon receptor/aryl hydrocarbon receptor nuclear translocator pathway causes developmental toxicity through a CYP1A-independent mechanism in zebrafish." Mol Pharmacol **66**(3): 512-521.

Carney, S. A., A. L. Prasch, et al. (2006). "Understanding dioxin developmental toxicity using the zebrafish model." Birth Defects Res A Clin Mol Teratol **76**(1): 7-18.

Cavalieri, E. L. and E. G. Rogan (1995). "Central role of radical cations in metabolic activation of polycyclic aromatic hydrocarbons." Xenobiotica **25**(7): 677-688.

Chew, G. L., A. Pauli, et al. (2013). "Ribosome profiling reveals resemblance between long non-coding RNAs and 5' leaders of coding RNAs." Development **140**(13): 2828-2834.

Choi, H., W. Jedrychowski, et al. (2006). "International studies of prenatal exposure to polycyclic aromatic hydrocarbons and fetal growth." Environ Health Perspect **114**(11): 1744-1750.

Ciganek, M., J. Neca, et al. (2004). "A combined chemical and bioassay analysis of traffic-emitted polycyclic aromatic hydrocarbons." Sci Total Environ **334-335**: 141-148.

Collins, J. F., J. P. Brown, et al. (1998). "Potency equivalency factors for some polycyclic aromatic hydrocarbons and polycyclic aromatic hydrocarbon derivatives." Regul Toxicol Pharmacol **28**(1): 45-54.

Couroucli, X. I., S. E. Welty, et al. (2002). "Regulation of pulmonary and hepatic cytochrome P4501A expression in the rat by hyperoxia: implications for hyperoxic lung injury." Mol Pharmacol **61**(3): 507-515.

Cubbage, C. C. and P. M. Mabee (1996). "Development of the cranium and paired fins in the zebrafish Danio rerio (Ostariophysi, cyprinidae)." Journal of Morphology **229**(2): 121-160.

Das, M., K. Garg, et al. (1994). "Attenuation of benzanthrone toxicity by ascorbic acid in guinea pigs." Fundam Appl Toxicol **22**(3): 447-456.

Denison, M. S. and S. R. Nagy (2003). "Activation of the aryl hydrocarbon receptor by structurally diverse exogenous and endogenous chemicals." Annu Rev Pharmacol Toxicol **43**: 309-334.

Detmar, J., M. Y. Rennie, et al. (2008). "Fetal growth restriction triggered by polycyclic aromatic hydrocarbons is associated with altered placental vasculature and AhR-dependent changes in cell death." Am J Physiol Endocrinol Metab **295**(2): E519-530.

Dong, P. D., C. A. Munson, et al. (2007). "Fgf10 regulates hepatopancreatic ductal system patterning and differentiation." Nat Genet **39**(3): 397-402.

Duarte-Salles, T., M. A. Mendez, et al. (2012). "Dietary benzo(a)pyrene and fetal growth: effect modification by vitamin C intake and glutathione S-transferase P1 polymorphism." Environ Int **45**: 1-8.

Durant, J. L., W. F. Busby, Jr., et al. (1996). "Human cell mutagenicity of oxygenated, nitrated and unsubstituted polycyclic aromatic hydrocarbons associated with urban aerosols." Mutat Res **371**(3-4): 123-157.

Durante, W. (2013). "Role of arginase in vessel wall remodeling." Front Immunol **4**: 111.

Dwivedi, N., M. Das, et al. (2001). "Role of biological antioxidants in benzanthrone toxicity." Archives of Toxicology **75**(4): 221-226.

Ebert, A. M., G. L. Hume, et al. (2005). "Calcium extrusion is critical for cardiac morphogenesis and rhythm in embryonic zebrafish hearts." Proc Natl Acad Sci U S A **102**(49): 17705-17710.

Eden, E., R. Navon, et al. (2009). "GOrilla: a tool for discovery and visualization of enriched GO terms in ranked gene lists." BMC Bioinformatics **10**: 48.

Ema, M., N. Ohe, et al. (1994). "Dioxin binding activities of polymorphic forms of mouse and human arylhydrocarbon receptors." J Biol Chem **269**(44): 27337-27343.

EPA, U. (2010). Development of a relative potency factor (RPF) approach for polycyclic aromatic hydrocarbon (PAH) mixtures (External review draft). U. S. E. P. Agency. Washington, DC.

EPA, U. (2012). "Integrated Risk Information System." from http://www.epa.gov/iris/.

Evans, B. R., S. I. Karchner, et al. (2008). "Repression of aryl hydrocarbon receptor (AHR) signaling by AHR repressor: role of DNA binding and competition for AHR nuclear translocator." Mol Pharmacol **73**(2): 387-398.

Fan, R., D. Wang, et al. (2012). "Preliminary study of children's exposure to PAHs and its association with 8-hydroxy-2'-deoxyguanosine in Guangzhou, China." Environ Int **42**: 53-58.

Fernandez-Salguero, P., T. Pineau, et al. (1995). "Immune system impairment and hepatic fibrosis in mice lacking the dioxin-binding Ah receptor." Science **268**(5211): 722-726.

Fleming, C. R. and R. T. Di Giulio (2011). "The role of CYP1A inhibition in the embryotoxic interactions between hypoxia and polycyclic aromatic hydrocarbons (PAHs) and PAH mixtures in zebrafish (Danio rerio)." Ecotoxicology **20**(6): 1300-1314.

Fukunaga, B. N. and O. Hankinson (1996). "Identification of a novel domain in the aryl hydrocarbon receptor required for DNA binding." J Biol Chem **271**(7): 3743-3749.

Fukunaga, B. N., M. R. Probst, et al. (1995). "Identification of functional domains of the aryl hydrocarbon receptor." J Biol Chem **270**(49): 29270-29278.

Gao, J., A. A. Voss, et al. (2005). "Ryanodine receptor-mediated rapid increase in intracellular calcium induced by 7,8-benzo(a)pyrene quinone in human and murine leukocytes." Toxicol Sci **87**(2): 419-426.

Garner, L. V. and R. T. Di Giulio (2012). "Glutathione transferase pi class 2 (GSTp2) protects against the cardiac deformities caused by exposure to PAHs but not PCB-126 in zebrafish embryos." Comp Biochem Physiol C Toxicol Pharmacol **155**(4): 573-579.

Gonzalez, F. J. and P. Fernandez-Salguero (1998). "The aryl hydrocarbon receptor: studies using the AHR-null mice." Drug Metab Dispos **26**(12): 1194-1198.

Goodale, B. C., S. C. Tilton, et al. (2013). "Structurally distinct polycyclic aromatic hydrocarbons induce differential transcriptional responses in developing zebrafish." Toxicol Appl Pharmacol.

Gu, Y. Z., J. B. Hogenesch, et al. (2000). "The PAS superfamily: sensors of environmental and developmental signals." Annu Rev Pharmacol Toxicol **40**: 519-561.

Guengerich, F. P. (2000). "Metabolism of chemical carcinogens." Carcinogenesis **21**(3): 345-351.

Gurbani, D., S. K. Bharti, et al. (2013). "Polycyclic aromatic hydrocarbons and their quinones modulate the metabolic profile and induce DNA damage in human alveolar and bronchiolar cells." Int J Hyg Environ Health.

Hahn, M. E. (1998). "The aryl hydrocarbon receptor: a comparative perspective." Comp Biochem Physiol C Pharmacol Toxicol Endocrinol **121**(1-3): 23-53.

Hahn, M. E. (2002). "Aryl hydrocarbon receptors: diversity and evolution." Chem Biol Interact **141**(1-2): 131-160.

Hahn, M. E. (2002). "Biomarkers and bioassays for detecting dioxin-like compounds in the marine environment." Sci Total Environ **289**(1-3): 49-69.

Hahn, M. E., S. I. Karchner, et al. (2006). "Unexpected diversity of aryl hydrocarbon receptors in non-mammalian vertebrates: insights from comparative genomics." J Exp Zool A Comp Exp Biol **305**(9): 693-706.

Hansch, C., Albert, L., Hoekman, D. (1995). Exploring QSAR: Volume 2: Hydrophobic, Electronic, and Steric Constants, American Chemical Society.

Hansen, A. M., L. Mathiesen, et al. (2008). "Urinary 1-hydroxypyrene (1-HP) in environmental and occupational studies--a review." Int J Hyg Environ Health **211**(5-6): 471-503.

Hecht, S. S., S. G. Carmella, et al. (2010). "Analysis of phenanthrene and benzo[a]pyrene tetraol enantiomers in human urine: relevance to the bay region diol epoxide hypothesis of benzo[a]pyrene carcinogenesis and to biomarker studies." Chem Res Toxicol **23**(5): 900-908.

Hermann, A., R. Donato, et al. (2012). "S100 calcium binding proteins and ion channels." Front Pharmacol **3**: 67.
Hernandez-Ochoa, I., B. N. Karman, et al. (2009). "The role of the aryl hydrocarbon receptor in the female reproductive system." Biochem Pharmacol **77**(4): 547-559.
Hertz-Picciotto, I., H. Y. Park, et al. (2008). "Prenatal exposures to persistent and non-persistent organic compounds and effects on immune system development." Basic Clin Pharmacol Toxicol **102**(2): 146-154.
Hofsteen, P., J. Plavicki, et al. (2013). "Sox9b is Required for Epicardium Formation and Plays a Role in TCDD-induced Heart Malformation in Zebrafish." Mol Pharmacol.
Hu, S. W., Y. J. Chan, et al. (2011). "Urinary levels of 1-hydroxypyrene in children residing near a coal-fired power plant." Environ Res **111**(8): 1185-1191.
Huang da, W., B. T. Sherman, et al. (2009). "Systematic and integrative analysis of large gene lists using DAVID bioinformatics resources." Nat Protoc **4**(1): 44-57.
Huang, L., C. Wang, et al. (2012). "Benzo[a]pyrene exposure influences the cardiac development and the expression of cardiovascular relative genes in zebrafish (Daniorerio) embryos." Chemosphere **87**(4): 369-375.
Hylland, K. (2006). "Polycyclic aromatic hydrocarbon (PAH) ecotoxicology in marine ecosystems." J Toxicol Environ Health A **69**(1-2): 109-123.
Incardona, J. P., M. G. Carls, et al. (2009). "Cardiac arrhythmia is the primary response of embryonic Pacific herring (Clupea pallasi) exposed to crude oil during weathering." Environ Sci Technol **43**(1): 201-207.
Incardona, J. P., M. G. Carls, et al. (2005). "Aryl hydrocarbon receptor-independent toxicity of weathered crude oil during fish development." Environ Health Perspect **113**(12): 1755-1762.
Incardona, J. P., T. K. Collier, et al. (2004). "Defects in cardiac function precede morphological abnormalities in fish embryos exposed to polycyclic aromatic hydrocarbons." Toxicol Appl Pharmacol **196**(2): 191-205.
Incardona, J. P., H. L. Day, et al. (2006). "Developmental toxicity of 4-ring polycyclic aromatic hydrocarbons in zebrafish is differentially dependent on AH receptor isoforms and hepatic cytochrome P4501A metabolism." Toxicol Appl Pharmacol **217**(3): 308-321.
Incardona, J. P., T. L. Linbo, et al. (2011). "Cardiac toxicity of 5-ring polycyclic aromatic hydrocarbons is differentially dependent on the aryl hydrocarbon receptor 2 isoform during zebrafish development." Toxicol Appl Pharmacol **257**(2): 242-249.
Isidoro Tavares, N., P. Philip-Couderc, et al. (2009). "Angiotensin II and tumour necrosis factor alpha as mediators of ATP-dependent potassium channel remodelling in post-infarction heart failure." Cardiovasc Res **83**(4): 726-736.
Jeng, H. A., C. H. Pan, et al. (2011). "Polycyclic aromatic hydrocarbon-induced oxidative stress and lipid peroxidation in relation to immunological alteration." Occup Environ Med **68**(9): 653-658.
Jennings, A. A. (2012). "Worldwide regulatory guidance values for surface soil exposure to carcinogenic or mutagenic polycyclic aromatic hydrocarbons." J Environ Manage **110**: 82-102.
Jennings, A. A. (2012). "Worldwide regulatory guidance values for surface soil exposure to noncarcinogenic polycyclic aromatic hydrocarbons." J Environ Manage **101**: 173-190.
Jensen, B. A., C. M. Reddy, et al. (2010). "Developing tools for risk assessment in protected species: Relative potencies inferred from competitive binding of halogenated

aromatic hydrocarbons to aryl hydrocarbon receptors from beluga (Delphinapterus leucas) and mouse." Aquat Toxicol **100**(3): 238-245.

Jia, Y., D. Stone, et al. (2011). "Estimated reduction in cancer risk due to PAH exposures if source control measures during the 2008 Beijing Olympics were sustained." Environ Health Perspect **119**(6): 815-820.

Jonsson, M. E., D. G. Franks, et al. (2009). "The tryptophan photoproduct 6-formylindolo[3,2-b]carbazole (FICZ) binds multiple AHRs and induces multiple CYP1 genes via AHR2 in zebrafish." Chem Biol Interact.

Jonsson, M. E., M. J. Jenny, et al. (2007). "Role of AHR2 in the expression of novel cytochrome P450 1 family genes, cell cycle genes, and morphological defects in developing zebra fish exposed to 3,3',4,4',5-pentachlorobiphenyl or 2,3,7,8-tetrachlorodibenzo-p-dioxin." Toxicol Sci **100**(1): 180-193.

Jules, G. E., S. Pratap, et al. (2012). "In utero exposure to benzo(a)pyrene predisposes offspring to cardiovascular dysfunction in later-life." Toxicology.

Jung, D., C. W. Matson, et al. (2011). "Genotoxicity in Atlantic killifish (Fundulus heteroclitus) from a PAH-contaminated Superfund site on the Elizabeth River, Virginia." Ecotoxicology **20**(8): 1890-1899.

Jung, K. H., J. H. Noh, et al. (2011). "Molecular signature for early detection and prediction of polycyclic aromatic hydrocarbons in peripheral blood." Environ Sci Technol **45**(1): 300-306.

Jyethi, D. S., P. S. Khillare, et al. (2013). "Risk assessment of inhalation exposure to polycyclic aromatic hydrocarbons in school children." Environ Sci Pollut Res Int.

Karchner, S. I., D. G. Franks, et al. (2005). "AHR1B, a new functional aryl hydrocarbon receptor in zebrafish: tandem arrangement of ahr1b and ahr2 genes." Biochem J **392**(Pt 1): 153-161.

Kerkvliet, N. I. (2009). "AHR-mediated immunomodulation: the role of altered gene transcription." Biochem Pharmacol **77**(4): 746-760.

Kerley-Hamilton, J. S., H. W. Trask, et al. (2012). "Inherent and Benzo[a]pyrene-Induced Differential Aryl Hydrocarbon Receptor Signaling Greatly Affects Life Span, Atherosclerosis, Cardiac Gene Expression, and Body and Heart Growth in Mice." Toxicol Sci **126**(2): 391-404.

Kim, K. T., T. Zaikova, et al. (2013). "Gold nanoparticles disrupt zebrafish eye development and pigmentation." Toxicol Sci **133**(2): 275-288.

Kimmel, C. B., W. W. Ballard, et al. (1995). "Stages of embryonic development of the zebrafish." Dev Dyn **203**(3): 253-310.

Kluver, N., L. Yang, et al. (2011). "Transcriptional response of zebrafish embryos exposed to neurotoxic compounds reveals a muscle activity dependent hspb11 expression." PLoS One **6**(12): e29063.

Knecht, A. L., B. C. Goodale, et al. (2013). "Comparative developmental toxicity of environmentally relevant oxygenated PAHs." Toxicol Appl Pharmacol.

Kojic, S., D. Radojkovic, et al. (2011). "Muscle ankyrin repeat proteins: their role in striated muscle function in health and disease." Crit Rev Clin Lab Sci **48**(5-6): 269-294.

Kraus, U., S. Breitner, et al. (2011). "Particle-associated organic compounds and symptoms in myocardial infarction survivors." Inhalation Toxicology **23**(7): 431-447.

Krieger, J. A., J. L. Born, et al. (1994). "Persistence of calcium elevation in the HPB-ALL human T cell line correlates with immunosuppressive properties of polycyclic aromatic hydrocarbons." Toxicol Appl Pharmacol **127**(2): 268-274.

Krieger, J. A., D. R. Davila, et al. (1995). "Inhibition of sarcoplasmic/endoplasmic reticulum calcium ATPases (SERCA) by polycyclic aromatic hydrocarbons in HPB-ALL human T cells and other tissues." Toxicol Appl Pharmacol **133**(1): 102-108.

Kriek, E., M. Rojas, et al. (1998). "Polycyclic aromatic hydrocarbon-DNA adducts in humans: relevance as biomarkers for exposure and cancer risk." Mutat Res **400**(1-2): 215-231.

Lahvis, G. P., R. W. Pyzalski, et al. (2005). "The aryl hydrocarbon receptor is required for developmental closure of the ductus venosus in the neonatal mouse." Mol Pharmacol **67**(3): 714-720.

Langrish, J. P., X. Li, et al. (2011). "Reducing personal exposure to particulate air pollution improves cardiovascular health in patients with coronary heart disease." Environ Health Perspect **120**(3): 367-372.

Layshock, J. A., G. Wilson, et al. (2010). "Ketone and Quinone-Substituted Polycyclic Aromatic Hydrocarbons in Mussel Tissue, Sediment, Urban Dust, and Diesel Particulate Matrices." Environmental Toxicology and Chemistry **29**(11): 2450-2460.

Le Mevel, J. C., F. Lancien, et al. (2008). "Central cardiovascular actions of angiotensin II in trout." Gen Comp Endocrinol **157**(1): 27-34.

Lee, M. S., S. Magari, et al. (2011). "Cardiac autonomic dysfunction from occupational exposure to polycyclic aromatic hydrocarbons." Occup Environ Med **68**(7): 474-478.

Li, X., Y. Feng, et al. (2012). "The dose-response decrease in heart rate variability: any association with the metabolites of polycyclic aromatic hydrocarbons in coke oven workers?" PLoS One **7**(9): e44562.

Livak, K. J. and T. D. Schmittgen (2001). "Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method." Methods **25**(4): 402-408.

Lundstedt, S., Y. Persson, et al. (2006). Transformation of PAHs during ethanol-Fenton treatment of an aged gasworks' soil. Chemosphere. England. **65:** 1288-1294.

Lundstedt, S., P. A. White, et al. (2007). "Sources, fate, and toxic hazards of oxygenated polycyclic aromatic hydrocarbons (PAHs) at PAH-contaminated sites." Ambio **36**(6): 475-485.

Mahler, B. J., P. C. Van Metre, et al. (2005). "Parking lot sealcoat: an unrecognized source of urban polycyclic aromatic hydrocarbons." Environ Sci Technol **39**(15): 5560-5566.

Mandrell, D., L. Truong, et al. (2012). "Automated zebrafish chorion removal and single embryo placement: optimizing throughput of zebrafish developmental toxicity screens." J Lab Autom **17**(1): 66-74.

Mao, C., L. Shi, et al. (2009). "Development of fetal brain renin-angiotensin system and hypertension programmed in fetal origins." Prog Neurobiol **87**(4): 252-263.

Mathew, L. K., E. A. Andreasen, et al. (2006). "Aryl hydrocarbon receptor activation inhibits regenerative growth." Mol Pharmacol **69**(1): 257-265.

Mathew, R., J. A. McGrath, et al. (2008). "Modeling polycyclic aromatic hydrocarbon bioaccumulation and metabolism in time-variable early life-stage exposures." Environ Toxicol Chem **27**(7): 1515-1525.

Matsumura, F., A. Puga, et al. (2009). "Biological functions of the arylhydrocarbon receptor: beyond induction of cytochrome P450s. Introduction to this special issue." Biochem Pharmacol **77**(4): 473.

Mattsson, A., B. Jernstrom, et al. (2009). "H2AX phosphorylation in A549 cells induced by the bulky and stable DNA adducts of benzo[a]pyrene and dibenzo[a,l]pyrene diol epoxides." Chem Biol Interact **177**(1): 40-47.

Mbewe-Campbell, N., Z. Wei, et al. (2012). "Genes and environment: novel, functional polymorphism in the human cathepsin L (CTSL1) promoter disrupts a xenobiotic response element (XRE) to alter transcription and blood pressure." J Hypertens.

McIntosh, B. E., J. B. Hogenesch, et al. (2010). "Mammalian Per-Arnt-Sim proteins in environmental adaptation." Annu Rev Physiol **72**: 625-645.

Meeker, N. D. and N. S. Trede (2008). "Immunology and zebrafish: spawning new models of human disease." Dev Comp Immunol **32**(7): 745-757.

Menzie, C. A., B. B. Potocki, et al. (1992). "Exposure to Carcinogenic Pahs in the Environment." Environmental Science & Technology **26**(7): 1278-1284.

Meylan, W. M. and P. H. Howard (1995). "Atom Fragment Contribution Method for Estimating Octanol-Water Partition-Coefficients." Journal of Pharmaceutical Sciences **84**(1): 83-92.

Moens, C. B., T. M. Donn, et al. (2008). "Reverse genetics in zebrafish by TILLING." Brief Funct Genomic Proteomic **7**(6): 454-459.

Morey, J. S., J. C. Ryan, et al. (2006). "Microarray validation: factors influencing correlation between oligonucleotide microarrays and real-time PCR." Biol Proced Online **8**: 175-193.

Mumtaz, M. and J. George (1995). Toxicological profile for polycyclic aromatic hydrocarbons (PAHs). Atlanta, GA, Agency for Toxic Substances and Disease Registry, U.S. Department of Health and Human Services.

Murk, A. J., J. Legler, et al. (1996). "Chemical-activated luciferase gene expression (CALUX): a novel in vitro bioassay for Ah receptor active compounds in sediments and pore water." Fundam Appl Toxicol **33**(1): 149-160.

Murray, I. A., C. A. Flaveny, et al. (2011). "Suppression of cytokine-mediated complement factor gene expression through selective activation of the Ah receptor with 3',4'-dimethoxy-alpha-naphthoflavone." Mol Pharmacol **79**(3): 508-519.

Nacci, D., M. Huber, et al. (2009). "Evolution of tolerance to PCBs and susceptibility to a bacterial pathogen (Vibrio harveyi) in Atlantic killifish (Fundulus heteroclitus) from New Bedford (MA, USA) harbor." Environ Pollut **157**(3): 857-864.

Narayanan, G. A., I. A. Murray, et al. (2012). "Selective aryl hydrocarbon receptor modulator-mediated repression of CD55 expression induced by cytokine exposure." J Pharmacol Exp Ther **342**(2): 345-355.

Narayanan, S. P., J. Suwanpradid, et al. (2011). "Arginase 2 deletion reduces neuro-glial injury and improves retinal function in a model of retinopathy of prematurity." PLoS One **6**(7): e22460.

Naumova, Y. Y., S. J. Eisenreich, et al. (2002). "Polycyclic aromatic hydrocarbons in the indoor and outdoor air of three cities in the U.S." Environ Sci Technol **36**(12): 2552-2559.

Nebert, D. W., T. P. Dalton, et al. (2004). "Role of aryl hydrocarbon receptor-mediated induction of the CYP1 enzymes in environmental toxicity and cancer." J Biol Chem **279**(23): 23847-23850.

Nebert, D. W., J. R. Robinson, et al. (1975). "Genetic expression of aryl hydrocarbon hydroxylase activity in the mouse." J Cell Physiol **85**(2 Pt 2 Suppl 1): 393-414.

Nebert, D. W., A. L. Roe, et al. (2000). "Role of the aromatic hydrocarbon receptor and [Ah] gene battery in the oxidative stress response, cell cycle control, and apoptosis." Biochem Pharmacol **59**(1): 65-85.

Nielsen, T., A. Feilberg, et al. (1999). "The variation of street air levels of PAH and other mutagenic PAC in relation to regulations of traffic emissions and the impact of atmospheric processes." Environmental Science and Pollution Research **6**(3): 133-137.

Nikolsky, Y., E. Kirillov, et al. (2009). "Functional analysis of OMICs data and small molecule compounds in an integrated "knowledge-based" platform." Methods Mol Biol **563**: 177-196.

O'Donnell, E. F., K. S. Saili, et al. (2010). "The anti-inflammatory drug leflunomide is an agonist of the aryl hydrocarbon receptor." PLoS One **5**(10).

Osgood, R. S., B. L. Upham, et al. (2013). "Polycyclic aromatic hydrocarbon-induced signaling events relevant to inflammation and tumorigenesis in lung cells are dependent on molecular structure." PLoS One **8**(6): e65150.

Ovrevik, J., V. M. Arlt, et al. (2010). "Differential effects of nitro-PAHs and amino-PAHs on cytokine and chemokine responses in human bronchial epithelial BEAS-2B cells." Toxicol Appl Pharmacol **242**(3): 270-280.

Pandini, A., M. S. Denison, et al. (2007). "Structural and functional characterization of the aryl hydrocarbon receptor ligand binding domain by homology modeling and mutational analysis." Biochemistry **46**(3): 696-708.

Pandini, A., A. A. Soshilov, et al. (2009). "Detection of the TCDD binding-fingerprint within the Ah receptor ligand binding domain by structurally driven mutagenesis and functional analysis." Biochemistry **48**(25): 5972-5983.

Park, K. and A. L. Scott (2010). "Cholesterol 25-hydroxylase production by dendritic cells and macrophages is regulated by type I interferons." J Leukoc Biol **88**(6): 1081-1087.

Patel, R. D., I. A. Murray, et al. (2009). "Ah receptor represses acute-phase response gene expression without binding to its cognate response element." Lab Invest **89**(6): 695-707.

Perera, F., W. Y. Tang, et al. (2009). "Relation of DNA methylation of 5'-CpG island of ACSL3 to transplacental exposure to airborne polycyclic aromatic hydrocarbons and childhood asthma." PLoS One **4**(2): e4488.

Perera, F. P., Z. Li, et al. (2009). "Prenatal Airborne Polycyclic Aromatic Hydrocarbon Exposure and Child IQ at Age 5 Years." Pediatrics.

Petersen, G. I. and P. Kristensen (1998). "Bioaccumulation of lipophilic substances in fish early life stages." Environmental Toxicology and Chemistry **17**(7): 1385-1395.

Peterson, R. E., H. M. Theobald, et al. (1993). "Developmental and reproductive toxicity of dioxins and related compounds: cross-species comparisons." Crit Rev Toxicol **23**(3): 283-335.

Pfaffl, M. W. (2001). "A new mathematical model for relative quantification in real-time RT-PCR." Nucleic Acids Res **29**(9): e45.

Planchart, A. and C. J. Mattingly (2010). "2,3,7,8-Tetrachlorodibenzo-p-dioxin upregulates FoxQ1b in zebrafish jaw primordium." Chem Res Toxicol **23**(3): 480-487.

Polidori, A., J. Kwon, et al. (2010). "Source proximity and residential outdoor concentrations of PM(2.5), OC, EC, and PAHs." J Expo Sci Environ Epidemiol **20**(5): 457-468.

Postlethwait, J., A. Amores, et al. (2004). "Subfunction partitioning, the teleost radiation and the annotation of the human genome." Trends Genet **20**(10): 481-490.

Prasch, A. L., H. Teraoka, et al. (2003). "Aryl hydrocarbon receptor 2 mediates 2,3,7,8-tetrachlorodibenzo-p-dioxin developmental toxicity in zebrafish." Toxicol Sci **76**(1): 138-150.

Price, R. L., W. Carver, et al. (1997). "The effects of angiotensin II and specific angiotensin receptor blockers on embryonic cardiac development and looping patterns." Dev Biol **192**(2): 572-584.

Puga, A., C. Ma, et al. (2009). "The aryl hydrocarbon receptor cross-talks with multiple signal transduction pathways." Biochem Pharmacol **77**(4): 713-722.

Ramesh, A., S. A. Walker, et al. (2004). "Bioavailability and risk assessment of orally ingested polycyclic aromatic hydrocarbons." Int J Toxicol **23**(5): 301-333.

Ramirez, N., A. Cuadras, et al. (2011). "Risk assessment related to atmospheric polycyclic aromatic hydrocarbons in gas and particle phases near industrial sites." Environ Health Perspect **119**(8): 1110-1116.

Reimers, M. J., J. K. La Du, et al. (2006). "Ethanol-dependent toxicity in zebrafish is partially attenuated by antioxidants." Neurotoxicol Teratol **28**(4): 497-508.

Ren, A., X. Qiu, et al. (2011). "Association of selected persistent organic pollutants in the placenta with the risk of neural tube defects." Proc Natl Acad Sci U S A **108**(31): 12770-12775.

Rennie, M. Y., J. Detmar, et al. (2011). "Vessel tortuousity and reduced vascularization in the fetoplacental arterial tree after maternal exposure to polycyclic aromatic hydrocarbons." Am J Physiol Heart Circ Physiol **300**(2): H675-684.

Reynaud, S. and P. Deschaux (2006). "The effects of polycyclic aromatic hydrocarbons on the immune system of fish: a review." Aquat Toxicol **77**(2): 229-238.

Saeed, A. I., V. Sharov, et al. (2003). "TM4: a free, open-source system for microarray data management and analysis." Biotechniques **34**(2): 374-378.

Safe, S. H. (1998). "Development validation and problems with the toxic equivalency factor approach for risk assessment of dioxins and related compounds." J Anim Sci **76**(1): 134-141.

Saili, K. S. (2012). Developmental neurobehavioral toxicity of bisphenol A in zebrafish (Danio rerio) [electronic resource] / by Katerine Schletz Saili. Corvallis, Or. :, Oregon State University.

Sarkar, A., D. Ray, et al. (2006). "Molecular Biomarkers: their significance and application in marine pollution monitoring." Ecotoxicology **15**(4): 333-340.

Sartor, M. A., M. Schnekenburger, et al. (2009). "Genomewide analysis of aryl hydrocarbon receptor binding targets reveals an extensive array of gene clusters that control morphogenetic and developmental programs." Environ Health Perspect **117**(7): 1139-1146.

Schmidt, J. V. and C. A. Bradfield (1996). "Ah receptor signaling pathways." Annu Rev Cell Dev Biol **12**: 55-89.

Schmidt, J. V., G. H. Su, et al. (1996). "Characterization of a murine Ahr null allele: involvement of the Ah receptor in hepatic growth and development." Proc Natl Acad Sci U S A **93**(13): 6731-6736.

Schoeny, R. a. K. P. (1993). Provisional guidance for quantitative risk assessment of polycyclic aromatic hydrocarbons. U. S. E. P. Agency. Washington, DC.

Scott, J. A., J. P. Incardona, et al. (2011). "AhR2-mediated, CYP1A-independent cardiovascular toxicity in zebrafish (Danio rerio) embryos exposed to retene." Aquat Toxicol **101**(1): 165-174.

Shannon, P., A. Markiel, et al. (2003). "Cytoscape: a software environment for integrated models of biomolecular interaction networks." Genome Res **13**(11): 2498-2504.

Shi, Z., N. Dragin, et al. (2010). "Organ-specific roles of CYP1A1 during detoxication of dietary benzo[a]pyrene." Mol Pharmacol **78**(1): 46-57.

Shimizu, Y., Y. Nakatsuru, et al. (2000). "Benzo[a]pyrene carcinogenicity is lost in mice lacking the aryl hydrocarbon receptor." Proc Natl Acad Sci U S A **97**(2): 779-782.

Singh, K. P., F. L. Casado, et al. (2009). "The aryl hydrocarbon receptor has a normal function in the regulation of hematopoietic and other stem/progenitor cell populations." Biochem Pharmacol **77**(4): 577-587.

Singh, R. P., R. Khanna, et al. (2003). "Comparative effect of benzanthrone and 3-bromobenzanthrone on hepatic xenobiotic metabolism and anti-oxidative defense system in guinea pigs." Archives of Toxicology **77**(2): 94-99.

Smith, B. W., S. S. Rozelle, et al. (2013). "The aryl hydrocarbon receptor directs hematopoietic progenitor cell expansion and differentiation." Blood.

Song, M. K., M. Song, et al. (2012). "Identification of molecular signatures predicting the carcinogenicity of polycyclic aromatic hydrocarbons (PAHs)." Toxicol Lett **212**(1): 18-28.

Suresh, R., A. Shally, et al. (2009). "Assessment of association of exposure to polycyclic aromatic hydrocarbons with bronchial asthma and oxidative stress in children: A case control study." Indian J Occup Environ Med **13**(1): 33-37.

Svoboda, K. R., A. E. Linares, et al. (2001). "Activity regulates programmed cell death of zebrafish Rohon-Beard neurons." Development **128**(18): 3511-3520.

Tang, D., T. Y. Li, et al. (2006). "PAH-DNA adducts in cord blood and fetal and child development in a Chinese cohort." Environ Health Perspect **114**(8): 1297-1300.

Tanguay, R. L., C. C. Abnet, et al. (1999). "Cloning and characterization of the zebrafish (Danio rerio) aryl hydrocarbon receptor." Biochim Biophys Acta **1444**(1): 35-48.

Teraoka, H., W. Dong, et al. (2003). "Induction of cytochrome P450 1A is required for circulation failure and edema by 2,3,7,8-tetrachlorodibenzo-p-dioxin in zebrafish." Biochem Biophys Res Commun **304**(2): 223-228.

Tian, B., D. E. Nowak, et al. (2005). "A TNF-induced gene expression program under oscillatory NF-kappaB control." BMC Genomics **6**: 137.

Tian, Y., S. Ke, et al. (1999). "Ah receptor and NF-kappaB interactions, a potential mechanism for dioxin toxicity." J Biol Chem **274**(1): 510-515.

Tilton, S. C., T. L. Tal, et al. (2012). "Bioinformatics resource manager v2.3: an integrated software environment for systems biology with microRNA and cross-species analysis tools." BMC Bioinformatics **13**(1): 311.

Timme-Laragy, A. R., C. J. Cockman, et al. (2007). "Synergistic induction of AHR regulated genes in developmental toxicity from co-exposure to two model PAHs in zebrafish." Aquat Toxicol **85**(4): 241-250.

Timme-Laragy, A. R., S. I. Karchner, et al. (2012). "Nrf2b, novel zebrafish paralog of oxidant-responsive transcription factor NF-E2-related factor 2 (NRF2)." J Biol Chem **287**(7): 4609-4627.

Timme-Laragy, A. R., L. A. Van Tiem, et al. (2009). "Antioxidant responses and NRF2 in synergistic developmental toxicity of PAHs in zebrafish." Toxicol Sci.

Trapnell, C., L. Pachter, et al. (2009). "TopHat: discovering splice junctions with RNA-Seq." Bioinformatics **25**(9): 1105-1111.
Trapnell, C., A. Roberts, et al. (2012). "Differential gene and transcript expression analysis of RNA-seq experiments with TopHat and Cufflinks." Nat Protoc **7**(3): 562-578.
Truong, L., S. L. Harper, et al. (2011). "Evaluation of embryotoxicity using the zebrafish model." Methods Mol Biol **691**: 271-279.
van Delft, J., S. Gaj, et al. (2012). "RNA-Seq provides new insights in the transcriptome responses induced by the carcinogen benzo[a]pyrene." Toxicol Sci **130**(2): 427-439.
Van den Berg, M., L. S. Birnbaum, et al. (2006). "The 2005 World Health Organization reevaluation of human and Mammalian toxic equivalency factors for dioxins and dioxin-like compounds." Toxicol Sci **93**(2): 223-241.
Van Metre, P. C. and B. J. Mahler (2005). "Trends in hydrophobic organic contaminants in urban and reference lake sediments across the United States, 1970-2001." Environ Sci Technol **39**(15): 5567-5574.
Van Metre, P. C. and B. J. Mahler (2010). "Contribution of PAHs from coal-tar pavement sealcoat and other sources to 40 U.S. lakes." Sci Total Environ **409**(2): 334-344.
Van Tiem, L. A. and R. T. Di Giulio (2011). "AHR2 knockdown prevents PAH-mediated cardiac toxicity and XRE- and ARE-associated gene induction in zebrafish (Danio rerio)." Toxicol Appl Pharmacol **254**(3): 280-287.
Vanwezel, A. P. and A. Opperhuizen (1995). "Narcosis Due to Environmental-Pollutants in Aquatic Organisms - Residue-Based Toxicity, Mechanisms, and Membrane Burdens." Critical Reviews in Toxicology **25**(3): 255-279.
Vlecken, D. H., J. Testerink, et al. (2009). "A critical role for myoglobin in zebrafish development." Int J Dev Biol **53**(4): 517-524.
Vogel, C. F., E. Sciullo, et al. (2004). "Dioxin increases C/EBPbeta transcription by activating cAMP/protein kinase A." J Biol Chem **279**(10): 8886-8894.
Walgraeve, C., K. Demeestere, et al. (2010). "Oxygenated polycyclic aromatic hydrocarbons in atmospheric particulate matter: Molecular characterization and occurrence." Atmospheric Environment **44**(15): 1831-1846.
Wan, B., J. W. Yarbrough, et al. (2008). "Structure-related clustering of gene expression fingerprints of thp-1 cells exposed to smaller polycyclic aromatic hydrocarbons." SAR QSAR Environ Res **19**(3-4): 351-373.
Wang, W., N. Jariyasopit, et al. (2011). "Concentration and photochemistry of PAHs, NPAHs, and OPAHs and toxicity of PM2.5 during the Beijing Olympic Games." Environ Sci Technol **45**(16): 6887-6895.
Wassenberg, D. M., A. L. Nerlinger, et al. (2005). "Effects of the polycyclic aromatic hydrocarbon heterocycles, carbazole and dibenzothiophene, on in vivo and in vitro CYP1A activity and polycyclic aromatic hydrocarbon-derived embryonic deformities." Environ Toxicol Chem **24**(10): 2526-2532.
Wei, S. L., B. Huang, et al. (2012). "Characterization of PM2.5-bound nitrated and oxygenated PAHs in two industrial sites of South China." Atmospheric Research **109**: 76-83.
White, S. S. and L. S. Birnbaum (2009). "An overview of the effects of dioxins and dioxin-like compounds on vertebrates, as documented in human and ecological epidemiology." J Environ Sci Health C Environ Carcinog Ecotoxicol Rev **27**(4): 197-211.
Wienholds, E., F. van Eeden, et al. (2003). "Efficient target-selected mutagenesis in zebrafish." Genome Res **13**(12): 2700-2707.

Wiens, G. D. and G. W. Glenney (2011). "Origin and evolution of TNF and TNF receptor superfamilies." Dev Comp Immunol **35**(12): 1324-1335.

Wilhelm, M., J. K. Ghosh, et al. (2012). "Traffic-related air toxics and term low birth weight in Los Angeles County, California." Environ Health Perspect **120**(1): 132-138.

Willett, K. L., D. Wassenberg, et al. (2001). "In vivo and in vitro inhibition of CYP1A-dependent activity in Fundulus heteroclitus by the polynuclear aromatic hydrocarbon fluoranthene." Toxicol Appl Pharmacol **177**(3): 264-271.

Wills, L. P., C. W. Matson, et al. (2010). "Characterization of the recalcitrant CYP1 phenotype found in Atlantic killifish (Fundulus heteroclitus) inhabiting a Superfund site on the Elizabeth River, VA." Aquat Toxicol **99**(1): 33-41.

Wilson, S. R., A. D. Joshi, et al. (2013). "The tumor suppressor Kruppel-like factor 6 is a novel aryl hydrocarbon receptor DNA binding partner." J Pharmacol Exp Ther **345**(3): 419-429.

Wirgin, I., N. K. Roy, et al. (2011). "Mechanistic basis of resistance to PCBs in Atlantic tomcod from the Hudson River." Science **331**(6022): 1322-1325.

Wittkopp, N., E. Huntzinger, et al. (2009). "Nonsense-mediated mRNA decay effectors are essential for zebrafish embryonic development and survival." Mol Cell Biol **29**(13): 3517-3528.

Wu, C., H. Lu, et al. (2011). "Molecular and Pathophysiological Features of Angiotensinogen: A Mini Review." N Am J Med Sci (Boston) **4**(4): 183-190.

Wu, M. T., T. C. Lee, et al. (2011). "Whole genome expression in peripheral-blood samples of workers professionally exposed to polycyclic aromatic hydrocarbons." Chem Res Toxicol **24**(10): 1636-1643.

Xiong, K. M., R. E. Peterson, et al. (2008). "Aryl hydrocarbon receptor-mediated down-regulation of sox9b causes jaw malformation in zebrafish embryos." Mol Pharmacol **74**(6): 1544-1553.

Xu, X., H. Hu, et al. (2013). "Studying the effects of polycyclic aromatic hydrocarbons on peripheral arterial disease in the United States." Sci Total Environ **461-462C**: 341-347.

Yu, H. (2002). "Environmental carcinogenic polycyclic aromatic hydrocarbons: photochemistry and phototoxicity." J Environ Sci Health C Environ Carcinog Ecotoxicol Rev **20**(2): 149-183.

Appendices

Appendix 1 - Developmental toxicity of DBT, BAA and PYR in *ahr2*^{hu3335} zebrafish

Objective

The developmental toxicity of BAA, DBT and PYR was compared between wild-type 5D embryos (*ahr2*$^{+}$) and embryos with non-functional AHR2 (*ahr2*^{hu3335}) in order to determine whether developmental toxicity of these three PAHs was mediated by *ahr2*.

Methods

Embryos were exposed to 25 uM benz(a)anthracene (BAA), dibenzothiophene (DBT), pyrene (PYR) or 1% DMSO vehicle control dissolved in embryo media from 6-48 hpf in glass vials. Exposures were conducted at 28C on a rocker, protected from light. At 48 hpf, exposure solutions were removed, embryos were rinsed 4 times and solution was replaced with 2 ml fresh embryo media. Embryos were incubated in the dark until 120 hpf, when they were evaluated for developmental malformations.

Results

As observed previously (Chapter 2), we observed significant malformation incidence in *ahr2*$^{+}$embryos exposed to 25μM BAA, DBT and PYR (Figure 1A). In *ahr2*^{hu3335} embryos, 25 uM BAA did not cause a significant increase in any malformations. DBT and PYR, however, did cause increases in malformations, suggesting that they cause toxicity through *ahr2*-independent mechanisms.

Conclusions

With these studies, we confirmed the dependence of BAA-induced developmental toxicity on AHR2. We also demonstrate that absence of AHR2 does not protect from DBT and PYR-induced tocicity. This has been previously observed in AHR2-deficient (AHR2 morpholino injected) embryos exposed to these compounds (Incardona, Day et al. 2006). Background malformations and low quantity of embryos precluded the use of the *ahr2*^{hu33335} line for the remaining studies in this dissertation. It is generally accepted that 5 outcrosses are required to sufficiently dilute background mutations in zebrafish lines generated by TILLING. At the writing of this thesis, the fourth outcrossed generation is spawning more reliably and better

quality eggs. The *ahr2*^{hu3335} shows promise for future work investigating AHR related questions in zebrafish.

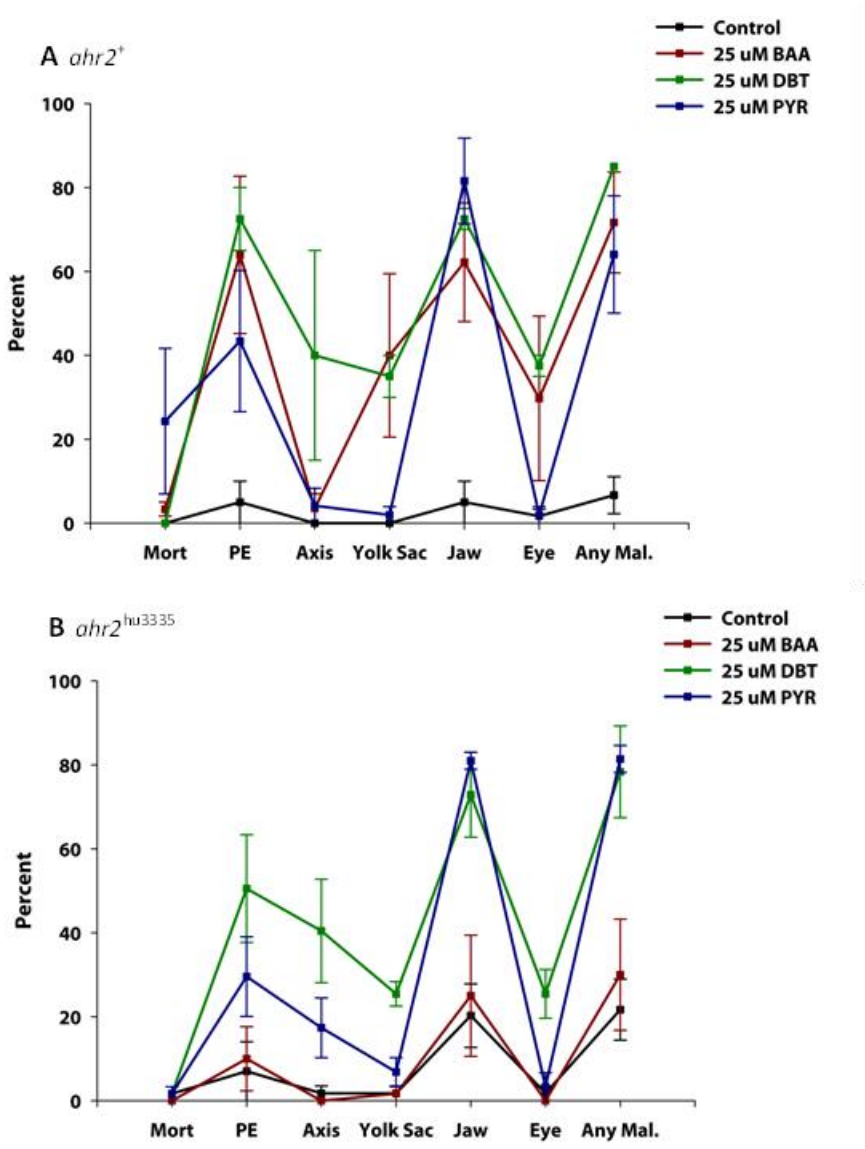


Figure A1-1

Profile plots of malformations observed at 5 dpf in PAH-treated wild-type and *ahr2*-null embryos indicate subtle differential responses between PAHs. PAH exposures did not induce significant mortality (Mort) in either fish line, but pericardial edema (PE), axis, yolk sac, jaw, and eye malformations were observed. All three PAH treatments caused a significant increase in the percent of wild-type embryos with at least one malformation (Any Mal.). BAA did not cause a significant increase in malformations in *ahr2*^{hu3335} embryos compared to control. Data represent 3 independent replicates analyzed by One-way Analysis of Variance with Tukey's post-test for pairwise comparisons.

Appendix 2 - Characterization of behavioral and neutrophil responses to PAH exposure

Objective

During developmental toxicity studies, we observed hyperactivity at 3 and 4 days post fertilization in embryos exposed to pyrene (PYR). By 120 hpf, when toxicity evaluations are conducted, behavior was no longer noticeable. We hypothesized that embryos were sensing and responding to PYR in a more immediate manner, and not because of effects on development. In this preliminary study we investigated whether zebrafish respond to acute PAH exposure at 120 hpf, when organ systems are fully developed.

In our microarray study (Chapter 2) we observed a large number of inflammatory-related transcripts misexpressed in response to PAH exposure. PAHs are also known skin irritants. We investigated whether localized inflammatory activity could be observed following PAH exposure using a transgenic zebrafish line (mpx:gfp) that expresses green fluorescent protein in neutrophils (Elks, Loynes et al. 2011).

Methods

Behavioral challenge assay

Zebrafish were dechorionated and placed individually into wells of a 96-well plate at 6 hpf in 100 µl embryo media. They were incubated with normal lighting conditions until 120 dpf. 100 ul of exposure solution (DBT, PYR or BAA) was then added to each well, to a final concentration of 1% DMSO. Embryos were exposed to 0, 1, 2.5, 5, 10 or 25 µM final concentrations of PAH. Behavior was recorded under lit conditions using Viewpoint Zebraboxes, starting 10 min following exposure for 5 hours. Distance traveled was calculated per minute for each fish. Data represent the mean of 32 fish per exposure group.

Imaging neutrophil response

GFP:mpo zebrafish were exposed to 25 uM PYR or 1% DMSO control in a 96-well plate at 72 hpf. 8 embryos from each group were anesthetized and imaged on a zeiss axiovert microscope using a 5X objective at 2, 4, 8 and 24 hours post exposure. Images were created from z-stack of 5 slices.

Results

Behavioral challenge assay

In this preliminary dataset, we observed distinct differences in behavioral patterns of fish exposed to DBT, PYR and BAA. Movement was heighted by exposure to all PAHs for the first hour of the study. Zebrafish exposed to DBT and BAA returned to activity levels comparable to controls after ~ 1.5 hr (Figure 1A, 1B). The 25uM DBT group remained more active, at 80 millimeters per minute, for almost 3 hours. In contrast, PYR-exposed zebrafish were less active immediately following exposure, but activity increased to ~120 millimeters per minute 1 hr after exposure in the 10 and 25 uM exposure groups (Figure 1C). Activity remained elevated for the duration of the study. We observed a concentration-response in hyperactivity, where zebrafish exposed to 2.5 uM PYR also had activity elevated above controls for the 5 hr exposure. This preliminary data demonstrates that zebrafish can immediately sense PAHs, and respond uniquely to PYR. The sensory mechanism and cause of activity remains unknown.

Imaging neutrophil response

From the whole-embryo images collected, we were not able to discern any specific areas of neutrophil activity in the PYR-exposed embryos. We found that neutrophil number and location was variable between fish. While a quantitative method may be able to detect small changes that are not readily observable, we were not able to identify a specific area in the fish that exhibited increased localization of these cells involved in the inflammatory response (Figure 2A). We note that we were only visualizing neutrophils; macrophages are also active at these developmental time points and could be more involved in response to PAH exposure. The waterborne exposure also would not necessarily be expected to result in a localized response.

Conclusions

In this preliminary data, we found that zebrafish can sense and immediately respond to PAH exposure with increased swimming behavior at 5 dpf. This was also observed anecdotally at 3 and 4 dpf. PYR induced a unique response, wherein zebrafish exhibited increased swimming activity for the duration of the study (5 hours). We test lower concentrations, and found increased activity at 2.5 uM, which is 10 fold lower than the concentration that

induced developmental malformations (in glass vial exposures). Fish have been shown to exhibit increased activity in response to water contaminants(Hellou 2011). The sensory mechanism, however, is not known. Given their structural similarity, the difference in response to PYR and BAA is particularly intriguing and under further investigation.

PAHs can cause skin irritation, and induce inflammatory activity in a number of model systems. We hypothesized that an inflammatory response to PYR might result in differential neutrophil localization. We did not observe any notable difference, however, in transgenic mpx:gfp zebrafish at 2, 4, 8 or 28 hours post exposure. Other cell types may be more involved in inflammatory response to PAHs, or this method may not be sufficient to discern differences.

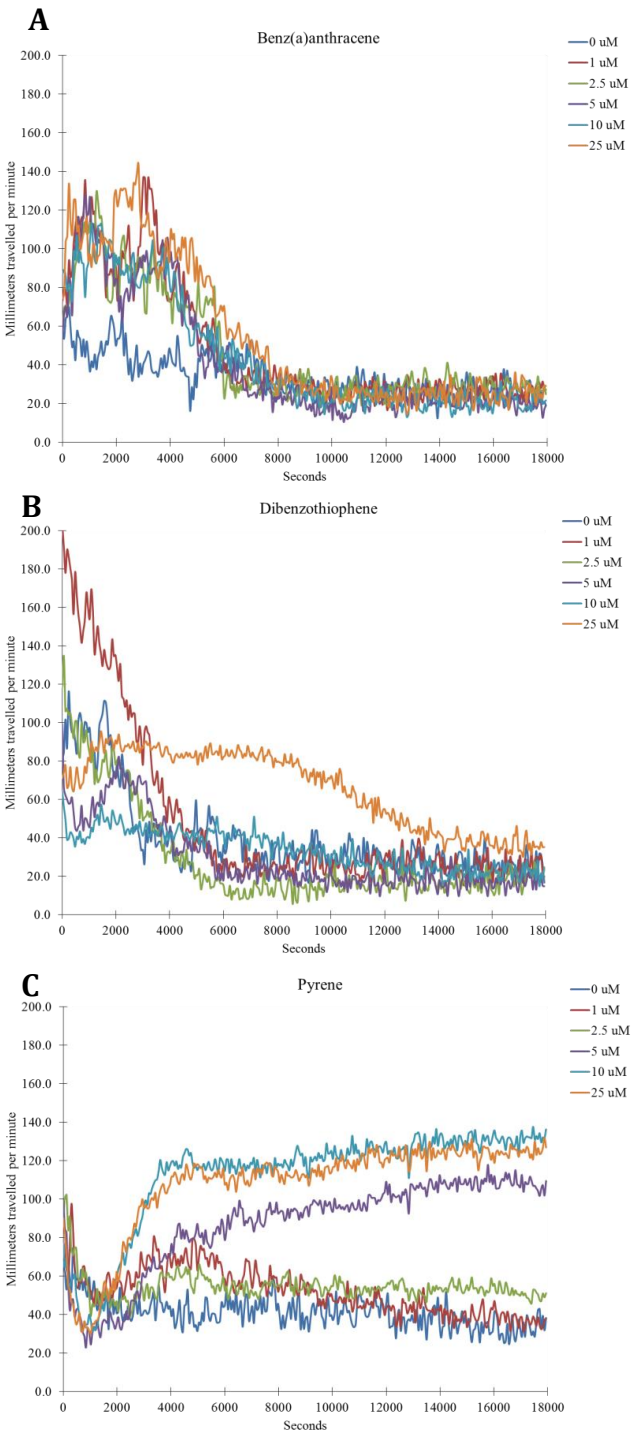


Figure A2-1

Activity of zebrafish (120 hpf) exposed to BAA (**A**), DBT (**B**), or PYR (**C**) was recorded for 5 hours following exposure.

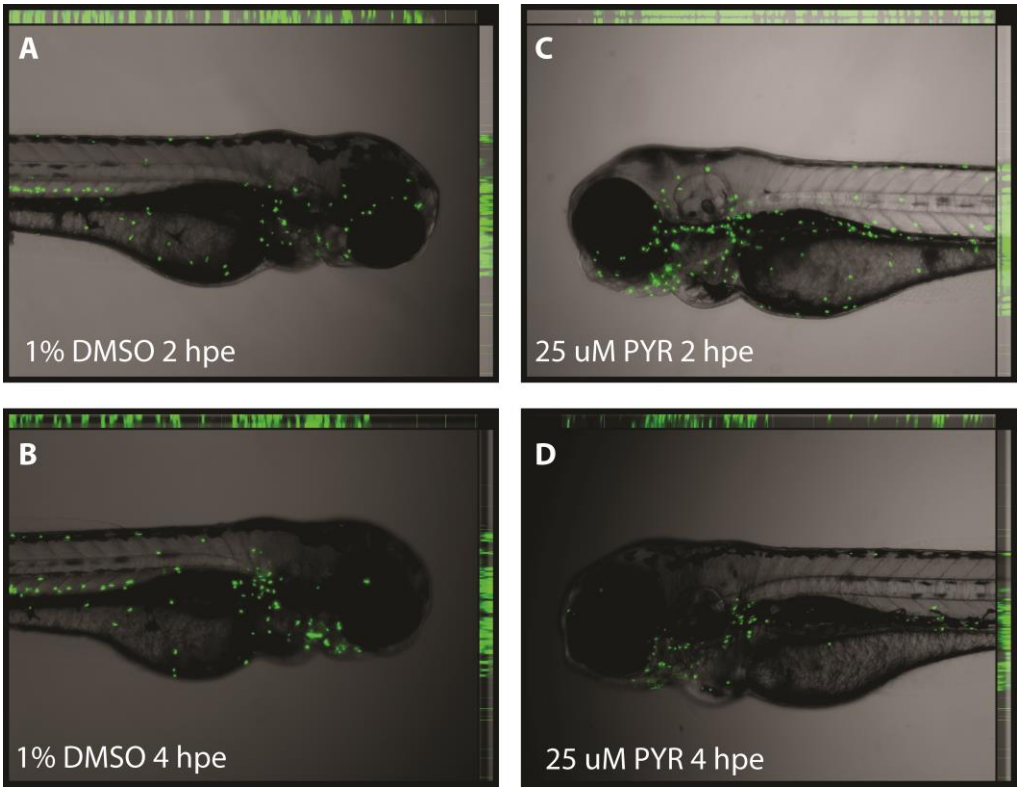


Figure A2-2 No treatment-related differences in neutrophil localization following PYR exposure

Representative images of 3 dpf zebrafish exposed to 1% DMSO at 2 and 4 hours post exposure (**A, B**). Treatment effects were not observed in embryos exposed to 25 µM PYR at 2 and 4 hpe (**C,D**).

References

Elks, P. M., C. A. Loynes, et al. (2011). "Measuring inflammatory cell migration in the zebrafish." Methods Mol Biol **769**: 261-275.

Hellou, J. (2011). "Behavioural ecotoxicology, an "early warning" signal to assess environmental quality." Environ Sci Pollut Res Int **18**(1): 1-11.

Incardona, J. P., H. L. Day, et al. (2006). "Developmental toxicity of 4-ring polycyclic aromatic hydrocarbons in zebrafish is differentially dependent on AH receptor isoforms and hepatic cytochrome P4501A metabolism." Toxicol Appl Pharmacol **217**(3): 308-321.

Management and Conservation Article

Greater Sage-Grouse Winter Habitat Selection and Energy Development

KEVIN E. DOHERTY,[1,2] *Wildlife Biology Program, College of Forestry and Conservation, University of Montana, Missoula, MT 59812, USA*
DAVID E. NAUGLE, *Wildlife Biology Program, College of Forestry and Conservation, University of Montana, Missoula, MT 59812, USA*
BRETT L. WALKER, *Wildlife Biology Program, College of Forestry and Conservation, University of Montana, Missoula, MT 59812, USA*
JON M. GRAHAM, *Department of Mathematical Sciences, University of Montana, Missoula, MT 59812, USA*

ABSTRACT Recent energy development has resulted in rapid and large-scale changes to western shrub-steppe ecosystems without a complete understanding of its potential impacts on wildlife populations. We modeled winter habitat use by female greater sage-grouse (*Centrocercus urophasianus*) in the Powder River Basin (PRB) of Wyoming and Montana, USA, to 1) identify landscape features that influenced sage-grouse habitat selection, 2) assess the scale at which selection occurred, 3) spatially depict winter habitat quality in a Geographic Information System, and 4) assess the effect of coal-bed natural gas (CBNG) development on winter habitat selection. We developed a model of winter habitat selection based on 435 aerial relocations of 200 radiomarked female sage-grouse obtained during the winters of 2005 and 2006. Percent sagebrush (*Artemisia* spp.) cover on the landscape was an important predictor of use by sage-grouse in winter. The strength of habitat selection between sage-grouse and sagebrush was strongest at a 4-km^2 scale. Sage-grouse avoided coniferous habitats at a 0.65-km^2 scale and riparian areas at a 4-km^2 scale. A roughness index showed that sage-grouse selected gentle topography in winter. After controlling for vegetation and topography, the addition of a variable that quantified the density of CBNG wells within 4 km^2 improved model fit by 6.66 Akaike's Information Criterion points (Akaike wt = 0.965). The odds ratio for each additional well in a 4-km^2 area (0.877; 95% CI = 0.834–0.923) indicated that sage-grouse avoid CBNG development in otherwise suitable winter habitat. Sage-grouse were 1.3 times more likely to occupy sagebrush habitats that lacked CBNG wells within a 4-km^2 area, compared to those that had the maximum density of 12.3 wells per 4 km^2 allowed on federal lands. We validated the model with 74 locations from 74 radiomarked individuals obtained during the winters of 2004 and 2007. This winter habitat model based on vegetation, topography, and CBNG avoidance was highly predictive (validation $R^2 = 0.984$). Our spatially explicit model can be used to identify areas that provide the best remaining habitat for wintering sage-grouse in the PRB to mitigate impacts of energy development. (JOURNAL OF WILDLIFE MANAGEMENT 72(1):187–195; 2008)

DOI: 10.2193/2006-454

KEY WORDS *Centrocercus urophasianus,* coal-bed natural gas, energy development, greater sage-grouse, habitat, land-use change, resource selection function, sagebrush, scale, winter.

Understanding landscape-scale habitat selection during critical life stages is essential for developing conservation plans for sensitive species. Studies of habitat selection at small scales further our ecological understanding of species–habitat relationships but do not convey spatially explicit information about habitat quality at a scale useful for prioritizing landscapes for conservation. Recent advances in modeling habitat selection from high-resolution satellite imagery using resource selection functions (RSF) offers the ability to rank specific areas by their relative probability of use (Manly et al. 2002). Resulting probability layers can then be mapped in a Geographic Information System (GIS) to identify regions where high-quality habitat is available. Further, these models allow cross-validation and testing against independent datasets to ensure that inferences regarding habitat selection are robust (Boyce et al. 2002, Johnson et al. 2006). The relative influence of variables thought to be important in habitat selection can also be assessed in a competing-model framework (Burnham and Anderson 2002).

Previously widespread, greater sage-grouse (*Centrocercus urophasianus*; hereafter sage-grouse) have been extirpated from approximately 50% of their original range in western North America (Schroeder et al. 2004), with an estimated range-wide population decline of 45–80% and local declines of 17–92% (Connelly and Braun 1997, Connelly et al. 2000, Aldridge and Brigham 2003). Despite increased concern for their populations, little effort has gone into measuring landscape-scale winter habitat selection by greater sage-grouse. Previous winter habitat studies have focused on the importance of micro-site vegetation features such as height, canopy cover, or crude protein levels of sagebrush (e.g., Eng and Schladweiler 1972, Beck 1977, Connelly et al. 2000, Crawford et al. 2004, Sauls 2006). In winter, sage-grouse inhabit areas with moderate to dense sagebrush (Eng and Schladweiler 1972, Homer et al. 1993, Connelly et al. 2000) and typically prefer areas with gentle (<10%), south- or west-facing slopes (Beck 1977, Hupp and Braun 1989). Previous demographic studies have documented high rates of winter survival (reviewed in Connelly et al. 2004). However, Moynahan et al. (2006) demonstrated that severe winters can have substantial population-level impacts. Birds also must often move long distances to find suitable winter habitat (Patterson 1952 *in* Connelly et al. 2004; Connelly et al. 1988; Robertson 1991). Impacts to wintering habitat may have disproportionate effects on regional population size and persistence. For example, Beck (1977) found that 80% of use sites occurred in <7% of the area of sagebrush available in northern Colorado, USA, suggesting that winter habitat may be

[1] *Present address: Wyoming Audubon, 358 N 5th Street, Unit A, Laramie, WY 82072, USA*
[2] *E-mail: k.doherty@audubon.org*

limited. The relationship between sagebrush and sage-grouse is arguably the closest during winter when birds switch from a diet of insects, forbs, and sagebrush to one composed of >96% sagebrush (Remington and Braun 1985, Welch et al. 1991, Connelly et al. 2000, Crawford et al. 2004). Heavy snowfall may even further reduce the amount of suitable habitat by limiting the abundance of sagebrush above the snow (Hupp and Braun 1989; Connelly et al. 2000, 2004).

Coal-bed natural gas (CBNG) development in the Powder River Basin (PRB) has caused rapid, large-scale changes to sagebrush habitats in Montana and Wyoming, USA. The sage-grouse sub-population in the PRB is a critical component of the larger Wyoming Basin population, which represents 25% of sage-grouse in the species' range (Connelly et al. 2004). The population in the PRB has a high density of active leks and serves as a link to populations in eastern Wyoming and western South Dakota, USA, and between the Wyoming Basin and central Montana, USA (Connelly et al. 2004). The CBNG field in the PRB is one of the largest developed energy fields in North America. In this region, approximately 29,000 CBNG wells have been drilled on public and private lands, and another approximately 37,000 wells are expected within a 2.4-million ha area, roughly the size of the state of New Hampshire (Bureau of Land Management 2003*a*, *b*). Drilling is typically authorized at a maximum density of 1 well per 32 ha on lands where federally owned gas reserves are extracted; however, there are no well-density restrictions placed on private or state-owned gas reserves. Wells, power lines, roads, vehicle traffic, pipelines, compressor stations, and water storage ponds within a gas field this size contribute to fragmentation of sagebrush habitats and may impact sagebrush obligates (Knick et al. 2003).

We investigated sage-grouse winter habitat use in the PRB as part of a larger study of the potential impacts of CBNG development on sage-grouse populations. Our objectives were to 1) create a robust habitat selection model for sage-grouse in winter, 2) evaluate the appropriate scale at which females select winter habitat, 3) spatially depict habitat suitability in a GIS to identify areas with a high probability of use, and 4) assess the influence of CBNG development on winter habitat selection.

STUDY AREA

Our study area in the PRB covered portions of Johnson, Sheridan, and Campbell counties in Wyoming, and Bighorn, Rosebud, and Powder River counties in Montana. Shrub-steppe habitat in the PRB was dominated by Wyoming big sagebrush (*Artemisia tridentata wyomingensis*) with an understory of native and nonnative grasses such as bluebunch wheatgrass (*Pseudoroegneria spicata*), western wheatgrass (*Agropyron smithii*), prairie junegrass (*Koeleria macrantha*), blue grama (*Bouteloua gracilis*), Japanese brome (*Bromus japonicus*), cheatgrass (*B. tectorum*), and crested wheatgrass (*Agropyron cristatum*). Plains silver sagebrush (*Artemisia cana cana*) was also present in drainages but at much lower abundance. Rocky mountain juniper (*Juniperus scopulorum*) and ponderosa pine (*Pinus ponderosa*) were located in wooded draws and formed forests across the extreme northern extent of the study area. Conifers were largely absent from the southern half of the study area. Land use was dominated by cattle ranching; only 4% of the landscape consisted of dry land or irrigated agriculture. The PRB typically was cold and dry in January with average temperatures of −6.0° C and 16.3 cm of snowfall. Winter weather conditions in 2004 and 2005 were almost identical to historical averages. The winter of 2006 was mild; in January, temperatures were 6.5° C above normal and snowfall was 15 cm below average. The January 2007 average temperature of −5.5° C was near historical norms; however, snowfall was 60% above normal.

METHODS

Marking and Monitoring Protocols

We captured sage-grouse by rocket-netting (Giesen et al. 1982) and spotlighting (Wakkinen et al. 1992) on and around leks in 3 study areas: 1) Bighorn County, Montana, 2) Campbell County, Wyoming, and 3) Johnson County, Wyoming during March–April and August of 2003–2006. We aged and sexed grouse and fitted females with a 21.6-g necklace-style radiocollar with a 4-hour mortality switch (model A4060; Advanced Telemetry Systems, Isanti, MN). Sage-grouse in the Bighorn and Campbell county study areas were nonmigratory. In contrast, many birds in the Johnson Country study area were migratory, with distinct breeding, summer, and winter ranges. In all study sites, we obtained winter locations after birds in our migratory population had moved to wintering areas but before they had moved back to the breeding grounds. We monitored sage-grouse via aerial radiotracking during the winters of 2005–2007. We used a fixed-wing airplane with aerial telemetry antennas mounted on both wings struts and connected to a switch box. We used a Global Positioning System (GPS) receiver to record locations of used sites as we circled sage-grouse at approximately 100–200-m elevation above the ground. We radiotracked sage-grouse on foot during the winter of 2004, and recorded their positions with a GPS receiver when we obtained visual sightings of radiomarked birds. We estimated the 95% error ellipse of aerial locations by relocating a transmitter placed in rolling sagebrush cover 40 times from the air in a blind trial. We then calculated a bivariate normal home range estimator (Jennrich and Turner 1969) using these relocations to quantify our maximum resolution to estimate the location of an unknown collar (78.2-m radius). The ability of our plane to tightly circle sage-grouse was not constrained by rugged areas or conifer-dominated landscapes in the PRB because birds were not located in these habitat features; thus, our test was representative of the maximum precision of our aerial telemetry locations in rolling sagebrush habitats. We did not quantify error for ground-based locations, but we assumed error estimates were smaller than aerial-based methods. Since we treated our aerial telemetry error test as a

maximum precision estimate, we conducted all analyses at scales ≥100 m to ensure that our inference was not confounded by location error.

Designation of Used and Available Sites

We employed a used–available design to evaluate sage-grouse habitat relationships in winter (Boyce et al. 2002, Manly et al. 2002, Johnson et al. 2006). We defined used points as the sites where we located radiomarked sage-grouse during radiotracking. We split sage-grouse used locations into those we analyzed to build a statistical model to quantify large-scale habitat relationships and those we analyzed to test the predictive ability of our spatially explicit winter habitat model. We located birds we used to build the model during 3 flights from 2 January to 25 January 2005 ($n = 292$ locations on 106 individuals) and on 3 flights from 24 December 2005 to 1 February 2006 ($n = 241$ locations on 94 individuals). To test the model, we used 87 locations collected on the ground from 15 to 18 January 2004 ($n = 30$ locations on 28 individuals) and on 2 flights on 18 and 26 January 2007 ($n = 57$ locations on 57 individuals). Of the 85 individuals used to test the model, 57 were not included among birds marked during 2005 or 2006. We found some radiomarked birds together in flocks. To avoid the possibility of dependency in our data, we retained only one used location per flock. The final data set contained 435 locations for building the model and 74 locations for testing the model.

We selected available points within circles that had a radius to the farthest winter used point and were centered on either the lek of capture or on the lek closest to where birds were captured via spotlighting. We merged circles that overlapped within each study area to create 3 nonoverlapping polygons that corresponded with our 3 study areas. We randomly selected available points from a spatial Poisson distribution (Beyer 2004) proportional to twice the number of used points within a polygon and year to ensure a representative sample of available habitats.

GIS Habitat Classification

We acquired SPOT-5 satellite imagery (Terra Image USA, Santa Barbara, CA) for the northern portion of the study area in August 2003 and for the southern portion in August 2004 when the project expanded to encompass a larger geographic area. We ortho-rectified SPOT-5 imagery to existing digital ortho-quads of the study area. The SPOT-5 panchromatic and multi-spectral images were combined into a single panchromatic, multi-spectral file. We then used the panchromatic 25-m^2-pixel image to perform pan-sharpening to reduce the multi-spectral image pixel size from 100 m^2 to 25 m^2, greatly increasing the resolution of our analysis. We used eCognition™ 4.0 software (Definiens Imaging, Munich, Germany) to cluster the pixels into regions representing spectrally similar ground features. We exported clusters into ArcGIS 9.2 software to create a polygon database. We collected field training points ($n =$ 7,092) that were stratified by space and landowner access to classify 5 habitat cover classes as sagebrush, conifer, grassland, riparian, and barren. Classification accuracy assessed by withholding subsamples of data (i.e., k-fold cross-validation with 10 folds; Boyce et al. 2002) was 83% for sagebrush, 77% for conifer, 76% for grassland, 70% for riparian, and 80% for barren with an overall accuracy of 78%. We removed urban areas and strip mines from analyses.

Vegetation, Topography, and Energy Development Variables

We quantified characteristics of vegetation, topography (e.g., Beck 1977, Remington and Braun 1985, Hupp and Braun 1989, Sauls 2006), and energy development around used and available points using a GIS to evaluate landscape predictors of sage-grouse winter habitat selection. We utilized used and available points to select individual 5 × 5-m raster pixels, which we then buffered by 100 m, 400 m, and 1,000 m. We quantified variables within a square centered on each used and available pixel at 3 spatial scales: 205 × 205-m (0.04-km^2), 805 × 805-m (0.65-km^2), and 2,005 × 2,005-m (4-km^2). We calculated the percent of total area covered by each of the 5 vegetation cover classes to quantify vegetation. To quantify topography, we processed a 900-m^2 resolution digital elevation model (DEM) using Spatial Analyst in ArcGIS 9.2 and used it to estimate slope and solar radiation for each pixel in the landscape. Solar radiation calculates how much sun a particular pixel receives dependent on slope and aspect. We estimated solar radiation using the hillshade command in Spatial Analyst using the angle and aspect of the sun during 15 January 2007 at 1300 hours (U.S. Navy Astronomical Applications Department 2007). We used the standard deviation of the DEM elevations within each buffer size to calculate an index to describe the roughness of the landscape. Elevation was not included as a predictor variable for GIS habitat modeling because elevational migration of sage-grouse does not occur in the PRB, and minor differences in elevation at used and available locations were biologically irrelevant. In the northern PRB, mean elevation was 1,210 m (SE = 3.8) for available locations and 1,248 m (SE = 3.9) for used locations. In the southern PRB, mean elevation was 1,363 m (SE = 4.1) for available locations and 1,378 m (SE = 3.4) for used locations. We used the density of CBNG wells as a measure of the extent of energy development. Wells are the only segment of the energy footprint accurately mapped and publicly available for the entire PRB from the Wyoming Oil and Gas Conservation Commission and Montana Board of Oil and Gas Conservation, and well density within a buffer is strongly correlated with other features of CBNG development such as roads, ponds, and power lines (D. E. Naugle, University of Montana, unpublished data).

Statistical Analyses

We employed logistic regression with used and available points for model selection and RSF model parameter estimates (Boyce et al. 2002, Manly et al. 2002, Johnson et al. 2006). We pooled used locations of individual animals

and made inferences at the population level (Design I; Erickson et al. 2001, Manly et al. 2002).

We first assigned variables into one of 3 model categories: vegetation, topography, or energy development. Because no published landscape-scale studies existed upon which to base a priori models (Burnham and Anderson 2002), we tested all variables individually and removed variables with odds ratios overlapping 1.0. We tested all buffer distances for each variable and identified the scale that best represented sage-grouse habitat selection for each variable using log-likelihood values. We then allowed the best scale for each variable to compete with all possible combinations of other variables within the same category to identify the most parsimonious model. We used information-theoretic methods (Burnham and Anderson 2002) to choose between competing models by converting log-likelihood values computed in logistic regression to Akaike's Information Criterion (AIC) values. We brought models within 2 AIC points to the next hierarchy of model selection. After identifying the top model(s) within vegetation, topography, and energy development, we allowed models to compete across categories to see if the additional information increased model fit.

We did not allow correlated predictors ($r \geq |0.7|$) in the same model at any level of model selection. If variables were correlated ($r \geq |0.7|$), we chose the variable we felt had the greatest biological meaning according to known characteristics of winter sage-grouse habitat from published studies. When variables were moderately correlated (i.e., $|0.3| \leq r < |0.7|$), we checked for stability and consistency of regression coefficient estimates as we added predictor variables to models. If a regression coefficient switched signs or standard errors increased substantially when correlated variables were in the same model, we removed one variable from analysis if the other was an important predictor.

We evaluated whether sage-grouse avoided energy development in winter by using AIC values to determine if the addition of CBNG wells/km^2 to the top habitat model explained more information than habitat alone. We then examined the resulting corresponding model coefficient for CBNG wells to determine if sage-grouse avoided or were attracted to energy development and to what degree. We performed a bootstrap analysis to quantify the change in odds of use with the introduction of CBNG wells in the form of 95% confidence intervals around the odds ratios for differences in the number of wells. Because the best approximating model had a high AIC weight ($w_i = 0.965$), we used beta coefficients from the best approximating model for all computations (see results; Burnham and Anderson 2002). For each bootstrap data set ($n = 5{,}000$) we calculated and stored model coefficients and the mean value for all used locations for each variable. We then repeated this bootstrap analysis, varying the number of CBNG wells in a 4-km^2 area from 0 wells to 22 wells, the full range of well density we observed in our original data set. For each of the 5,000 simulations we computed the odds of use with the logistic equation. We then ordered these ratios and used a rankit adjustment (Chambers et al. 1983) to compute 2.5% and 97.5% percentiles for the upper and lower 95% confidence interval bounds.

We then used the same bootstrap technique to quantify how the amount of sagebrush within a 4-km^2 area affected the odds of use in winter with and without CBNG development (12.3 wells/4 km^2 and 0.0 wells/4 km^2, respectively). We used the logistic equation to generate odds of use for each bootstrap dataset ($n = 5{,}000$) by applying stored model coefficients to mean values of parameters at used locations while systematically varying percent sagebrush within 4 km^2 from 0% to 100% at 0.0 and 12.3 wells per 4 km^2. To test if the odds of use were significantly different with the addition of CBNG we computed the difference in odds generated from each bootstrap data set with and without CBNG. Again, we ordered odds ratios with and without CBNG and their differences and used a rankit adjustment (Chambers et al. 1983) to compute 2.5% and 97.5% percentiles for the upper and lower 95% confidence interval bounds.

To turn our statistical model into a spatially explicit GIS habitat model, we employed a RSF model that had the form:

$$w(x) = \exp(\beta_1 x_1 + \beta_2 x_2 + \cdots + \beta_k x_k), \qquad (1)$$

where $w(x)$ is the raw RSF value for each pixel in the landscape, and $x_1, x_2, \ldots x_k$ represent values for vegetation, topography, and energy development generated from a moving-window analysis for each pixel, and $\beta_1, \ldots, \beta_k$ are the model parameters estimated with logistic regression (Boyce et al. 2002, Manly et al. 2002, Johnson et al. 2006). We applied β-coefficients from equation 1 to GIS layers in ArcView Spatial Analyst. The output was a new GIS layer that represents the RSF values generated from equation 1 for each individual 25-m^2 pixel for the entire landscape. We created each component GIS layer by moving-window analyses for key vegetation, topographic, and energy development variables identified in model selection. These analyses resulted in summary statistics for each pixel in the GIS layer at the desired scale. We resampled sagebrush to a 900-m^2 pixel size because the time required to process a 4-km^2 area for 625 million pixels exceeded our computational capacity. Sagebrush resampled well and little information was lost when evaluating the 900-m^2 resampled sagebrush layer versus the original 25-m^2 resolution sagebrush layer ($r = 0.934$). Conifer resampled poorly ($r = 0.793$) so we kept this variable at the original pixel size.

We categorized RSF values into 5 ordinal 20% quantile bins representing progressively selected habitats. We validated our spatial model with the test data set of sage-grouse locations collected during the winters of 2004 and 2007. We regressed the observed proportion of the test data set in each RSF bin against the expected proportion of use from the original RSF model to evaluate model fit (Johnson et al. 2006). A good model fit should have a high validation R^2 value, a slope not different from 1.0, and an intercept not different from zero (Johnson et al. 2006).

Table 1. Vegetation, topographic, and energy development variables that we evaluated as potential landscape predictors of sage-grouse winter habitat selection, Powder River Basin, Montana and Wyoming, USA, 2005 and 2006. We used log-likelihoods to identify best scale at which selection occurred for individual variables along with confidence intervals of odds ratios that did not overlap 1 to choose variables for model selection.

Model category	Variable	Buffer area (km^2)	Log-likelihood	Odds ratio	Upper 95% CI	Lower 95% CI
Vegetation[a]	Sagebrush	4	−799.550	1.052	1.060	1.044
	Sagebrush	0.65	−814.010	1.048	1.043	1.034
	Sagebrush	0.04	−825.694	1.030	1.035	1.024
	Grass	4	−877.583	0.972	0.980	0.964
	Grass	0.04	−878.044	0.982	0.987	0.976
	Grass	0.65	−884.551	0.980	0.987	0.973
	Conifer	0.65	−813.051	0.765	0.822	0.712
	Conifer	0.04	−833.587	0.793	0.859	0.732
	Conifer	4	−818.951	0.810	0.850	0.772
	Riparian	4	−851.246	0.843	0.882	0.805
	Riparian	0.65	−860.729	0.870	0.909	0.833
	Riparian	0.04	−889.368	0.958	0.979	0.938
	Barren	0.65	−890.643	0.897	0.940	0.856
	Barren	4	−890.197	0.866	0.919	0.816
	Barren	0.04	−898.349	0.960	0.987	0.934
Topography	Roughness	0.65[b]	−838.257	0.888	0.909	0.868
	Roughness	0.04	−844.885	0.815	0.850	0.782
	Roughness	4	−848.668	0.921	0.936	0.905
	Solar radiation	0.0009	−902.677	0.997	1.002	0.992
	Slope	0.0009	−863.384	0.879	0.907	0.852
Energy Development	Distance to nearest well		−865.638	1.000	1.002	0.997
	No. wells	4	−857.717	0.961	0.985	0.939
	No. wells	0.65	−859.699	0.833	0.943	0.736
	No. wells	0.04	−863.083	0.434	1.102	0.171

[a] We excluded grass from further habitat models because of its correlation with sagebrush ($r = -0.78$).
[b] Roughness = index calculated using the SD of a digital elevation model.

RESULTS

Sagebrush at the 4-km^2 scale was the dominant variable in univariate space (Table 1). Sagebrush and grassland accounted for >95% of the total vegetation cover at used locations, which explains their strong negative correlation ($r = -0.78$). Within a 4-km^2 area, used sites contained >75% sagebrush cover intermixed with grassland. There was 14.5% more sagebrush at used (76.0%, SE = 0.55) than at available sites (61.5%, SE = 0.61). Sage-grouse used sites averaged 19.1% (SE = 0.53) grassland cover within a 4-km^2 area.

The best model for sage-grouse vegetation use consisted of sagebrush and riparian (4-km^2 scale), as well as conifer and barren (0.65-km^2 scale; Table 2). The roughness index at a 0.65-km^2 scale and slope were both important topographic predictors of sage-grouse use (Table 2). The number of CBNG wells within a 4-km^2 area was the best model to represent energy development (Table 1).

Model fit increased when the best approximating models from vegetation, topography, and energy development were combined (Table 3). We removed barren ground from the final vegetation model because it lacked stability and

Table 2. Log-likelihood (LL), number of parameters (K), Akaike's Information Criterion value (AIC), change in AIC value from the top model (ΔAIC), and Akaike weight (w_i) of sage-grouse winter habitat selection for vegetation and topography models, Powder River Basin, Montana and Wyoming, USA, winters of 2005 and 2006.

Model	LL	K	AIC	ΔAIC	w_i
Vegetation models					
Sagebrush[a] + Conifer + Riparian + Barren	−716.337	5	1,442.674	0.000	0.998
Sagebrush + Conifer + Riparian	−723.772	4	1,455.544	12.870	0.002
Sagebrush + Conifer + Barren	−744.539	4	1,497.078	54.404	0.000
Sagebrush + Conifer	−749.355	3	1,504.710	62.036	0.000
Sagebrush + Riparian + Barren	−780.350	4	1,568.700	126.026	0.000
Sagebrush + Riparian	−787.762	3	1,581.524	138.850	0.000
Sagebrush + Barren	−799.877	3	1,605.754	163.080	0.000
Topography models					
Roughness[b] + Slope[c]	−835.881	3	1,677.762	0.000	0.798
Roughness	−838.257	2	1,680.514	2.752	0.202
Slope	−863.384	2	1,730.768	53.006	0.000

[a] Vegetation variables = % cover of each Geographic Information System vegetation category within a selected buffer distance (% sagebrush, barren and riparian within 4 km^2 + % conifer and roughness within 0.65 km^2).
[b] Roughness = index calculated using the SD of a digital elevation model (DEM).
[c] Slope = slope of pixel calculated using a DEM.

Table 3. Log-likelihood (LL), number of parameters (K), Akaike's Information Criterion value (AIC), change in AIC value from the top model (ΔAIC), and Akaike weight (w_i) of sage-grouse winter habitat model selection, Powder River Basin, Montana and Wyoming, USA, winters of 2005 and 2006.

Model[a]	LL	K	AIC	ΔAIC	w_i
Vegetation[b] + Topography[c] + CBNG[d]	−683.644	7	1,381.288	0.000	0.965
Vegetation + Topography	−687.974	6	1,387.948	6.660	0.035
Vegetation + CBNG	−718.083	5	1,446.166	64.878	0.000
Vegetation	−723.772	4	1,455.544	74.256	0.000
Topography + CBNG	−826.657	3	1,659.314	278.026	0.000
Topography	−835.881	3	1,677.762	296.474	0.000
CBNG	−857.717	2	1,719.434	338.146	0.000

[a] Models represent the AIC best combination of variables within each model category.
[b] Vegetation = % sagebrush and riparian within 4 km^2 + % conifer within 0.65 km^2.
[c] Topography = roughness of land within 0.65 km^2 + slope.
[d] CBNG = no. of coal-bed natural gas wells/4 km^2.

consistency due to its correlation with roughness ($r = 0.32$). When roughness and barren ground were in the same model, the coefficient for barren ground switched from a negative to a positive effect and its standard error increased, causing the odds ratio interval to overlap 1.0 (odds 0.96–1.06). Roughness was a more stable predictor and was unaffected by the inclusion of barren ground. The final combined model was 1.96 AIC points better when barren ground was removed.

Sage-grouse selected large expanses of sagebrush with gentle topography and avoided conifer, riparian, and energy development (Table 4). The addition of the average number of wells per 4-km^2 improved model fit by 6.66 AIC points (Table 3). An Akaike weight ($w_i = 0.965$) indicated that the model with both habitat and energy variables had overwhelming support (Table 3). The resulting model coefficients from the habitat and energy model indicate that after adjusting for sage-grouse habitat preference, birds avoid CBNG development in otherwise suitable habitat (Table 4).

Our bootstrap analysis demonstrated that current legal maximum well density on federal lands (approx. 12.3 wells/4 km^2, or 32-ha spacing) decreased the odds of sage-grouse use by 0.30 compared to the average landscape selected by our radiomarked sage-grouse (odds 0.57 vs. 0.87; Fig. 1). Sage-grouse were 1.3 times more likely to use winter habitat if CBNG development was not present. The odds of sage-grouse winter habitat use increased with greater percentage sagebrush cover within 4 km^2 (Fig. 2a). The difference in odds of use with and without CBNG development was statistically significant at all levels of sagebrush ($P < 0.05$); however, these differences were more pronounced in high-quality winter habitats dominated by sagebrush cover (Fig. 2b). Avoidance of CBNG was not relevant to winter habitat selection at low levels of sagebrush cover because sage-grouse showed strong avoidance of those areas prior to development (Fig. 2a).

The best approximating model including vegetation, topography, and energy variables accurately predicted an independent data set of 74 winter locations (validation $R^2 = 0.98$; Fig. 3). Using 6-, 7-, or 8-bin ordinal RSF models with quantile breaks did not change the strength or pattern of model validation. The slope of observed versus expected values did not differ from 1.0 (slope = 1.14, 95% CI = 0.87–1.41) and the intercept did not differ from zero (−2.85, 95% CI = −1.06–4.9). The top 2 RSF classes accounted for 86.6% of the 435 locations used to build the RSF model and 90.5% of the 74 locations used to test the winter habitat model (Fig. 3).

DISCUSSION

Our study is the first to show that abundance of sagebrush at a landscape scale influences sage-grouse habitat selection in

Table 4. Logistic regression β-coefficients (SE) and odds ratios from the best model (Akaike wt = 0.965) describing winter habitat selection and energy avoidance for sage-grouse, Powder River Basin, Montana and Wyoming, USA, 2005 and 2006.

Parameters	Estimate	SE	Odds ratio	Upper 95% CI	Lower 95% CI
Constant	−1.106	0.369			
Roughness[a]	−0.039	0.017	0.962	0.994	0.931
Slope[b]	−0.102	0.022	0.903	0.943	0.865
Conifer[c]	−0.203	0.033	0.816	0.871	0.765
Sagebrush[d]	0.028	0.004	1.028	1.037	1.020
Riparian[e]	−0.131	0.026	0.877	0.923	0.834
CBNG wells[f]	−0.035	0.014	0.966	0.992	0.940

[a] Roughness = topographic index calculated as the SD of a digital elevation model (DEM) within 0.65 km^2.
[b] Slope = slope of pixel calculated from DEM.
[c] Conifer = % conifer cover within 0.65 km^2.
[d] Sagebrush = % sagebrush cover within 4 km^2.
[e] Riparian = % riparian cover within 4 km^2.
[f] CBNG = no. of coal-bed natural gas wells within 4 km^2.

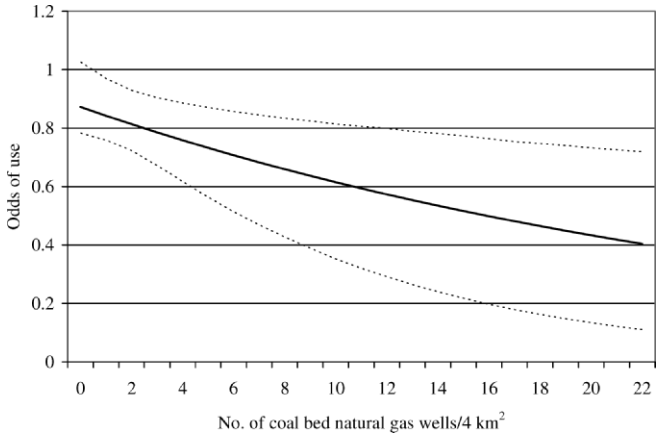


Figure 1. Reduction in the odds of sage-grouse winter habitat use versus available habitat with increasing coal-bed natural gas (CBNG) well density, Powder River Basin, Montana and Wyoming, USA, 2005–2006. Odds (solid line) and 95% confidence intervals (dashed line) are based on 5,000 bootstrap samples with densities varying between 0–22 wells per 4 km^2, the range of CBNG development we observed in our sample of used and available points.

winter. Recent advances in RSF modeling and habitat mapping using satellite imagery enabled us to document what all major reviews on sage-grouse habitat requirements have suggested (Schroeder et al. 1999; Connelly et al. 2000, 2004; Crawford et al. 2004). At the largest scale evaluated (4 km^2), sage-grouse selected for sagebrush and grassland landscapes (>95% area) that were dominated by sagebrush (>75%) with little tolerance for other cover types. Conversion of sagebrush negatively influences sage-grouse populations (Leonard et al. 2000, Smith et al. 2005). Sage-grouse avoided riparian areas at the 4-km^2 scale and conifer habitats and rugged landscapes at a 0.65-km^2 scale, relationships that would have been less discernible at broader spatial scales. Our roughness index was a much stronger predictor than the rest of our suite of topographic variables, but slope further increased model fit. Roughness is readily calculated from available DEMs and may be applicable to other life stages for sage-grouse. In the only other sage-grouse landscape study that has evaluated habitat selection at multiple scales, birds selected large expanses (>1 km^2) of sagebrush and avoided anthropogenic edge during the breeding season (Aldridge and Boyce 2007). Our findings from winter in conjunction with those of Aldridge and Boyce (2007) highlight the need for landscape-scale research to gain further insight into sage-grouse ecology.

Our habitat model was highly predictive. We built our model using sage-grouse locations collected during mild to average winter conditions and validated it in years with average temperatures or above-average snowfall. We do not know whether we defined winter habitat broadly enough to include refugia necessary for birds to survive a 50- or 100-year winter storm event (Moynahan et al. 2006), but we believe the model is useful to identify habitat available in most winters. Extreme events may move birds into rugged landscapes as they search for exposed sagebrush, thermal

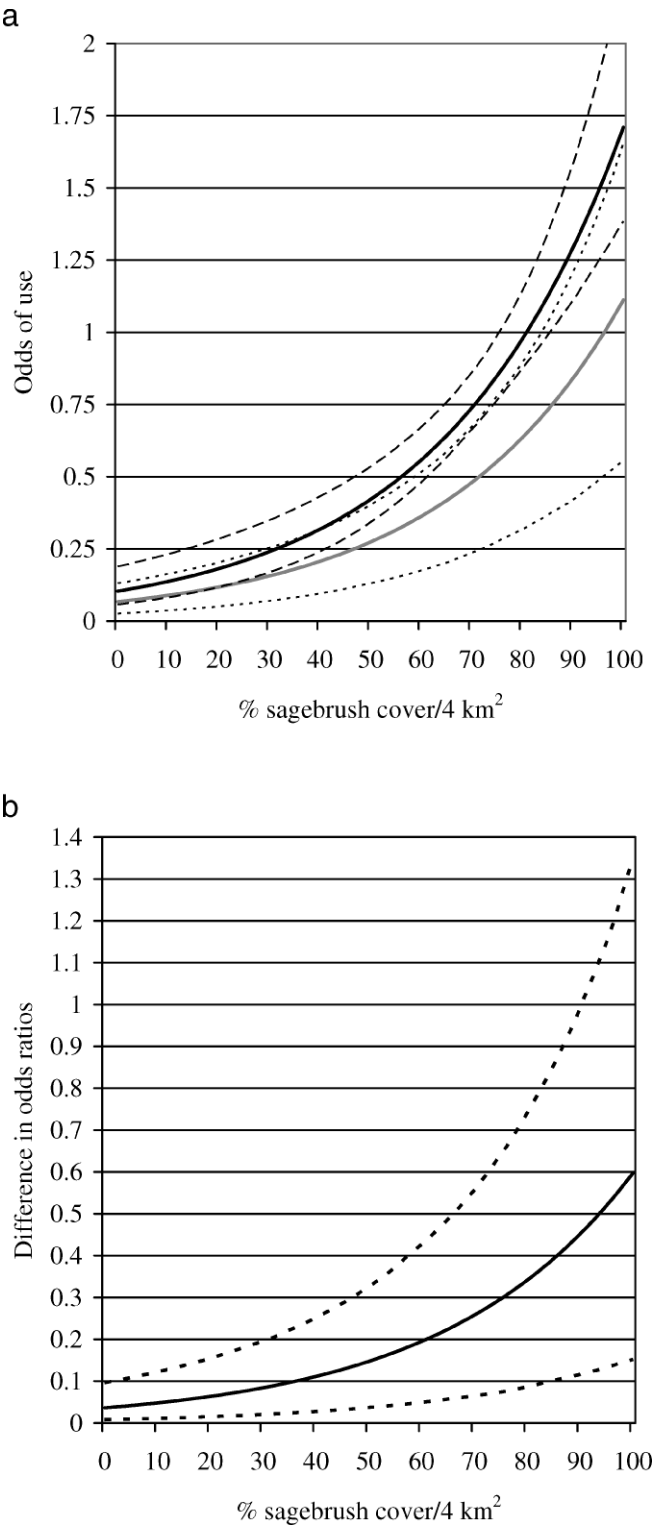


Figure 2. Odds of sage-grouse winter habitat use in relation to percent sagebrush cover per 4 km^2, Powder River Basin, Montana and Wyoming, USA, 2005–2006. Odds and 95% confidence intervals are based on 5,000 bootstrap samples with sagebrush varying from 0% to 100%, with and without coal-bed natural gas (CBNG) development. a) The gray line represents CBNG development (12.3 wells/4 km^2, 95% CI small dashed line) and the black line represents no CBNG development (0.0 wells/4 km^2, 95% CI large dashed line). b) The difference of means for odds of use with and without CBNG (black line minus grey line from part [a] above) is plotted against varying amounts of sagebrush cover per 4 km^2 (95% CI dashed line).

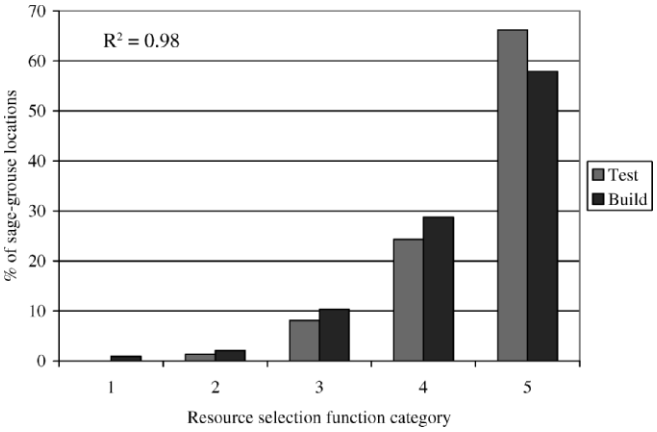


Figure 3. Percent of sage-grouse use locations in each of 5 ordinal resource-selection function bins we used to build (black bars, $n = 436$ locations from 2005 to 2006) and test (gray bars, $n = 74$ locations from 2004 and 2007) the winter habitat model, Powder River Basin, Montana and Wyoming, USA.

cover, and protection from high winds (Beck 1977, Hupp and Braun 1989, Robertson 1991, Connelly et al. 2004).

A multi-scale approach is needed to understand the relative importance of local and landscape factors influencing sage-grouse habitat selection. Local vegetation measures have been the primary focus of sage-grouse habitat research to date (Eng and Schladweiler 1972, Beck 1977, Connelly et al. 2000, Crawford et al. 2004, Sauls 2006). Ideally, local variables should compete against landscape factors in an AIC framework to predict sage-grouse habitat use. Examination of ecological processes at the landscape scale does not eliminate the need to understand habitat relationships at local scales; rather, it will likely require a combination of scales to completely understand how sage-grouse respond to their environment.

Our spatially explicit habitat model provides resource managers with a practical tool to guide conservation planning. Effective planning requires that we know which habitats are selected at landscape scales, where those habitats are located, and how species respond to disturbances. Recent advances in wildlife ecology enable biologists to develop RSF models that link resource use with changes in habitat quality and potential stressors (Manly et al. 2002, Johnson et al. 2004). Moreover, RSFs estimate the strength of selection and enable predictive equations to be linked in a GIS to depict spatial relationships across a planning region (Manly et al. 2002, Johnson et al. 2004). Spatially explicit planning tools should be used to prioritize landscapes with the highest probability of supporting populations. Once identified, local biologists provide on-site recommendations for how to best deliver on-the-ground conservation.

After adjusting for sage-grouse habitat preference, sage-grouse avoided energy development in otherwise suitable habitats in winter. Previous research has shown that breeding sage-grouse in oil and gas fields avoid development, experience higher rates of mortality, or both (Holloran 2005, Kaiser 2006, Aldridge and Boyce 2007). Accumulating evidence of the impacts of energy development in sagebrush-steppe ecosystems extends beyond that of sage-grouse. Mule deer (*Odocoileus hemionus*) avoided otherwise suitable habitats within 2.7–3.7 km of gas wells (Sawyer et al. 2006) and densities of Brewer's sparrow (*Spizella breweri*) and sage sparrow (*Amphispiza belli*) declined 36–57% within 100-m of dirt roads in gas fields (Ingelfinger and Anderson 2004). Some suitable winter habitat remains undeveloped for sage-grouse in the PRB (RSF bins 4 and 5; Fig. 3), but the anticipated addition of another 37,000 CBNG wells at 32-ha spacing has the potential to affect >1.18 million ha of land. As remaining winter habitats are developed, and sage-grouse can no longer avoid CBNG, it is unclear whether birds will be able to adapt to a disturbance of this magnitude.

MANAGEMENT IMPLICATIONS

Sage-grouse avoidance of energy development in winter shows that a comprehensive strategy is needed to maintain suitable habitats in all seasons. Identifying and setting aside areas of undeveloped, high-quality habitat within the project area should be top priority. Currently, only 0.5-km^2 (quarter-mile buffer) of land surrounding a lek is excluded from development, an area that is 8 times smaller than the scale at which individual sage-grouse selected winter habitats (i.e., 4 km^2). Timing stipulations that restrict CBNG development within 3.2 km of a lek during the breeding season (15 Mar–15 Jun) are insufficient because they do not prevent infrastructure from displacing sage-grouse in winter. An additional stipulation in Montana that restricts new drilling activities within crucial winter range (1 Dec–31 Mar) only protects sage-grouse habitat during the winter in which the drilling is scheduled. Current stipulations leave only a small fraction of the land undeveloped, place no restrictions on the location of wells in winter habitat, and allow human access to all areas throughout the life of the producing gas field. Our spatially explicit winter habitat model can be used to identify areas in the PRB that provide the best remaining habitat for sage-grouse in winter.

ACKNOWLEDGMENTS

Comments by J. W. Hupp, J. W. Connelly, C. L. Aldridge and one anonymous reviewer greatly improved this manuscript. We thank landowners in the Powder River Basin that granted access to private lands. We thank J. Hess for her outstanding efforts in the field. Funding for this work came from Bureau of Land Management offices in Montana and Wyoming. Additional project support came from the Bureau of Land Management (Washington, D.C.), United States Department of Energy, Montana Department of Fish, Wildlife and Parks, Wyoming Game and Fish Department, National Fish and Wildlife Foundation, National Science Foundation (EPS–CORE program), Montana Cooperative Wildlife Research Unit, Petroleum Association of Wyoming, Western Gas Resources Inc., Wolf Creek Charitable Foundation, Bighorn Environmental Consulting, Anheuser-Busch Companies, Inc., and the University of Montana.

LITERATURE CITED

Aldridge, C. L., and M. S. Boyce. 2007. Linking occurrence and fitness to persistence: a habitat-based approach for endangered greater sage-grouse. Ecological Applications 17:508–526.

Aldridge, C. L., and R. M. Brigham. 2003. Distribution, abundance, and status of the greater sage-grouse, *Centrocercus urophasianus*, in Canada. Canadian Field-Naturalist 117:25–34.

Beck, T. D. I. 1977. Sage grouse flock characteristics and habitat selection in winter. Journal of Wildlife Management 41:18–26.

Beyer, H. L. 2004. Hawth's analysis tools for ArcGIS. <http://www.spatialecology.com/htools>. Accessed 15 May 2006.

Boyce, M. S., P. R. Vernier, S. E. Nielsen, and F. K. A. Schmiegelow. 2002. Evaluating resource selection functions. Ecological Modeling 157: 281–300.

Bureau of Land Management. 2003*a*. Final environmental impact statement and proposed plan amendment for the Powder River Basin Oil and Gas Project. <http://www.wy.blm.gov/nepa/prb-feis/index.htm>. Accessed 18 Mar 2007.

Bureau of Land Management. 2003*b*. Montana statewide final oil and gas EIS and amendment of the Powder River and Billings Resource Management Plans. <http://www.mt.blm.gov/mcfo/CBNG/eis/index.html>. Accessed 18 Mar 2007.

Burnham, K. P., and D. R. Anderson. 2002. Model selection and inference: a practical information-theoretic approach. Second edition. Springer-Verlag, New York, New York, USA.

Chambers, J. M., W. S. Cleveland, B. Kleiner, and P. A. Tukey. 1983. Graphical methods for data analysis. Duxbury Press, Boston, Massachusetts, USA.

Connelly, J. W., and C. E. Braun. 1997. Long-term changes in sage grouse *Centrocercus urophasianus* populations in western North America. Wildlife Biology 3:229–234.

Connelly, J. W., H. W. Browers, and R. J. Gates. 1988. Seasonal movements of sage grouse in southeastern Idaho. Journal of Wildlife Management 52:116–122.

Connelly, J. W., S. T. Knick, M. A. Schroeder, and S. J. Stiver. 2004. Conservation assessment of greater sage-grouse and sagebrush habitats. Western Association of Fish and Wildlife Agencies, Cheyenne, Wyoming, USA.

Connelly, J. W., M. A. Schroeder, A. R. Sands, and C. E. Braun. 2000. Guidelines to manage sage grouse populations and their habitats. Wildlife Society Bulletin 28:967–985.

Crawford, J. A., R. A. Olson, N. E. West, J. C. Moseley, M. A. Schroeder, T. D. Whitson, R. F. Miller, M. A. Gregg, and C. S. Boyd. 2004. Ecology and management of sage-grouse and sage-grouse habitat. Journal of Range Management 57:2–19.

Eng, R. L., and P. Schladweiler. 1972. Sage grouse winter movements and habitat use in central Montana. Journal of Wildlife Management 36:141–146.

Erickson, W. P., T. L. McDonald, K. G. Gerow, S. Howlin, and J. W. Kern. 2001. Statistical issues in resource selection studies with radio-marked animals. Pages 209–242 *in* J. J. Millspaugh and J. M. Marzluff, editors. Radiotracking and animal populations. Academic Press, San Diego, California, USA.

Giesen, K. M., T. J. Schoenberg, and C. E. Braun. 1982. Methods for trapping sage grouse in Colorado. Wildlife Society Bulletin 10:224–231.

Holloran, M. J. 2005. Greater sage-grouse (*Centrocercus urophasianus*) population response to natural gas field development in western Wyoming. Dissertation, University of Wyoming, Laramie, USA.

Homer, C. G., T. C. Edwards, Jr., R. D. Ramsey, and K. P. Price. 1993. Use of remote sensing methods in modeling sage grouse winter habitat. Journal of Wildlife Management 57:78–84.

Hupp, J. W., and C. E. Braun. 1989. Topographic distribution of sage grouse foraging in winter. Journal of Wildlife Management 53:823–829.

Ingelfinger, F., and S. Anderson. 2004. Passerine response to roads associated with natural gas extraction in a sagebrush steppe habitat. Western North American Naturalist 64:385–395.

Jennrich, R. I., and F. B. Turner. 1969. Measurement of non-circular home range. Journal of Theoretical Biology 22:227–237.

Johnson, C. J., S. E. Nielsen, E. H. Merrill, T. L. McDonald, and M. S. Boyce. 2006. Resource selection functions based on use–availability data: theoretical motivation and evaluation methods. Journal of Wildlife Management 70:347–357.

Johnson, C. J., D. R. Seip, and M. S. Boyce. 2004. A quantitative approach to conservation planning: using resource selection functions to map the distribution of mountain caribou at multiple spatial scales. Journal of Applied Ecology 41:238–251.

Kaiser, R. C. 2006. Recruitment by greater sage-grouse in association with natural gas development in western Wyoming. Thesis, University of Wyoming, Laramie, USA.

Knick, S. T., D. S. Dobkin, J. T. Rotenberry, M. A. Schroeder, W. M. Vander Haegen, and C. Van Riper, III. 2003. Teetering on the edge or too late? Conservation and research issues for avifauna of sagebrush habitats. Condor 105:611–634.

Leonard, K. M., K. P. Reese, and J. W. Connelly. 2000. Distribution, movements and habitats of sage grouse, *Centrocercus urophasianus,* on the Upper Snake River Plain of Idaho: changes from the 1950s to the 1990s. Wildlife Biology 6:265–270.

Manly, B. F. J., L. L. McDonald, D. L. Thomas, T. L. McDonald, and W. P. Erickson. 2002. Resource selection by animals: statistical design and analysis for field studies. Second edition. Kluwer Academic, Dordrecht, The Netherlands.

Moynahan, B. J., M. S. Lindberg, and J. W. Thomas. 2006. Factors contributing to process variance in annual survival of female greater sage-grouse in north-central Montana. Ecological Applications 16:1529–1538.

Patterson, R. L. 1952. The sage grouse in Wyoming. Wyoming Game and Fish Commission and Sage, Denver, Colorado, USA.

Remington, T. E., and C. E. Braun. 1985. Sage grouse food selection in winter, North Park, Colorado. Journal of Wildlife Management 49:1055–1061.

Robertson, M. D. 1991. Winter ecology of migratory sage grouse and associated effects of prescribed fire in southern Idaho. Thesis, University of Idaho, Moscow, USA.

Sauls, H. S. 2006. The role of selective foraging and cecal microflora in sage-grouse nutritional ecology. Thesis, University of Montana, Missoula, USA.

Sawyer, H., R. M. Nielson, F. Lindzey, and L. L. McDonald. 2006. Winter habitat selection of mule deer before and during development of a natural gas field. Journal of Wildlife Management 70:396–403.

Schroeder, M. A., C. L. Aldridge, A. D. Apa, J. R. Bohne, C. E. Braun, S. D. Bunnell, J. W. Connelly, P. A. Diebert, S. C. Gardner, M. A. Hilliard, G. D. Kobriger, and C. W. McCarthy. 2004. Distribution of sage-grouse in North America. Condor 106:363–376.

Schroeder, M. A., J. R. Young, and C. E. Braun. 1999. Sage grouse (*Centrocercus urophasianus*). Account 425 *in* A. Poole and F. Gill, editors. The birds of North America. The Academy of Natural Sciences, Philadelphia, Pennsylvania, and The American Ornithologists' Union, Washington, D.C., USA.

Smith, J. T., L. D. Flake, K. F. Higgins, G. D. Kobriger, and C. G. Homer. 2005. Evaluating lek occupancy of greater sage-grouse in relation to landscape cultivation in the Dakotas. Western North American Naturalist 65:310–320.

U.S. Navy Astronomical Applications Department. 2007. Altitude and azimuth of the sun during one day. <http://aa.usno.navy.mil/>. Accessed 7 Mar 2007.

Wakkinen, W. L., K. P. Reese, J. W. Connelly, and R. A. Fischer. 1992. An improved spotlighting technique for capturing sage grouse. Wildlife Society Bulletin 20:425–426.

Welch, B. L., F. J. Wagstaff, and J. A. Roberson. 1991. Preference of wintering sage grouse for big sagebrush. Journal of Range Management 44:462–465.

Associate Editor: Hupp.

OPEN ACCESS Freely available online

PLoS one

Prioritizing Conservation of Ungulate Calving Resources in Multiple-Use Landscapes

Matthew R. Dzialak*, Seth M. Harju, Robert G. Osborn¤, John J. Wondzell, Larry D. Hayden-Wing, Jeffrey B. Winstead, Stephen L. Webb

Hayden-Wing Associates, LLC, Natural Resource Consultants, Laramie, Wyoming, United States of America

Abstract

Background: Conserving animal populations in places where human activity is increasing is an ongoing challenge in many parts of the world. We investigated how human activity interacted with maternal status and individual variation in behavior to affect reliability of spatially-explicit models intended to guide conservation of critical ungulate calving resources. We studied Rocky Mountain elk (*Cervus elaphus*) that occupy a region where 2900 natural gas wells have been drilled.

Methodology/Principal Findings: We present novel applications of generalized additive modeling to predict maternal status based on movement, and of random-effects resource selection models to provide population and individual-based inference on the effects of maternal status and human activity. We used a 2×2 factorial design (treatment vs. control) that included elk that were either parturient or non-parturient and in areas either with or without industrial development. Generalized additive models predicted maternal status (parturiency) correctly 93% of the time based on movement. Human activity played a larger role than maternal status in shaping resource use; elk showed strong spatiotemporal patterns of selection or avoidance and marked individual variation in developed areas, but no such pattern in undeveloped areas. This difference had direct consequences for landscape-level conservation planning. When relative probability of use was calculated across the study area, there was disparity throughout 72–88% of the landscape in terms of where conservation intervention should be prioritized depending on whether models were based on behavior in developed areas or undeveloped areas. Model validation showed that models based on behavior in developed areas had poor predictive accuracy, whereas the model based on behavior in undeveloped areas had high predictive accuracy.

Conclusions/Significance: By directly testing for differences between developed and undeveloped areas, and by modeling resource selection in a random-effects framework that provided individual-based inference, we conclude that: 1) amplified selection or avoidance behavior and individual variation, as responses to increasing human activity, complicate conservation planning in multiple-use landscapes, and 2) resource selection behavior in places where human activity is predictable or less dynamic may provide a more reliable basis from which to prioritize conservation action.

Citation: Dzialak MR, Harju SM, Osborn RG, Wondzell JJ, Hayden-Wing LD, et al. (2011) Prioritizing Conservation of Ungulate Calving Resources in Multiple-Use Landscapes. PLoS ONE 6(1): e14597. doi:10.1371/journal.pone.0014597

Editor: Michael Somers, University of Pretoria, South Africa

Received June 23, 2010; **Accepted** December 15, 2010; **Published** January 26, 2011

Funding: Pioneer Natural Resources Company funded this work. Hayden-Wing Associates, LLC provided in-kind contributions including travel costs associated with dissemination of this work, and materials associated with data collection and analysis including radio-telemetry equipment, GPS devices, and statistical analysis software. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: This work was funded by a commercial source, Pioneer Natural Resources Company. Hayden-Wing Associates, LLC provided in-kind contributions including travel costs associated with dissemination of this work, and materials associated with data collection and analysis including radio-telemetry equipment, GPS devices, and statistical analysis software. Having received funding from this source, the consultancy could reasonably be perceived as a financial competing interest. This does not alter the authors' adherence to all the PLoS ONE policies on sharing data and materials.

* E-mail: matt@haydenwing.com

¤ Current address: New Mexico Game and Fish Department, Santa Fe, New Mexico, United States of America

Introduction

Identifying resources associated with critical life-history phases in ungulates is a conservation priority. Winter range, parturition areas, and migration routes are important seasonal habitats in North America that provide resources necessary for survival and reproduction such as high-quality forage, reduced exposure to inclement conditions, and reduced risk of predation [1], [2]. In the Intermountain West USA, these habitats have become increasingly fragmented. Here, energy development is of broad conservation interest because its prominence has increased in recent decades along with concern about its potential impact on wildlife and their habitats [3]. Understanding how human activity such as resource extraction interacts with wildlife, and developing tools to guide conservation planning in areas where human activity is widespread or increasing are ongoing challenges in conservation science. In this paper, we investigated interactions between human activity associated with energy development and resource selection by female Rocky Mountain elk (*Cervus elaphus*) during calving season with the larger goal of informing conservation planning for ungulates in places where human activity is widespread or increasing. We asked: 1) what are the relative influences of maternal status (parturiency) and human activity on resource selection, 2) to what extent does behavior vary among individuals,

3) how does individual variation interact with human activity, and 4) how can our findings be applied in conservation planning and decision making? First, we describe a novel application of generalized additive models for designating maternal status using movement data. Model-based methods to designate maternal status were necessary because data bearing directly on maternal status were unavailable for most elk. Second, we estimated group-dependent random-effects resource selection functions (RSFs) to identify how resource selection patterns differed relative to maternal status and human activity, and how behavior varied among individuals. We applied results by developing and validating group-dependent predictive maps of critical calving resources, and quantifying discrepancies among maps relative to predictive accuracy.

A key approach in studying wildlife-human interaction is resource selection modeling [4]. Resource selection is a fundamental ecological process that structures animal movement and distribution [5]. The choices animals make as they move throughout the landscape reflect trade-offs between selecting resources that meet their needs for survival and reproduction, and minimizing perceived risk of harm – such risk often is a function of interaction with predators or humans [6], [7], [8]. A set of analytical methods used with increasing frequency for investigating resource selection in animals is the estimation of RSFs [9]. RSFs describe the relative probability of occurrence of animals as a function of behavioral responses to features of the environment. The probability of occurrence is described as relative because RSFs are estimated in a use-versus-availability framework in which selection is quantified relative to available but presumed non-used features. Environmental features can include a wide range of variables such as vegetation, terrain, group/herd size, risk of predation, or human-modifications of the landscape [10], [11]. RSFs have strong application in conservation planning where wildlife-human interaction is a concern; specifically, in establishing a spatially explicit basis from which to prioritize conservation action such as reclamation, mitigation, or minimizing human activity in particular habitats.

Areas associated with parturition are important because female ungulates make resource-related choices that affect offspring development during gestation and provisioning of recently born calves that are susceptible to malnutrition and predation [12], [13]. While there is little evidence of consistent parturition site fidelity in many ungulates, strong fidelity among females to seasonal ranges, particularly around calving time, has been demonstrated [2], [14]. New light has been shed on the adaptive significance of resource selection during the period that encompasses reproductive activity in many vertebrates through the study of maternal effects – developmental mechanisms by which parents translate their environmental experience into adaptive variation in their offspring [15], [16], [17]. The adaptive significance of resource selection during reproductive periods suggests that conservation strategies designed around ungulate parturition areas might be most reliable when based on parturient females rather than samples including both parturient and non-parturient individuals.

Another key feature of animal ecology that warrants further attention as part of conservation planning is variation in behavior among individuals. Individual variation is widespread and well-known in many animal species [18] and can reflect long-term selection for a given trait or learned behavior [19], [20]. Fitness is influenced by the choices individuals make in terms of resource selection because each resource type has particular costs and benefits to the individual [21]. In risky or rapidly changing environments such as those in which human activity is increasing, optimal behavioral strategies may vary among individuals on a situation-specific basis [22] making it difficult to generalize behavior across the population and thus effectively guide conservation planning.

Methods

Study area

The 1845 km^2 study area encompassed northern portions of the Raton basin in south-central Colorado, USA. Topography is rugged with steep slopes, rocky outcrops, ridges, and valleys ranging in elevation from 2000–3000 m. Mean annual precipitation is about 40–53 cm depending on elevation [23]. Vegetation includes conifer forest, montane shrub, and grassland. Dominant species include ponderosa pine (*Pinus ponderosa*), one-seed juniper (*Juniperus monosperma*), two-needle pinyon (*Pinus edulis*), Gambel oak (*Quercus gambelii*) which commonly forms shrub-thickets on southern aspects, antelope bitterbrush (*Purshia tridentata*), skunkbush sumac (*Rhus trilobata*), and willow (*Salix* spp.) in riparian areas. Predators of elk (including neonates) include black bear (*Ursus americanus*), mountain lion (*Felis concolor*), and coyote (*Canis latrans*); no wolf (*Canis lupus*) pack occurred in the study area. The study area encompassed historic and ongoing energy development. Bituminous coal mining was a dominant land use during 1873–1970. Coal-bed methane development was initiated in Raton basin in 1982 and accelerated in the late 1990s [24], [25]. In 2009, there were about 2900 wells associated with methane development in the Basin (Figure 1).

Capturing elk

In February and March 2006–2009, helicopter net-gunning was used to capture yearling (1.5 years) and adult ($\geq$2.5 years) female elk throughout and adjacent to the gas field. Elk were fitted with Global Positioning Systems (GPS) collars (TGW-3590, Telonics, Inc., Mesa, AZ 85204) configured with store-on-board and Very High Frequency (VHF) beacon options. Twenty-five female elk were fitted with GPS collars in 2006, 40 in 2007, 50 in 2008, and 50 in 2009. GPS collars attempted to record location information every 3 h resulting in a maximum of 8 locations/elk/day. Age of elk was estimated based on dental eruption and wear patterns [26]. Blood samples were collected from captured elk in 2008 and 2009 to determine pregnancy, but not from elk captured in previous years. Animal capture and handling protocols were approved by the Colorado Division of Wildlife (Permit #s 06TR1083, 07TR1083, 08TR1083 and 09TR1083A001).

Grouping Elk Relative to Maternal Status and Human Activity

We predicted maternal status (parturient versus non-parturient) by using generalized additive models (GAMs) to parameterize response curves depicting daily movement of elk during calving season. GAMs are semi-parametric extensions of generalized linear models [27]. The central concept is that the function of a covariate is estimated nonparametrically from the data by means of scatterplot smoothers. The functional form of the relationship between the response and covariate(s) is therefore determined by the data rather than being restricted to a parametric form [28]. Formally, the linear regression model

$$y = \alpha + \sum_{i=1}^{I} \beta_i(X_i) + \varepsilon$$

is generalized by modeling y as being related to covariates additively by

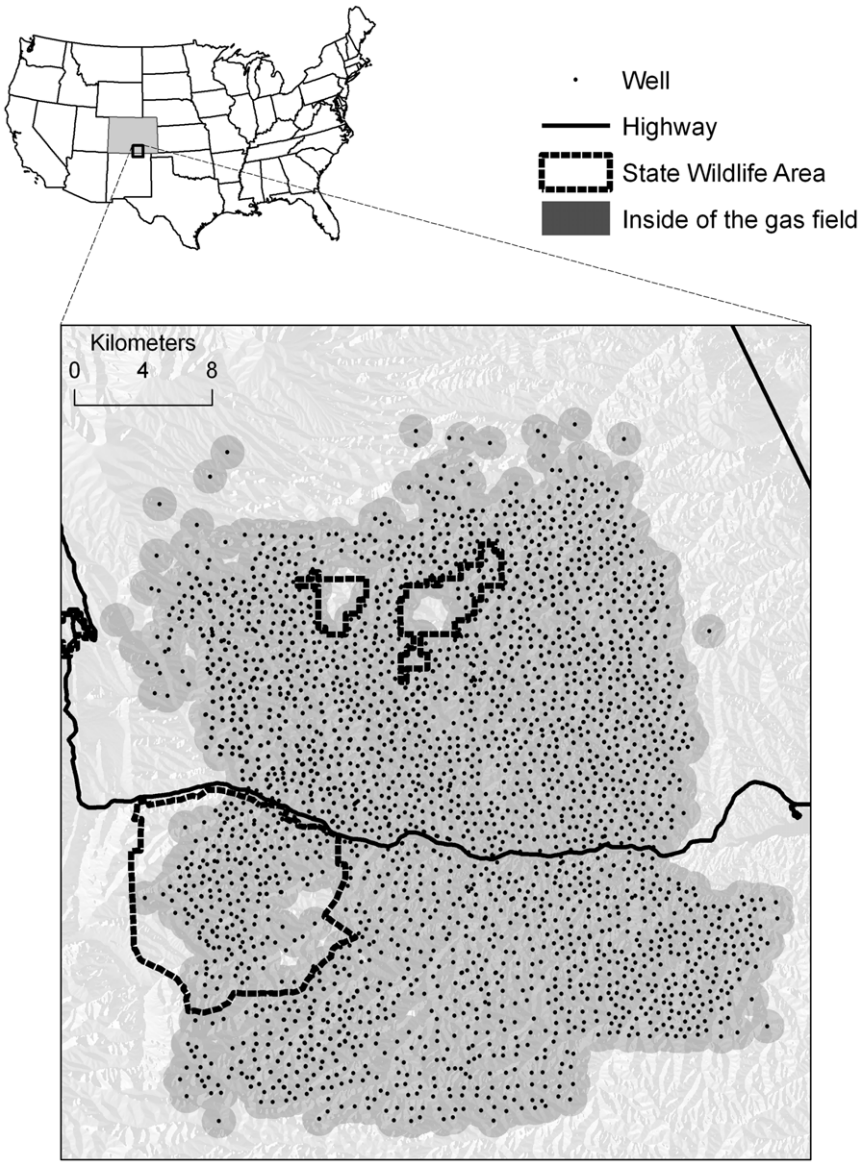


Figure 1. The Raton Basin gas field and adjacent areas in south-central Colorado, USA. Each natural gas well is encircled by a 1-km buffer (shaded region). We designated elk locations occurring within the buffered region to be ''inside of the gas field'' whereas locations adjacent but external to the buffered region were designated ''outside of the gas field''.
doi:10.1371/journal.pone.0014597.g001

$$g(y) = \alpha + \sum_{i=1}^{I} f_i(X_i) + \varepsilon$$

where g is the link function and ε is a random error term. Functions f_i may be linear or nonparametric functions defined by smoothers such as smoothing splines or locally estimated scatterplot smoothers (loess; also referred to as locally estimated, or weighted, polynomial regression). Smoothers provide a series of data summaries of the response that are specific to regions of the covariates; a well known smoother is the moving average [29]. The amount of smoothing is calibrated by the size of the neighborhood, or percentage of the data points, over which averaging is done; a quantity known as span. A larger span yields a smoother data summary (less curvature) whereas a smaller span yields a less smooth data summary (more curvature). This data summary (*i.e.*, the effective number of parameters of a smoother) is described by the quantity equivalent degrees of freedom (*df*). Span is related inversely to *df* so as span increases *df* decreases [27]. Data that are best described by a straight line (a single parameter comprises the smoother) correspond to a span of 100% and thus 1 *df*. Conversely, data that are best described by gradients or turning points (several or many parameters comprise the smoother) correspond to a smaller span and thus to a larger number of *df* [27], [30]. The analyst may set the span by specifying *df* based on visual examination of the data, or implement generalized cross validation methods in which appropriate *df* are identified automatically given the data. From a GAM perspective, movement of female ungulates during the calving season likely contains information on maternal status. Restlessness and the seeking of solitude characterize imminent parturition [31], [32], [33] and establish a general pattern of increased daily movement pre-partum and decreased movement post-partum relative to barren females (Figure 2a; [34],

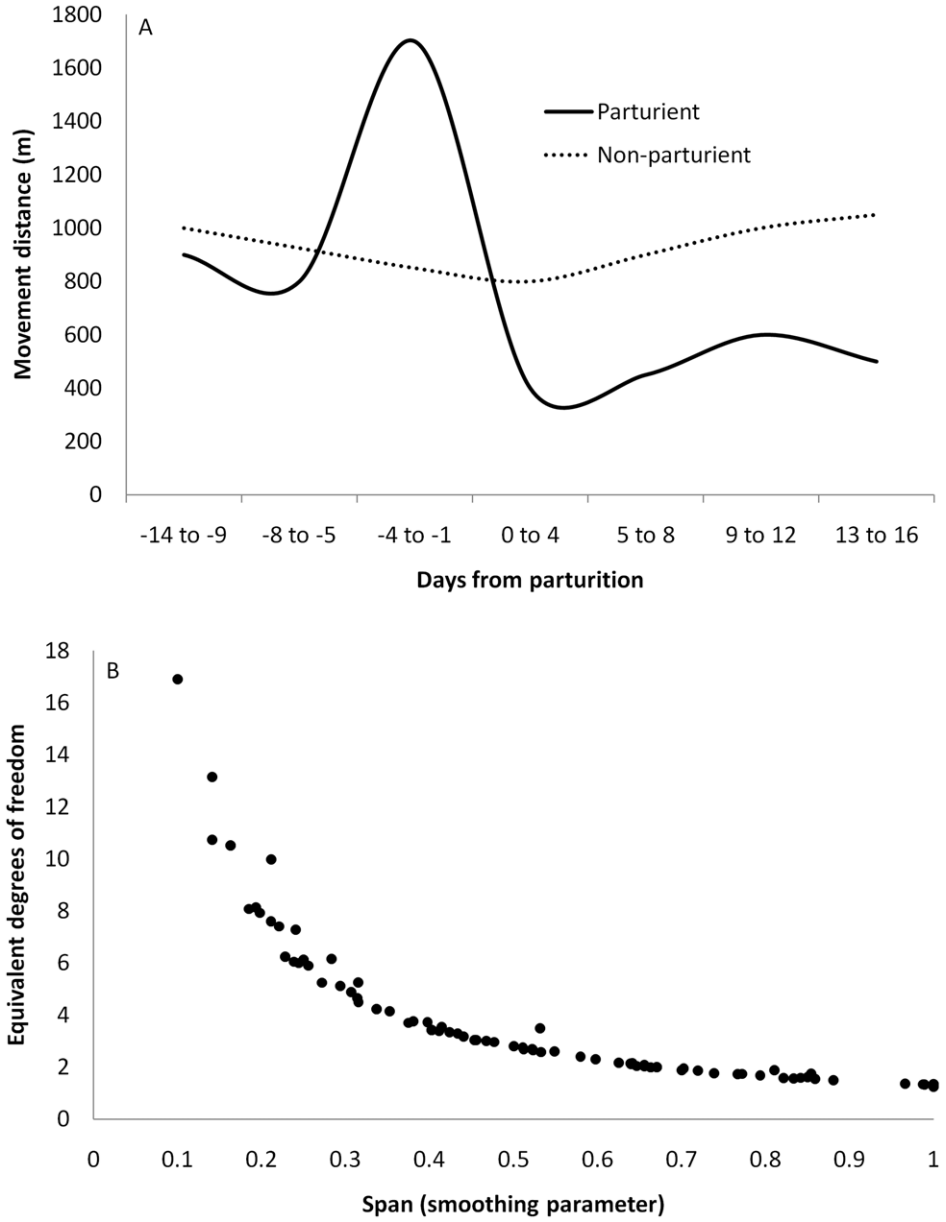


Figure 2. Movement in female ungulates during calving time. Generalized from the literature [34], [35], [36], parturient females often will make long-distance movements associated with pre-parturient "restlessness" within days of parturition and then exhibit reduced movement associated with provisioning the neonate (a). This pattern may provide a quantifiable distinction between parturient and non-parturient females. Also shown (b) is parameterization (equivalent degrees of freedom) as a function of span based on generalized additive models of distance moved between consecutive locations in 103 female Rocky Mountain elk. Loess smoothing and automated calculation of degrees of freedom using the generalized cross validation method were specified.
doi:10.1371/journal.pone.0014597.g002

[35], [36]). We hypothesized that during calving time, movement associated with restlessness and solitude seeking in parturient elk provides a general pattern of complexity that is not observed in non-parturient females, and we predicted that smoothers associated with movement data on parturient elk consistently would be comprised of more parameters and thus more *df* than smoothers associated with non-parturient elk.

We used GAMs to regress distance traveled within a 24 h period (using locations recorded at 1200 h on consecutive days) against date. Date encompassed the 15 May – 1 July calving season [33] in each of the 4 years comprising the study period. We used the generalized cross validation option to assign *df* to response curves depicting daily changes in the distance moved by elk between successive locations. We used PROC GAM in SAS® (SAS Institute, Inc., Cary, North Carolina, USA), specifying automated calculation of *df* using the generalized cross validation method and loess smoothing, to assign *df* to response curves depicting calving season movement patterns in each elk. We established the following prediction: movement described by <3 *df* in GAMs would depict non-parturiency whereas movement described by ≥ 3 *df* would depict parturiency. This prediction was based on two observations. First, the shape of the response curve depicting relatively simple movement, as in non-parturient individuals, appears to correspond with ≤ 2 regions that differ in

slope, each of which being calibrated over a span of ≥50% of the data (Figure 2a; [34], [35]). Second, by plotting *df* as a function of span, our data show that a span of 50% corresponds to ~3 *df* (Figure 2b).

We tested the GAM approach using blood samples obtained from elk during capture in 2008 and 2009, and field observation of females and calves in those same years. Blood sera were tested for presence of pregnancy-specific protein-B (PSPB; BioTracking, LLC, Moscow, Idaho, USA; [37]). We conducted field observation from dawn to 0900 and from 1800 until dusk using binoculars and spotting scopes to watch for behavior that suggested a maternal bond between a female and calf. This behavior included nursing and licking bouts, traveling as a female/calf unit, and heightened attentiveness between a female and calf [13], [38]. Females for which PSPB testing indicated pregnancy and for which field observation suggested a strong female-calf bond were designated as parturient. Determining non-parturiency is never definitive; however, we designated females as non-parturient with negative PSPB results and for which field observation was unable to associate the female with a calf [13]. We thus established two groups of elk relative to maternal status by which we analyzed resource selection.

Similarly, we established two groups of elk relative to human activity: elk occupying developed areas versus elk occupying undeveloped areas. Elk locations occurring within 1 km of a gas well were considered to be in the developed area (Figure 1). Human activity was apparent in the areas we called undeveloped including some ranching and residences; however, no industrial development occurred in undeveloped areas and human activity was limited relative to developed areas. Given the short temporal window within which we conducted these analyses, calving season use areas (100% minimum convex polygons) generally occurred wholly within developed areas or wholly within undeveloped areas. Only a small number of elk occupied both areas during the calving season – we discuss these elk separately. We estimated minimum convex polygons because the temporal window of the study was relatively short, calving season use areas comprised a portion of the annual use areas, and we wanted to err on the side of inclusiveness rather than potentially omitting a portion of critical calving range from the analysis. In comparing resource selection between elk occupying developed versus undeveloped areas, a key assumption is that human activity associated with energy development is the primary difference between areas and that other factors to which elk respond were similar between areas. We compared landscape and habitat covariate values between areas to inform this assumption.

Random-effects Resource Selection Modeling

Modeling variables as random effects can improve our understanding of resource selection, which will enhance the practical application of RSFs in management decisions [4], [39]. Random effects models assume that sample units are drawn at random from a larger population and that the data are structured hierarchically (*i.e.*, within subject responses are more similar than between-subject responses). Mechanistically, assumptions of random-effects models are: (1) the random effects are distributed normally with mean equal to 0 and unknown variance, (2) within-group correlation is constant through time, and (3) the analyst has correctly specified the variance-covariance structure (see [39] for a review of the application of random-effects models in resource selection analysis). In RSF models, it is appropriate to model intercepts and/or covariates as random effects when variance among sample units is of interest, animal response to gradients in a resource is suspected, or population-level inference is of interest. Random intercepts can account for unbalanced data, correlation among observations, and provide improved model fit and parameter estimation [39] (*but see* [11]). Random-effects (or mixed-effects) RSF models provide information on individual behavior, how individuals contribute to population-level observations, and how their responses to a resource may change as a function of its availability – a process known as a functional response [40], [41]. Analytical approaches to model functional responses in resource selection are particularly important when there is a trade-off in selection for a particular resource [4], [40], which would be the case if human activity is perceived as a risk of harm [7].

We incorporated random effects into the use versus availability design [9] in which covariates representing important resources are compared at used and available (but presumed non-used) locations using

$$\hat{w}(x) = \exp(\hat{\beta}_0 + \hat{\beta}_1 x_1 + \hat{\beta}_2 x_2 + \ldots + \hat{\beta}_n x_n)$$

where $\hat{w}(x)$ is the relative probability of use as a function of covariates x_n with coefficients $\hat{\beta}_n$ estimated from logistic regression. Availability was defined for each elk by including random locations within 100% minimum convex polygon seasonal use area estimates; the number of random locations generated was 3 times the number of used locations for each elk. We examined resource selection within seasonal use areas (*i.e.*, 3rd order selection). We modeled resource selection separately during day and night because we expected behavior of elk to differ between day and night. We assigned time of day at random to available locations for day versus night comparisons; times assigned to available locations corresponded to times associated with used locations (e.g., every 3 hours on the sampled hour). Day models included the times 0900, 1200, 1500, and 1800 h whereas night models included 0000 and 0300 h. Using a Geographic Information System (GIS; ArcGIS 9.2), we calculated 7 covariates at used and random locations (Table 1). Four of these covariates including cover type, slope, elevation, and habitat edge density were calculated at locations both within and outside of the gas field. Three covariates including road density, distance to a human-built structure, and industrial development footprint (Table 1) were calculated only within the gas field because human activity in areas adjacent but outside of the gas field was less intense and not associated with industrial development. Raster data for cover type were developed from annual aerial photography of the study area, terrain covariates were calculated from a 30-m resolution digital elevation model, and human activity covariates including roads, structures, and industrial development footprint were heads-up-digitized from aerial photography and analyzed as year-specific covariates (Table 1). We used Spatial Analyst in ArcGIS to extract values from raster data for all covariates.

We estimated a three-level random-effects model in which locations $i = 1 \ldots I$ occurred within strata representing individual elk $j = 1 \ldots J$ [39]. Considering a random intercept and random coefficients, the RSF is estimated by

$$g(x) = \beta_0 + \beta_1 x_{1ij} + \ldots + \beta_k x_{kij} + \gamma_{kj} x_{kj} + \gamma_{0j}$$

where covariates k ($k = 1 \ldots K$) have values x, γ_{0j} is the random intercept and γ_{kj} is the random coefficient of x_k for elk j, which is the difference in the intercept and coefficient for elk j from the mean population-level intercept β_0 and coefficient β_{kj}, respectively. We estimated models using the GLIMMIX procedure in SAS®. We specified the conditional probability distribution of the data as

Table 1. Covariates used in random-effects resource selection models and their descriptions.

Covariate	Description
Slope	Digital elevation model (DEM) provided at a resolution of 1.52 m and re-sampled for covariate calculation to a resolution of 30 m and measured in degrees. Values for slope calculated at point locations in our study ranged from 0.02 (flat ground) to 41.8 degrees (very steep).
Elevation	DEM re-sampled for covariate calculation to a resolution of 30 m and measured in m. Elevation at sample points in our study ranged from 1918.7–2970.0 m.
Cover type	Raster dataset compiled from 1-foot resolution aerial imagery of the study area using Image Analysis™ and re-sampled to 30 m resolution for covariate calculation. From an elk-centric perspective, habitat in the Raton basin functioned in one of two ways: as security cover or as forage resources. Raster cells were assigned one of two values (binary covariate) representing habitat that functioned to provide cover versus habitat that did not function to provide cover. All tree or oak-thicket dominated habitats were considered security cover, whereas all shrub and grassland dominated habitats were considered non-cover.
Edge density	Density of line features depicting the interface of cover and non-cover habitat calculated for the central grid cell within a 990 m^2 moving window. Values in our study were 22.2–136.0 km/km^2.
Road density	Density of line features depicting roads calculated for grid cells at the center of a 990 m^2 moving window. Road density was calculated as a year-specific variable – that is, new road features were added to the data set as annual aerial imagery became available. Values in our study, calculated within the gas field only, were 0.0–7.6 km/km^2.
Distance to structure	Linear distance from a sample location to a human-built structure including houses, agricultural facilities, and industrial facilities. Distance to structure was a year-specific variable and was analyzed as natural log transformed distance +0.1 to allow its magnitude to decrease with increasing distance. Values in our study, calculated within the gas field only, were 0.0–5854.9 m.
Industrial development footprint	Area (density) of physically modified ground calculated from aerial photography as a year-specific variable. Physically modified ground primarily reflected industrial development including well pads, pipe yards, pipelines, construction areas, or clearing for facilities development. Industrial development footprint was calculated for the central grid cell within a 990 m^2 moving window. This covariate excludes physical disturbance associated with roads. Values in our study, calculated only within the gas field, were 0.0–0.62 km^2/km^2.

doi:10.1371/journal.pone.0014597.t001

binomial and used a logit link function. We included ordinal date as a class variable and specified a variance components covariance structure in which random intercepts for ordinal date were nested within each individual to address within-day autocorrelation among locations. R-side, or marginal, random-effects models estimated using GLIMMIX provide conditional coefficient estimates for each individual, that is, estimates for individual animals that are conditional on the distribution of coefficient estimates across all individuals in the population. To estimate marginal (*i.e.*, population-level) coefficients we assumed that conditional coefficients for each elk represented a random sample from a normal distribution with the mean of that distribution representing the population-level effects of covariates on the probability of use [42]. We estimated marginal coefficients using

$$\hat{\bar{\beta}}_k = \frac{1}{J}\sum_{j=1}^{J}\hat{\beta}_{kj}$$

where $\hat{\beta}_{kj}$ was the estimate of coefficient k for elk j [43], [44], and we estimated variance using

$$\mathrm{var}\left(\hat{\beta}_k\right) = \frac{1}{J-1}\sum_{j=1}^{J}(\hat{\beta}_{kj} - \hat{\bar{\beta}}_k)^2$$

Population-level coefficient estimates ($\hat{\bar{\beta}}_k$) are similar to the average of the conditional estimates because the conditional coefficient estimates are constrained to have means $\hat{\beta}_k$ [44]. In all, five groups of elk were available for comparison: 1) all sample elk inside of the gas field, 2) elk predicted to be parturient based on GAMs inside the gas field, 3) field-observed parturient elk inside of the gas field, 4) field-observed non-parturient elk inside of the gas field, and 5) all sample elk outside of the gas field (sample size was too low to develop maternal groups; see Results).

Mapping Responses

We applied the results from random-effects models to map relative probability of use across the study area which encompassed developed and undeveloped areas. We used marginal coefficients from logistic regression to derive an RSF at a resolution of 30 m using

$$w(x) = \exp\left(\sum_{k=1}^{K}\beta_k^* x_k\right)$$

where covariates k ($k = 1 \ldots K$) have values x [9]. We were interested in differences in resource selection patterns depending on maternal status and between elk that occupied developed versus undeveloped areas. It is important to note that, although we analyzed an independent group of field-observed parturient elk to facilitate comparison of marginal estimates, these elk were withheld from final RSF development as a validation sample. Thus, we examined differences in how the relative probability of use was assigned throughout the landscape depending on whether this probability was based on models of: 1) all sample elk inside the gas field, 2) elk predicted to be parturient based on GAMs inside the gas field, and 3) all sample elk outside of the gas field (groups 1,2, and 5 above). We conducted this examination using the following methods separately for day and night. We developed annual RSF maps for each of the 3 groups identified above. We estimated quantiles in SAS® (PROC RANK, PROC MEANS) by which pixels comprising the raster surface were partitioned into 5 equal-sized subsets based on pixel value. In GIS we reclassified RSF values based on quantiles establishing 5 ranks of the relative probability of use (1 = low probability, 5 = high probability). We summed within-year maps across all years and ranked relative probability of use as described above yielding 6 multi-year predictive maps (day map and night map for each group) with relative probability of use ranging from 1 (low) to 5 (high). We validated predictive maps using locations from 24 elk that were observed in the field to be

parturient (see below); these elk were withheld from final RSF development so they represent an independent validation sample. Locations from these elk were plotted on multi-year predictive maps. We tested whether the number of locations that occurred within each predicted probability of use rank (1–5) differed from expectation using a chi-square test for specified proportions (PROC FREQ, SAS®). To provide a measure of the amount to which a map that validated well differed from a map that validated poorly, we calculated the number of pixels comprising the raster surface that differed between maps in terms of relative probability rank.

Results

Capturing Elk and the Environment Inside versus Outside of the Gas Field

We fitted 25 female elk with GPS collars in 2006, 40 in 2007, 50 in 2008, and 50 in 2009. The entire within-year sample was unavailable for these analyses because the analyses spanned a relatively short period during calving (Table 2), and in each year several elk moved from the study area to alpine habitat for parturition. Inadequate sample size (*i.e.*, $\leq$2 elk comprising maternal groups) in undeveloped areas restricted a more comprehensive assessment. Differences in elevation, slope, edge density, and cover type between developed and undeveloped areas were minimal. Based on 25,290 GIS-generated random sample points (12,645 inside and 12,645 outside of the gas field, respectively), $\bar{x} \pm$ SD values inside versus outside of the gas field respectively were 2,347.7 $\pm$ 146.4 m and 2,382.6 $\pm$ 243.4 m for elevation, 10.6 $\pm$ 6.2 degrees and 9.4 $\pm$ 7.0 degrees for slope, and 82.0 $\pm$ 10.2 km/km^2 and 77.5 $\pm$ 12.7 km/km^2 for edge density. The proportion of sample points that occurred within security habitat was 0.84 and 0.80 inside versus outside of the gas field, respectively. Topographic covariates (elevation and slope) were not ground truthed; however, vegetation attributes were ground-truthed based on field-established polygons ($n = 1177$) of known vegetation type.

Table 2. Resource selection by Rocky Mountain elk during calving time; sample size and number of locations used in analyses.

		#Elk		#Locations	
Year	**Group**[a]	**Day**	**Night**	**Day**	**Night**
2006	Inside – all sample elk	11	10	1,759	879
	Inside – GAM-predicted parturient	5	5	917	458
	Outside – all sample elk	7	7	122	61
2007	Inside – all sample elk	26	24	3,281	1,641
	Inside – GAM-predicted parturient	9	9	1,174	587
	Outside – all sample elk	16	14	900	451
2008	Inside – all sample elk	19	19	2,056	1,455
	Inside – GAM-predicted parturient	7	7	776	438
	Inside – field-observed parturient	12	12	1,598	802
	Inside – field-observed non-parturient	5	5	425	227
	Outside – all sample elk	19	16	417	230
2009	Inside – all sample elk	15	15	2,735	1,482
	Inside – GAM-predicted parturient	8	8	1,381	757
	Inside – field-observed parturient	12	12	1,976	1,062
	Inside – field-observed non-parturient	5	5	685	496
	Outside – all sample elk	16	14	411	215

[a]Inside refers to areas within the gas field and outside refers to areas adjacent but external to the gas field. GAM-predicted parturient refers to female elk that we predicted to be parturient based on results of generalized additive modeling (GAM) of movement data. Field-observed refers to elk for which we observed behavior in the field to assess parturition status.

Total sample fitted with Global Positioning Systems collars was 25, 40, 50, and 50 in 2006, 2007, 2008, and 2009, respectively. Within-year deviation from the available sample arises from 3 sources: 1) some collared elk moved out of the study area during calving, 2) the GAM-predicted sample was nested within the total sample, and 3) some individuals occupied areas both inside and outside of the gas field.

doi:10.1371/journal.pone.0014597.t002

Generalized Additive Modeling to Designate Maternal Status

We applied results from PSPB and field observation of female elk in 2008 and 2009 to test the GAM approach, in which movement patterns parameterized by $<3df$ were predicted to depict non-parturiency whereas movement patterns parameterized by ≥ 3 *df* were predicted to depict parturiency. Based on PSPB results and 788 hours of field observation, we assigned maternal status to 34 elk; 24 were identified as parturient and 10 as non-parturient. Movement during calving time was highly variable, especially among parturient elk, with parameterization ranging from 1.24–16.9 *df*. However, among elk observed to be parturient in the field, 93% (13/14) were parameterized by ≥ 3 *df* (Table 3).

Random-effects Resource Selection Modeling – Marginal Inference

We found little evidence of lack of fit or overdispersion in RSF models; generalized chi-square/degrees of freedom was 0.79–0.92 for night time models and 0.87–0.97 for day time models. The most notable response by elk inside of the gas field was strong avoidance of the industrial development footprint during the day among all groups (at the population-level; Figure 3a). Other notable behavior in field-observed parturient elk included strong selection for cover and avoidance of high road density during the day relative to other groups (Figure 3b,c). While field-observed parturient elk showed some day-time preference for flatter areas and lower elevation relative to the larger sample, differences among maternal groups in selection for these and other resources were small, inconsistent, or not apparent.

Table 3. Parameterizing movement using generalized additive models.

Year/Group	$df<2$[a]	$2<df<3$	$df\geq 3$
2008 Field-observed parturient	2	1	9
2009 Field-observed parturient	5	3	4
2008 Field-observed non-parturient	2	2	1
2009 Field-observed non-parturient	3	2	0

[a]As part of generalized additive modeling we specified the generalized cross validation option to assign parameters (*df*) to polynomials depicting daily changes in the distance moved by elk between successive locations.

Maternal status was assigned to 34 female Rocky Mountain elk (10 non-parturient and 24 parturient) using information from blood samples (pregnancy-specific protein-B) and field observation (788 h). We assessed how generalized additive models of location data parameterized movement, and how parameterization corresponded with calf status as determined in the field. Results show that among 14 elk for which movement was parameterized by ≥ 3 *df*, 13 were observed in the field to be with calf.

doi:10.1371/journal.pone.0014597.t003

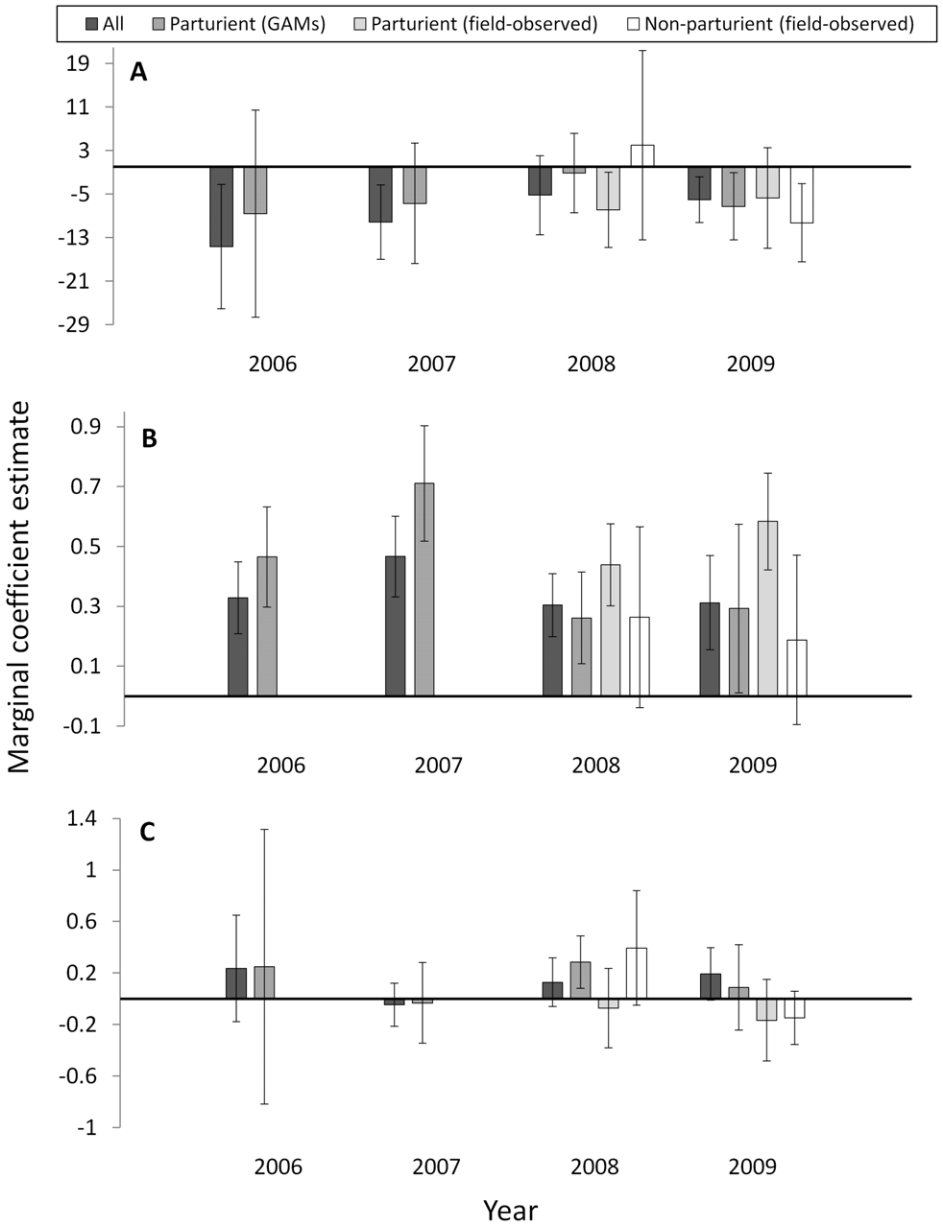


Figure 3. Group-dependent population-level responses: inside of the gas field. Marginal coefficient estimates ± 95% CL of selection for human disturbance (a), security cover (b), and road density (c) by female elk during 2006-2009 in Raton Basin, Colorado, USA. Day time results are displayed among maternal statuses for elk inside of the gas field.
doi:10.1371/journal.pone.0014597.g003

We estimated selection for 4 landscape/habitat covariates in both developed and undeveloped areas including security cover, slope, elevation, and habitat edge density. Considering population-level coefficient estimates, selection for resources that functioned to provide security cover differed markedly between elk that occupied developed areas versus undeveloped areas (Figure 4). Elk inside of the gas field consistently showed strong selection for security cover during the day (Figure 4a) and strong selection for non-cover or forage habitats during the night (Figure 4b) throughout the study period whereas elk occupying areas outside of the gas field generally selected randomly for security cover with coefficient estimates near zero. Another difference between elk inside versus outside of the gas field involved selection for elevation. Elk outside of the gas field generally selected randomly for elevation with coefficient estimates always near zero. Elk inside of the gas field generally selected for higher elevation during the day (Figure 4c; however, 95% CL overlapped zero), and for lower elevation at night (Figure 4d). Diurnal patterns in selection for slope and edge density were apparent but differences in selection for these features between elk inside versus outside of the gas field generally were small (Table 4).

Random-effects Resource Selection Modeling – Conditional Inference

Conditional estimates revealed a complex association between human activity and individual variation in response to environmental and anthropogenic features (Figure 5). Elk inside the gas field showed greater heterogeneity among individuals relative to elk outside of the

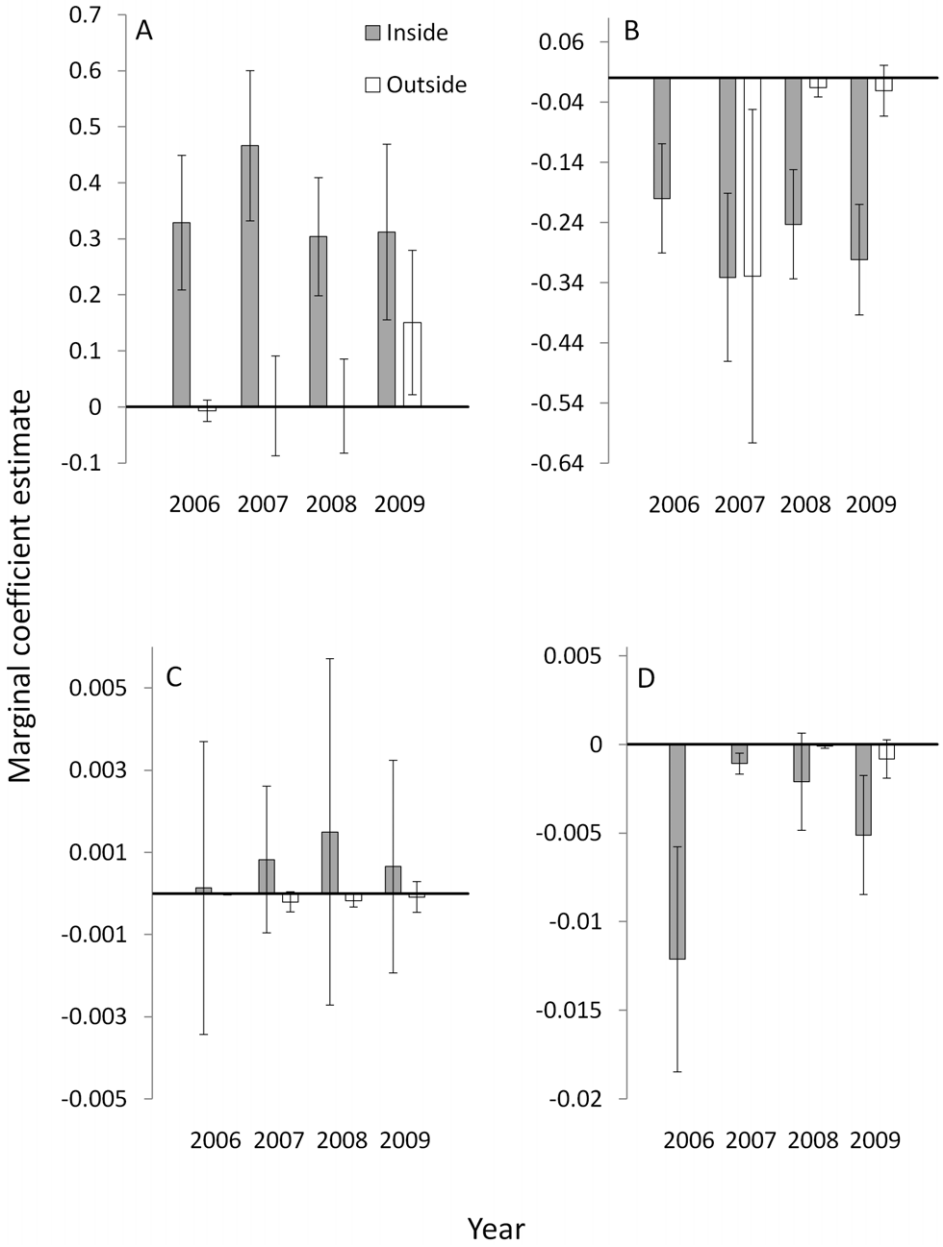


Figure 4. Population-level responses: inside versus outside of the gas field. Marginal coefficient estimates ±95% CL of day-time (a) and night-time (b) selection for security cover, and day-time (c) and night-time (d) selection for elevation by female elk during 2006-2009 in Raton Basin, Colorado, USA. Inside refers to elk occupying an active gas field whereas outside refers to elk occupying adjacent undeveloped areas; maternal status is not considered (*i.e.*, all sample elk are grouped depending on whether they occurred inside versus outside of the gas field).
doi:10.1371/journal.pone.0014597.g004

gas field in their responses to edge density and elevation. Selection for slope (Figure 5a,b) and edge density (Figure 5c, d) was estimated more precisely among elk inside of the gas field; however, elk outside the gas field selected (randomly) for elevation with high precision (Figure 5e, f). Also apparent inside the gas field but not outside was a temporal trend of increasing avoidance of high edge density (Figure 5c). Within-year marginal estimates of day time selection for road density were positive (*i.e.*, elk selected for higher road density) in 3 of 4 years. However, conditional estimates revealed that, across all years, only 37% of sampled elk selected for relatively high road density; 32% selected neither for nor against high road density, and 31% avoided high road density (Figure 5g).

Seasonal use areas of 15 elk overlapped both developed and undeveloped areas. Individuals showed notable behavioral differences, including diurnal variation, relative to selection for security cover and elevation depending on whether they were in developed versus undeveloped areas (Figure 6). During the day, 13 of 15 elk showed stronger selection for security cover when inside the gas field, but at night this pattern broke down with no apparent consistent behavioral response. Selection for elevation was variable inside the gas field during day and night with some elk showing relatively strong selection for higher or lower elevations. When elk were outside of the gas field they generally used elevation at random (Figure 6).

Table 4. Marginal coefficient estimates ($\hat{\bar{\beta}}_n$[SE]) for two-level random-effects models of resource selection by Rocky Mountain elk during calving time (15 May - 1 July), 2006–2009.

	Day		Night	
Effect/Year	**Inside**	**Outside**	**Inside**	**Outside**
Slope 2006	−0.001 (0.002)	−0.003 (0.034)	−0.064 (0.013)	-
Slope 2007	0.011 (0.006)	0.005 (0.013)	−0.030 (0.010)	−0.040 (0.016)
Slope 2008	−0.003 (0.007)	0.003 (0.003)	−0.035 (0.007)	−0.045 (0.008)
Slope 2009	0.012 (0.005)	0.024 (0.014)	−0.023 (0.011)	−0.027 (0.013)
Edge Density 2006	0.015 (0.016)	−0.022 (0.004)	−0.007 (0.021)	-
Edge Density 2007	0.001 (0.013)	−0.003 (0.012)	0.006 (0.011)	−0.013 (0.004)
Edge Density 2008	−0.026 (0.009)	−0.008 (0.003)	−0.026 (0.008)	−0.010 (0.002)
Edge Density 2009	−0.027 (0.009)	−0.006 (0.010)	−0.036 (0.012)	−0.044 (0.021)

- Model failed to converge.

doi:10.1371/journal.pone.0014597.t004

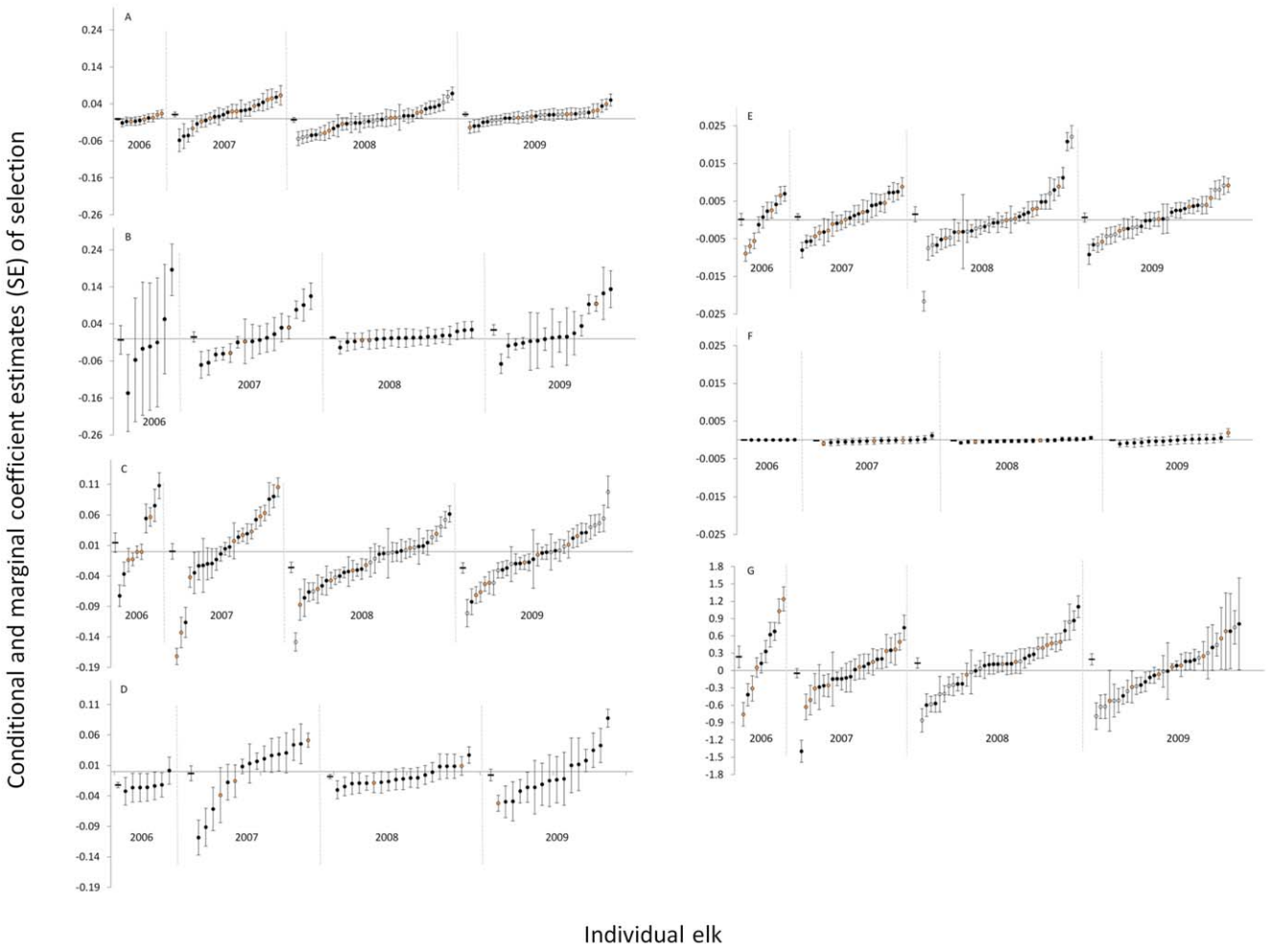


Figure 5. Population-level and individual responses. Marginal (dash) and conditional (circles) coefficient estimates (SE) of day-time selection for slope (a, b) edge density (c, d), elevation (e, f), and road density (g) by female elk during 2006-2009 in Raton basin, Colorado, USA. Results are displayed by year for elk that occupied developed (panels a, c, e, and g) and undeveloped (panels b, d, and f) areas. Orange depicts elk that were predicted to be parturient using generalized additive modeling; open circles (○) indicate conditional estimates for field-observed parturient elk in 2008 and 2009.

doi:10.1371/journal.pone.0014597.g005

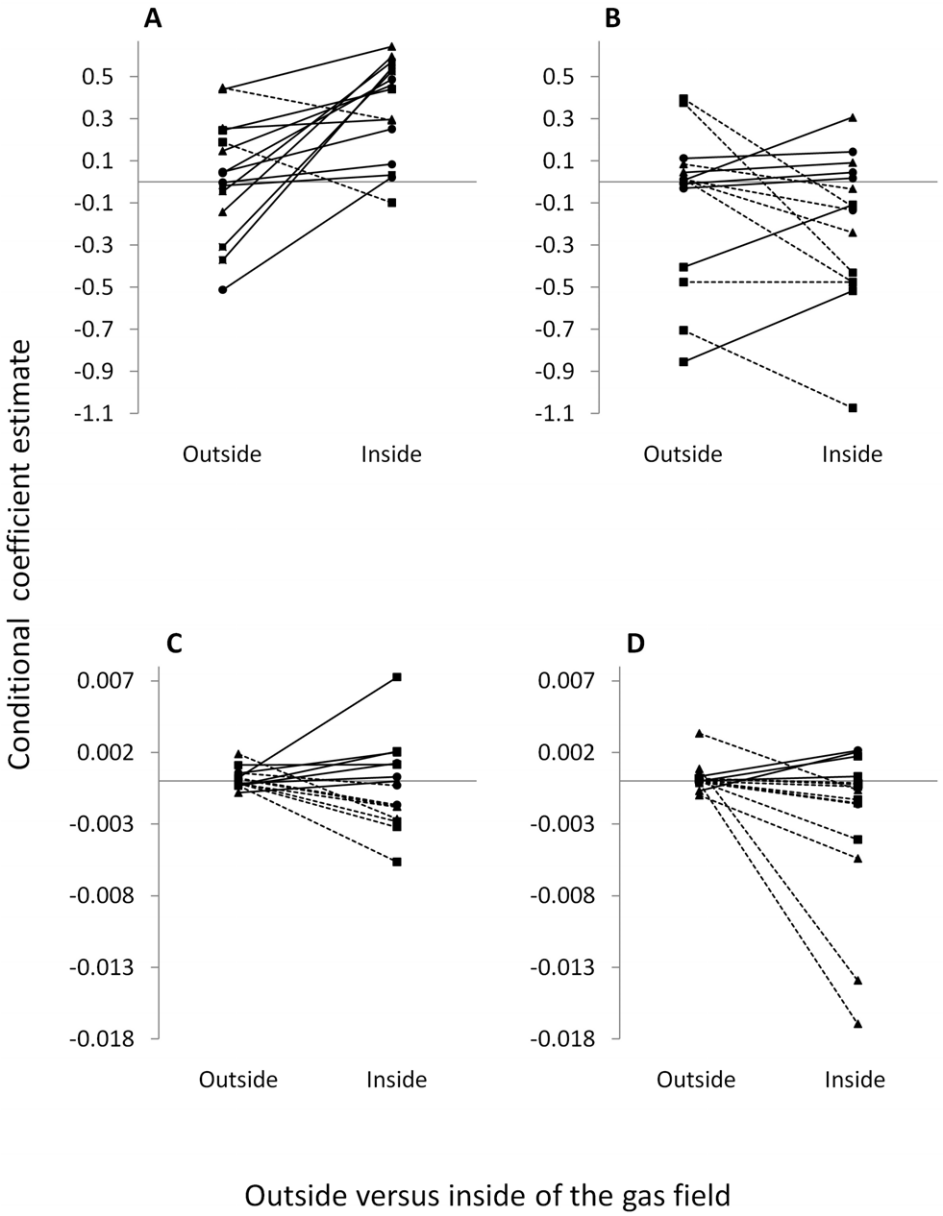


Figure 6. Movement from within the gas field to areas adjacent to the gas field. A subsample of female elk ($n = 15$) occupied areas within the gas field as well as areas outside but adjacent to the gas field. This figure shows differences in day-time (a) and night-time (b) selection for habitat that provides security cover, and day-time (c) and night-time (d) selection for elevation among these elk depending on whether they were inside versus outside of the gas field. Symbols represent conditional coefficient estimates (y-axes) for each elk in 2007 (squares; $n = 6$), 2008 (circles; $n = 4$), and 2009 (triangles; $n = 5$); lines show how selection changed within each elk depending on whether it was inside versus outside of the gas field. Solid lines depict larger selection coefficients inside the gas field whereas dashed lines depict larger coefficients outside of the gas field. Coefficients are informed by 36-293 location per elk inside the gas field, and 20-248 locations per elk outside the gas field.
doi:10.1371/journal.pone.0014597.g006

Functional Responses

Consistent with a functional response in resource selection, elk selected randomly for disturbed areas when disturbance was minimal. As human activity increased (*i.e.*, across individual elk calving season areas, or through time), elk showed stronger avoidance of the industrial development footprint during the day but not at night; this spatiotemporal pattern of avoidance revealed that elk continued to use physically disturbed areas but modified their behavior to avoid human activity (Figure 7a). One way elk avoided human activity in the day was by modifying selection for security cover. In developed areas elk were constrained to select resources near physical disturbance and, during the day, showed a stronger functional response to the proportion of cover than did elk in undeveloped areas (Figure 7b, c).

Mapping Responses

We developed six models (day and night separately for each of three groups): the first was based on all sample elk that occurred inside of the gas field; the second was a subset of the first group for which GAMs predicted females to be parturient; and the third was based on all sample elk that occurred outside of the gas field. We validated day and night models separately (1830 night locations

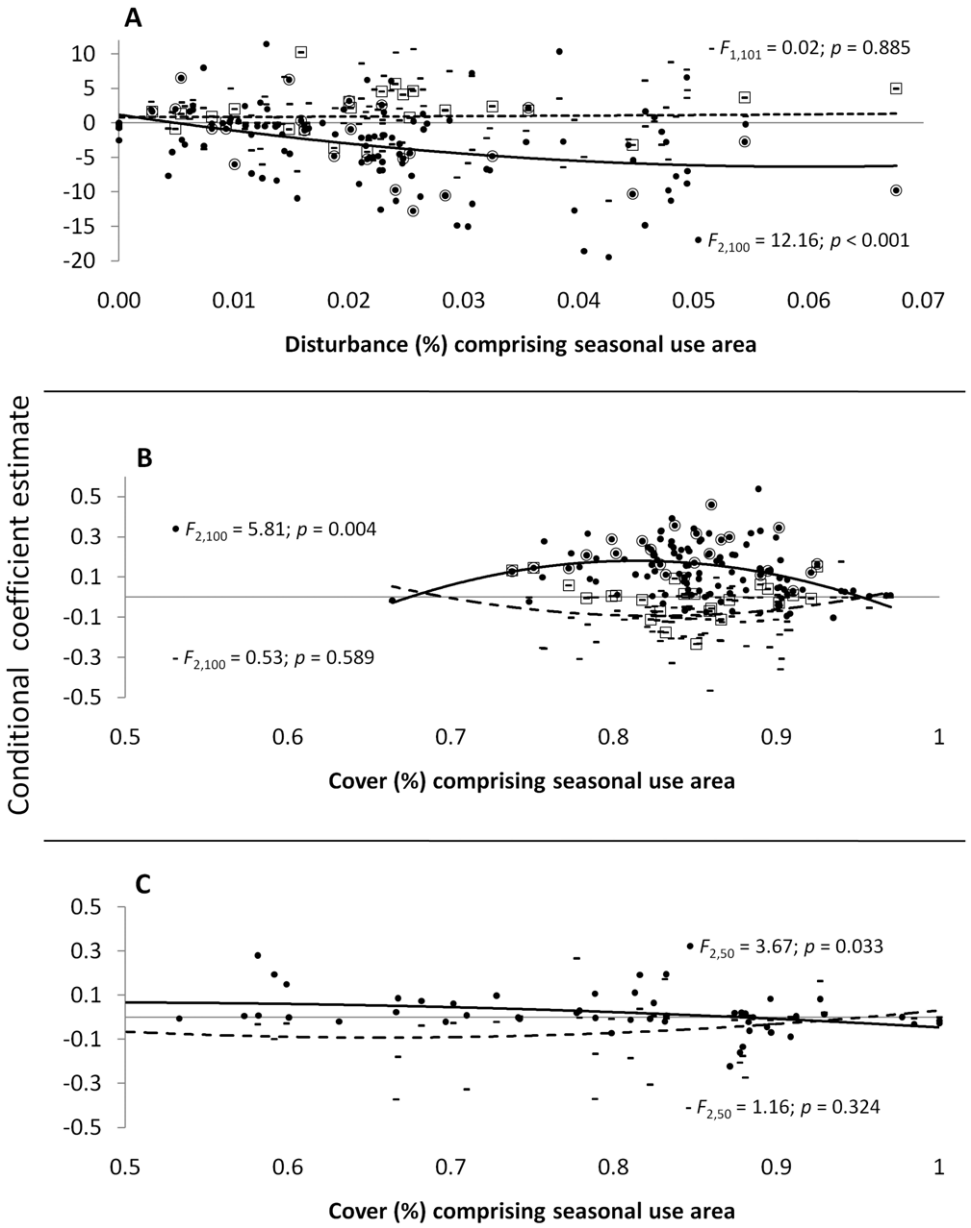


Figure 7. Functional responses in resource selection by female elk. Selection for the human disturbance footprint (a), security cover inside of the gas field (b), and security cover outside of the gas field (c) change as a function of availability. Availability was calculated at the seasonal use area level and conditional coefficients were estimated from generalized linear random-effects resource selection models. Dashes (-) and dashed lines symbolize night-time resource selection, and circles (•) and solid lines symbolize day-time resource selection. Dashes and circles outlined by open circles (○) or boxes (□) indicate conditional estimates for field-observed parturient elk in 2008 and 2009.
doi:10.1371/journal.pone.0014597.g007

and 3505 day locations) and then summed day and night values within the three groups of interest to estimate overall within-group model performance. For both models based on elk behavior inside of the gas field (Figure 8a), habitat with low predicted probability of use was modeled accurately; however, validation elk used habitat predicted to have high probability of use only as much as would be expected if elk used all predictive classes equally (Figure 8c). The model based on elk behavior outside of the gas field (Figure 8b) validated well with few locations occurring in lowest ranked areas and many locations occurring in highest ranked areas (Figure 8d). The proportion of pixels ranked differently between the model that validated most poorly and the model that validated best was 0.72–0.88 depending on year.

Discussion

We successfully integrated GAMs, field observation, and random-effects RSF modeling to designate parturiency, describe individual and population-level resource selection, and determine the relative influence of maternal status and human activity on the reliability of spatially explicit models intended to guide conservation of critical ungulate calving resources. We assumed that planning tools such as spatially explicit models would be more reliable if they account for adaptive behavior which, among parturient elk, should be reflected in movement and resource selection [45]. This was the basis for using GAMs to designate parturiency based on movement; our thinking was that ungulate

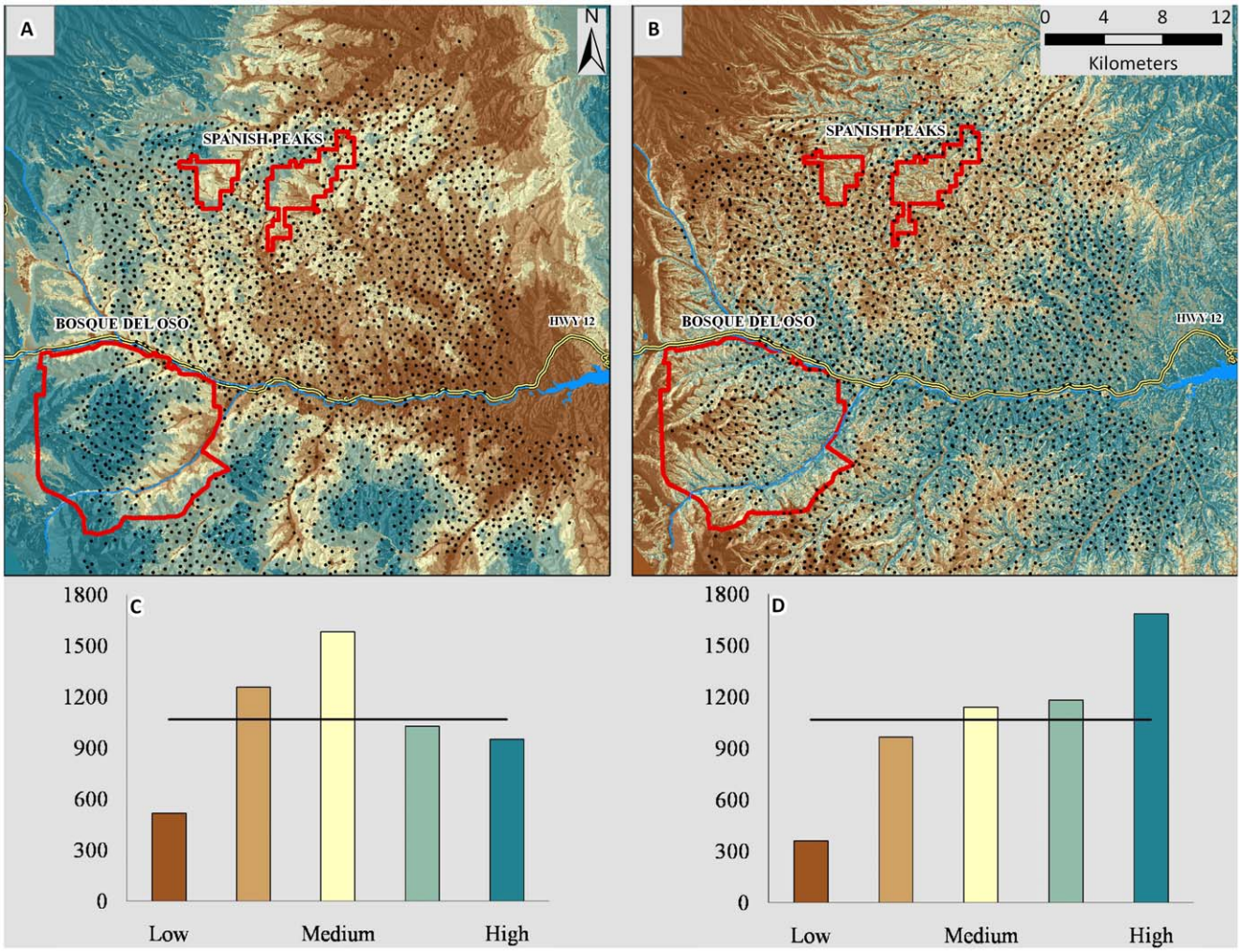


Figure 8. Assigning the relative probability of use throughout the landscape. Comparison of how the relative predicted probability of use was assigned throughout the landscape depending on whether RSF mapping was based on resource selection behavior among female elk that occupied a natural gas field (a) or occupied areas adjacent to but outside of a natural gas field (b). Maps depict day-time probability of use. Wells are depicted as black dots. Borders of wildlife areas managed by the state of Colorado are depicted in red. Probability of use is scaled from low to high with each of 5 ordinal bins representing quantiles of the total number of pixels (30-m resolution) comprising the area. Charts (c, d) display map validation with columns depicting the number of locations from an independent sample of elk that, when plotted on RSF maps (a, b), occurred within each ordinal bin. The black horizontal line depicts expectation if resources were selected at random. The map based on behavior inside of the gas field validated poorly (c; $\chi^2 = 577.46$, $df = 4$, $p < 0.001$) relative to the map based on behavior outside of the gas field (d; $\chi^2 = 849.18$, $df = 4$, $p < 0.001$) with the distribution of validation locations differing significantly from random in both cases. RSF maps (a, b) are based on all sample elk inside and outside of the gas field, respectively; not shown is the RSF map based on elk predicted to be parturient using generalized additive models.
doi:10.1371/journal.pone.0014597.g008

behavior contains information on reproductive status and GAMs would reveal a difference in the shape and relative complexity of the response curve between parturient and non-parturient females. Maternal status was evident in elk movement with our application of GAMs correctly predicting parturiency 93% of the time. We note, however, the GAM approach as presented would require further development if improved sensitivity (reduced false negative rate) is desirable. GAMs have been a standard tool in epidemiologic analyses and have found broad application in ecology for modeling tolerance thresholds and spatial distributions [46], [47], [48], [49]. Using movement data to designate functional groups, seasonality, or behavior in animals has increased in prevalence as GPS-based research has become more frequent [50], [51]. Although we found some potentially important differences in resource selection patterns between parturient and non-parturient elk (see below), behavior in these groups was more similar than expected. Had maternal status been more apparent in shaping resource selection, methods to designate status might have found better application in landscape-level mapping of the relative predicted probability of use. Here, both parturient and non-parturient elk were examined inside the gas field where any behavior distinctly associated with parturiency was obscured by apparent risk-aversive behavior related to human activity during the day time.

At the population-level parturient elk, while avoiding roads and selecting for security cover more strongly than other elk, conformed to a general pattern of avoiding human activity during the day by occupying upland forest or *Q. gambelii* thickets, and selecting forage resources in valley bottoms at night regardless of proximity to infrastructure. Trade-offs that structure resource selection in many ungulates, most notably between forage requirements and risk avoidance [6], [52], often are amplified in

parturient females through nutritional demands to support body condition, lactation, and neonatal defense [53]. Here, responses to human activity were more apparent than differences in resource selection as a function of parturiency with elk exhibiting a clear spatiotemporal avoidance of human activity at the population-level and modified patterns of selection for environmental features, notably security cover and elevation, in developed areas relative to undeveloped areas. Research has shown that ungulates exhibit avoidance behavior relative to human development, recreation, hunting, and other activities [54]. Modeling resource selection separately during day and night offered insight into whether the physical presence of infrastructure versus the operation and maintenance of such infrastructure (only occurring during the day) was more important in avoidance behavior. Elk inside the gas field used cover and elevation to modulate avoidance of human activity during the day [55] whereas cover and elevation were less influential outside of the gas field suggesting that human activity during the day was the factor to which elk were responding [3]. At night during calving time female elk showed no aversion to infrastructure and selected for areas characterized by valley-bottoms and foraging resources that in many instances were in close proximity to, or directly associated with, disturbances such as well pads and roads [56]. Day time refugia characterized by minimal human disturbance, security cover, and upper slope positions will be critical for maintenance of calving and perhaps other seasonal habitats in multiple-use landscapes.

To our knowledge, fully random-effects RSF models do not appear in the published literature. The random-effects framework provided insight into individual behavior and how elk modified patterns of calving season resource selection relative to development. Individuals that moved between developed and undeveloped areas (Figure 6) were spatially aware and showed marked changes in their behavior that were consistent with the hypothesis that human activity during the day was perceived as a source of risk [6]. An observation that warrants further attention in wildlife-human interaction studies is that heterogeneity among individuals in response to their environment was apparent, and this variation was amplified in developed areas relative to undeveloped areas. Associated with this observation was generally more precise estimation of selection within individuals in developed areas (except selection for elevation). Among-individual variation can comprise the majority of a population's niche width, and only when the within-individual component of total niche width is constrained does between-individual variation become prominent. Of particular relevance here is that trade-offs remain among the most plausible mechanisms for the observation of limited within-individual variation [18]. If ungulates in developed areas must make trade-offs associated with avoiding human activity, particularly during daylight hours, we might expect constrained within-individual variation and thus more heterogeneity among individuals. Human activity functioning to constrain decision-making in ungulates is consistent with the notion that risky environments impose pressures that disallow animals to respond to other features as they otherwise would [57]. This could make it difficult to establish or predict general patterns of resource selection during periods in which ungulates show fidelity to historic ranges, yet human activity rapidly modifies the landscape. In fact, we showed that RSF maps based on day-time resource selection behavior in developed areas had poor predictive accuracy.

The study of behavioral syndromes offers a relevant framework within which to discuss the conservation implications of individual variation in human-wildlife interaction studies [58]. A behavioral syndrome is a suite of correlated behaviors reflecting among-individual consistency across multiple contexts. Within a syndrome, individuals have a behavioral type such as risk-aversion (*i.e.*, more risk-averse versus less risk-averse types; [58]). The notion that individuals can be more or less risk aversive implies a limit to their range of behavioral plasticity. From a conservation perspective, animals exhibiting limited plasticity in environments undergoing rapid change, such as those affected by industrial development, may be less able to adapt. Elk clearly show some ability to adapt to human activity [59]. Nonetheless, if the effect of human activity is a threshold phenomenon [60], we might expect there to exist a limit of physical disturbance corresponding to a limit in the range of behavioral plasticity in ungulates, beyond which redistribution, social, or demographic effects may be observed [61], [62].

Examining only average responses across populations obscures variability among individuals that may have important implications for management or provide new ecological insight. For example, management strategies designed to conserve a resource that is important, on average, to the population may overlook resources that are critical to individuals that comprise a smaller demographic segment that functions disproportionately in population persistence. It has been stated "information on individual resource use is necessary if we are to make the transition from phenomenological models of population dynamics to mechanistic models in which the dynamics of a population are predicted from the properties of its components" [18]. Conditional estimates also provide information on why marginal estimates may be counter-intuitive, or how individuals assemble to comprise the marginal estimate. For example, the marginal estimate of selection for road density in our study indicated that elk selected for higher road density in 3 of the 4 years comprising the study period (Figure 5). This observation is counter to most research on roads and ungulates [63], [64]. Conditional estimates revealed that $\sim$1/3 of elk showed a positive association with higher road density but just as many avoided high road density; this observation sets the stage for examining potential links between a particular behavioral strategy such as road avoidance and demographic responses. The increase in the within-year proportion of elk occupying areas with higher road density from 2007 to 2009 (Figure 5d), concurrent with an increase in new road development, suggests that elk maintained relatively consistent calving season use areas during the study period, but modified their behavior as they became increasingly constrained to select resources in proximity to development. Such modification was consistent with a functional response in resource selection; elk response to human activity changed as a function of availability and with time of day. During the day when humans are active elk showed avoidance behavior that strengthened with increasing disturbance. This response spilled over to elk selection for security cover which appeared to have high importance to elk inside of the gas field as a day time refugium. Outside of the gas field elk showed relatively weak day time selection for cover and, as might be expected, selection weakened as the proportion of cover within the seasonal use area increased (*sensu* [40], Figure 7). These observations are consistent with the hypothesis that ungulates face a trade-off that is mediated by human activity in multiple-use landscapes – that is, the strength of the trade-off varies in direct relation to resource availability which is driven by human activity [41].

Spatially explicit models of relative predicted probability of use validated poorly when they were based on resource selection behavior inside of the gas field. The model based on behavior outside of the gas field validated well. To our knowledge, this treatment/control concept is a novel approach in RSF-based conservation planning. Typically, quantifying relative predicted probability of use in human-modified areas is based on animal

behavior in those areas. This approach has been used effectively; it has been shown that predictive maps based on mule deer (*Odocoileus hemionus*) behavior in human-modified areas validated well [3]. In their study [3], variation among individuals was present but limited relative to our findings. We suggest that ungulates in developed areas often respond to human activity in situation-specific ways. In our study, the local attributes of human activity varied in space and time within the seasonal use area of each elk making it difficult to generalize across the population. Situation-specific responses, including individual variation in the strength of selection or avoidance response, induced heterogeneity which complicated the application of models based on such behavior for conservation planning.

Protecting unmodified habitat in multiple-use landscapes typically is not an option [65]; therefore, measures to account for or reconcile changes in animal behavior across gradients of human-modification might be considered. If conservation objectives include establishing zones within such landscapes intended to function as refugia from human activity and promote long-term population persistence, information on resource selection patterns in existing refugia would be expected to provide valuable guidance in prioritizing the creation of new zones in modified landscapes. Establishing refugia based on resource selection patterns that reflect responses to human activity risks uncertainty in the performance of such refugia once human disturbance pressure is released allowing animals to respond to features as they otherwise would have [57]. In our study, models based on resource selection patterns that reflected responses to human activity classified the relative probability of use differently from the models based on behavior in the absence of industrial development throughout extensive portions of the landscape, potentially resulting in large errors in where conservation action would have its greatest impact. In population response research it is always desirable to include spatial and temporal controls (before-after, control-impact design), and demographic responses. Availability of such components is uncommon in large-scale wildlife-human interaction studies. In such situations the approach we describe, including efforts to account for possible adaptive behavior among reproductive groups, attention to the treatment/control concept, and a random-effects modeling framework, should have general application in human-wildlife interaction research particularly among species that inhabit places where human activity is intense, or among special status species for which little information on resource needs exists.

Acknowledgments

We thank Pioneer Natural Resources Company for supporting this work. We thank C.R. Anderson, C.J. Bishop, B.P. Dreher, V.J. Dreitz, P.M. Lukacs, and M.B. Rice, of the Colorado Division of Wildlife for providing insightful comments on an earlier version of this paper. We thank J.J. Cox and B. Augustine of the University of Kentucky Department of Forestry for providing comments on earlier versions. We thank landowners in Raton Basin who participated in this effort for their patience and ongoing enthusiasm for this work.

Author Contributions

Conceived and designed the experiments: MRD SMH RGO LDHW JBW. Performed the experiments: MRD SMH. Analyzed the data: MRD SMH SLW. Wrote the paper: MRD SMH SLW. Field observation: MRD SMH RGO JJW. Data collection: SMH RGO JJW.

References

1. Bowyer RT, Van Ballenberghe V, Kie JG, Maier JK (1999) Birth-site selection by Alaskan moose: maternal strategies for coping with a risky environment. Journal of Mammalogy 80: 1070–1083.
2. White PJ, Davis TL, Barnowe-Meyer KK, Crabtree RL, Garrott RA (2006) Partial migration and philopatry of Yellowstone pronghorn. Biological Conservation 135: 502–510.
3. Sawyer H, Kauffman MJ, Nielsen RM (2009) Influence of well pad activity on winter habitat selection patterns of mule deer. Journal of Wildlife Management 73: 1052–1061.
4. Hebblewhite M, Merrill E (2008) Modelling wildlife-human relationships for social species with mixed-effects resource selection models. Journal of Applied Ecology 45: 834–844.
5. Fortin D, Courtois R, Etcheverry P, Dussault C, Gingras A (2008) Winter selection of landscapes by woodland caribou: behavioral response to geographical gradients in habitat attributes. Journal of Applied Ecology 45: 1392–1400.
6. Frid A, Dill L (2002) Human-caused disturbance stimuli as a form of predation risk. Conservation Ecology 6: 11–25.
7. Beale CM, Monaghan P (2004) Human disturbance: people as predation-free predators? Journal of Applied Ecology 41: 335–343.
8. Creel S, Christianson D, Liley S, Winnie JA (2007) Predation risk affects reproductive physiology and demography of elk. Science 315: 960.
9. Manly BFJ, McDonald LL, Thomas DL, McDonald TL, Erickson WP (2002) Resource selection by animals: statistical design and analysis for field studies. Norwell: Kluwer Academic Publishers. 240 p.
10. Kittle AM, Fryxell JM, Desy GE, Hamr J (2008) The scale-dependent impact of wolf predation risk on resource selection by three sympatric ungulates. Oecologia 157: 163–175.
11. Fortin D, Fortin ME, Beyer HL, Duchesne T, Courant S, et al. (2009) Group-size-mediated habitat selection and group fusion-fission dynamics of bison under predation risk. Ecology 90: 2480–2490.
12. Singer FJ, Harting A, Symonds KK, Coughenour MB (1997) Density dependence, compensation, and environmental effects on calf mortality in Yellowstone National Park. Journal of Wildlife Management 61: 12–25.
13. Phillips GE, Alldredge AW (2000) Reproductive success of elk following disturbance by humans during calving season. Journal of Wildlife Management 64: 521–530.
14. Schaefer JA, Bergman CM, Luttich SN (2000) Site fidelity of female caribou at multiple spatial scales. Landscape Ecology 15: 731–739.
15. Bernardo J (1996) Maternal effects in animal ecology. Am Zool 36: 83–105.
16. Mousseau TA, Fox CW (1998) The adaptive significance of maternal effects. Trends in Ecology and Evolution 13: 403–407.
17. Blount JD, Surai PF, Nager RG, Houston DC, Møller AP, et al. (2002) Carotenoids and egg quality in the lesser black-backed gull *Larus fuscus*: a supplemental feeding study of maternal effects. Proceedings of the Royal Society B 269: 29–36.
18. Bolnick DI, Svanbäck R, Fordyce JA, Yang LH, Davis JM, et al. (2003) The ecology of individuals: incidence and implications of individual specialization. The American Naturalist 161: 1–28.
19. Austin D, Bowen WD, McMillan JI (2004) Intraspecific variation in movement patterns: modeling individual behavior in a large marine predator. Oikos 105: 15–30.
20. Thompson MJ, Henderson RE (1998) Elk habituation as a credibility challenge for wildlife professionals. Wildlife Society Bulletin 26: 477–483.
21. Estes JA, Riedman ML, Staedler MM, Tinker MT, Lyon BE (2003) Individual variation in prey selection by sea otters: patterns, causes, and implications. Journal of Animal Ecology 72: 144–155.
22. Gillespie RG, Caraco T (1987) Risk-sensitive foraging strategies of two spider populations. Ecology 68: 887–899.
23. Western Regional Climate Center (2010) Available: http://www.wrcc.dri.edu/coopmap/. Accessed 2010 Jan 14.
24. Hemborg HT (1998) Spanish Peak Field, Las Animas County, Colorado: Geologic setting and early development of a coalbed methane reservoir in the central Raton basin. Colorado Geological Survey Department of Natural Resources, Resources Series 33.
25. Vitt A (2007) Trinchera Data Analysis Unit E-33, Game Management Units 83, 85, 140, 851, Elk Management Plan. Colorado Division of Wildlife, Pueblo.
26. Quimby DC, Gaab JE (1957) Mandibular dentition as an age indicator in Rocky Mountain elk. Journal of Wildlife Management 21: 435–451.
27. Hastie TJ, Tibshirani RJ (1990) Generalized additive models. London: Chapman and Hall. 335 p.
28. Guisan A, Edwards Jr. TC, Hastie T (2002) Generalized linear and generalized additive models in studies of species distributions: setting the scene. Ecological Modelling 157: 89–100.
29. Beck N, Jackman S (1997) Getting the mean right is a good thing: generalized additive models. Society for Political Methodology Working Papers.
30. Fewster RM, Buckland ST, Siriwardena GM, Baillie SR, Wilson JD (2000) Analysis of population trends for farmland birds using generalized additive models. Ecology 81: 1970–1984.
31. Clutton-Brock TH, Guinness FE (1975) Behaviour of red deer (*Cervus elaphus* L.) at calving time. Behaviour 55: 287–299.
32. Cowie GM, Moore GH, Fisher MW, Taylor MJ (1985) Calving behavior of farmed red deer. Proceedings of a Deer Course for Veterinarians 2:1 43-154.

33. Hudson RJ, Haigh JC (2002) Physical and physiological adaptations. In: Toweill DE, Thomas JW, eds. North American elk: ecology and management. Washington DC: Smithsonian Institution Press. pp 199–257.
34. Vore JM, Schmidt EM (2001) Movements of female elk during the calving season in northwest Montana. Wildlife Society Bulletin 29: 720–725.
35. Poole KG, Serrouya R, Stuart-Smith K (2007) Moose calving strategies in interior montane ecosystems. Journal of Mammalogy 88: 139–150.
36. Long RA, Kie JG, Bowyer RT, Hurley MA (2009) Resource selection and movements by female mule deer *Odocoileus hemionus*: effects of reproductive stage. Wildlife Biology 15: 288–298.
37. Noyes JH, Sasser RG, Johnson BK, Byrant LD, Alexander B (1997) Accuracy of pregnancy detection by serum protein (PSPB) in elk. Wildlife Society Bulletin 25: 695–698.
38. Johnson DE (1951) Biology of the elk calf, *Cervus elaphus nelsoni*. Journal of Wildlife Management 15: 396–410.
39. Gillies CS, Hebblewhite M, Nielsen SE, Krawchuk MA, Aldridge C, et al. (2006) Application of random effects to the study of resource selection by animals. Journal of Animal Ecology 75: 887–898.
40. Mysterud A, Ims RA (1998) Functional responses in habitat use: availability influences relative use in trade-off situations. Ecology 79: 1435–1441.
41. Godvik IMR, Loe LE, Vik JO, Veiberg V, Langvatn R, et al. (2009) Temporal scales, trade-offs, and functional responses in red deer habitat selection. Ecology 90: 699–710.
42. Sawyer H, Nielson RM, Lindzey F, McDonald LL (2006) Winter habitat selection of mule deer before and during development of a natural gas field. Journal of Wildlife Management 70: 396–403.
43. Marzluff JM, Millspaugh JJ, Hurvitz P, Handcock MS (2004) Relating resources to a probabilistic measure of space use: forest fragments and Steller's jays. Ecology 85: 1411–1427.
44. Thomas DL, Johnson D, Griffith B (2006) A Bayesian random effects discrete-choice model for resource selection: population-level selection inference. Journal of Wildlife Management 70: 404–412.
45. Geist V (2002) Adaptive behavioral strategies. In: Toweill DE, Thomas JW, eds. North American elk: ecology and management. Washington DC: Smithsonian Institution Press. pp 389–433.
46. Dominici F, McDermott A, Zeger SL, Samet JM (2002) On the use of generalized additive models in time-series studies of air pollution and health. American Journal of Epidemiology 156: 193–203.
47. Yuan LL (2004) Assigning macroinvertebrate tolerance classifications using generalized additive models. Freshwater Biology 49: 662–677.
48. Potvin MJ, Drummer TD, Vucetich JA, Beyer Jr. DE, Peterson RO, et al. (2005) Monitoring and habitat analysis for wolves in upper Michigan. Journal of Wildlife Management 69: 1660–1669.
49. Winter AG, Swartzman GL (2006) Interannual changes in distribution of age-0 walleye Pollock near the Pribilof Islands, Alaska, with reference to the prediction of Pollock year-class strength. Journal of Marine Science 63: 1118–1135.
50. Franke A, Caelli T, Kuzyk G, Hudson RJ (2006) Prediction of wolf (*Canis lupus*) kill-sites using hidden Markov models. Ecological Modelling 197: 237–246.
51. Vander Wal E, Rodgers AR (2009) Designating seasonality using rate of movement. Journal of Wildlife Management 73: 1189–1196.
52. Bowyer RT, Kie JG, Van Ballenberghe V (1998) Habitat selection by neonatal black-tailed deer: climate, forage, or risk of predation? Journal of Mammalogy 79: 415–425.
53. Keech MA, Bowyer RT, Ver Hoef JM, Boertje RD, Dale BW, et al. (2000) Life-history consequences of maternal condition in Alaskan moose. Journal of Wildlife Management 64: 450–462.
54. Morrison JR, de Vergie WJ, Alldredge AW, Byrne AE, Andree WW (1995) The effects of ski area expansion on elk. Wildlife Society Bulletin 23: 481–489.
55. Edge WD, Marcum CL (1991) Topography ameliorates the effects of roads and human disturbance on elk. In: Christensen AG, Lyon LJ, Lonner TN, editors. Proceedings of a symposium on elk vulnerability. Bozeman: Montana State University. pp 132-137.
56. Anderson DP, Turner MG, Forester JD, Zhu J, Boyce MS, et al. (2005) Scale-dependent summer resource selection by reintroduced elk in Wisconsin, USA. Journal of Wildlife Management 69: 298–310.
57. Winnie Jr. J, Christianson D, Creel S, Maxwell B (2006) Elk decision-making is simplified in the presence of wolves. Behavioral Ecology and Sociobiology 61: 277–289.
58. Sih A, Bell A, Johnson JC (2004) Behavioral syndromes: and ecological and evolutionary overview. Trends in Ecology and Evolution 19: 372–378.
59. Edge WD, Marcum CL (1985) Movements of elk in relation to logging disturbances. Journal of Wildlife Management 49: 926–930.
60. Harju SM, Dzialak MR, Taylor RC, Hayden-Wing LD, Winstead JB (2010) Thresholds and time lags in the effects of energy development on greater sage-grouse populations. Journal of Wildlife Management 74: 437–448.
61. Cameron RD, Reed DJ, Dau JR, Smith WT (1992) Redistribution of calving caribou in response to oil field development on the Arctic slope of Alaska. Arctic 45: 338–342.
62. Manor R, Saltz D (2003) Impact of human nuisance disturbance on vigilance and group size of a social ungulate. Ecological Applications 13: 1830–1834.
63. Rowland MM, Wisdom MJ, Johnson BK, Kie JG (2000) Elk distribution and modeling in relation to roads. Journal of Wildlife Management 64: 672–684.
64. Friar JL, Merrill EH, Beyer HL, Morales JM (2008) Thresholds in landscape connectivity and mortality risks in response to growing road networks. Journal of Applied Ecology 45: 1504–1513.
65. Moilanen A, Franco AMA, Early RI, Fox R, Wintle B, Thomas CD (2005) Prioritizing multiple-use landscapes for conservation: methods for large multi-species planning problems. Proceedings of The Royal Society B 272: 1885–1891.

Available online at www.sciencedirect.com
ScienceDirect
Energy Procedia 1 (2009) 1297–1304
Energy Procedia
www.elsevier.com/locate/procedia

GHGT-9

Environmental impact of amines

Ingvild Eide-Haugmo[a] *, Odd Gunnar Brakstad[b], Karl Anders Hoff[b], Kristin Rist Sørheim[b], Eirik Falck da Silva[b], Hallvard F. Svendsen[a]

[a]*Norwegian University of Science and Technology, 7491 Trondheim, Norway*
[b]*SINTEF Materials and Chemistry, 7465 Trondheim, Norway*

Abstract

The process of post combustion CO_2-capture by chemical absorption relies on large scale use of chemicals, typically amines in aqueous solution. In such operations, emissions of amines may occur through the cleaned exhaust gas, as degraded solvent and as accidental spills. It is thus important that the chemicals used have low or no environmental effects. To check this, standard ecotoxicity and biodegradability tests for a marine environment were performed on more than 40 amines, including both solvents already in use for CO_2-removal and new promising chemicals. The results form a database for environmental risk assessment of common absorption solvents and will be used to correlate chemical structure of the solvents to degradation and toxicity data for use in solvent screening. Some of the solvents used for carbon capture, have been shown to have low biodegradability. The tertiary amines which have been tested do not degrade easily, while the amino acids tested both have low toxicity and degrade easily. The fate estimation model EPI Suite™ [US EPA, Washington DC, USA] has also been used to estimate the biodegradation and toxicity of the chemicals. It was compared to the experimental results to investigate if this tool could be used for future solvent screening. For the biodegradability the predictions showed agreement with 48% of the tested compounds, while for ecotoxicity the predictions showed agreement with 66%.

Keywords: amine; marine environment; biodegradability; degradation; toxicity; EPI Suite™; solvent;

* Corresponding author. Tel.:+47-7355-0346; fax: +47-7359-4080.
E-mail address: ingvild.eide.haugmo@chemeng.ntnu.no.

doi:10.1016/j.egypro.2009.01.170

1. Introduction

Amine based processes have been used commercially for removal of acid gas impurities from process gas stream, and is currently the most popular way to remove CO_2 in industry[1]. For natural gas sweetening operations, typically alkanolamines like monoethanolamine (MEA), diethanolamine (DEA), and N-methyldiethanolamine (MDEA), as well as mixtures of alkanolamines are used. Capture by absorption relies on large scale use of chemicals and emissions of the solvent may occur through the cleaned exhaust gas, as degraded solvent and as accidental spills. It is thus important that the chemicals used have low or no environmental effect. In this work, the main focus is on emissions to the marine environment.

An environmental assessment of the alkanolamines have been done by Davis and Carpenter [2], reviewing their potential for degradation as well as aquatic toxicology and bioconcentration potential. The alkanolamines were said to be highly susceptible to biodegradation and it was further stated that the toxicity was low for the majority of species. However, the assessment was based on soil and freshwater, not on the marine environment. Calamari *et al.*[3] conducted a series of biodegradation and ecotoxicity tests on 8 amines using three different inocula in aqueous solution, wherein two amines, tert-butylamine and morpholine, showed no degradation at all. The rest of the compounds - aniline, dimethylamine, diethylamine, di-n-butylamine, diisopropanolamine and cyclohexylamine – underwent degradation to some extent. Price *et al.* [4] performed biodegradation tests both in seawater and freshwater, however the bacterial content used in the tests was higher than that expected in the ocean. The amines tested were diethanolamine, diethylenetriamine, ethylenediamine and triethanolamine. In general the rate of degradation in the seawater biodegradation tests was found to be slower than in the freshwater system, and only the most degradable compounds from the freshwater system were tested. Diethylenetriamine was the only amine showing no degradation in freshwater, while ethylenediamine showed poor degradation in seawater.

2. Experimental

Two tests, a biodegradation and an ecotoxicity test, have been performed to determine the environmental fate of the chemicals in the marine environment. Biodegradation was determined by a marine biodegradation test, conducted according to the OECD guideline 306, “Biodegradability in seawater” [5]. The chemicals were diluted in normal seawater which had been aged for 3-5 days, and essential nutrients for the bacteria in the seawater were added. Seawater without chemicals was used for blank samples, and as a reference solution, aniline diluted in seawater was used. The solutions were distributed as duplicates in bottles with stoppers, and incubated in the dark for 28 days at 20 ± 2°C. A dissolved oxygen meter was used to measure the concentration of dissolved oxygen in solution: At the start of the experiment and after 5, 15 and 28 days bottles in each series with blank, test and reference solutions were removed for measurements. After measurment the bottles were discharged. The biological oxygen demand (BOD) was used as a measure for biodegradation. The BOD values were calculated as the difference in dissolved oxygen between solution with blank and test solutions, and the percentage of the theoretical oxygen demand (ThOD) was calculated. The method for estimating the theoretical oxygen demand is given in equation 1. Here c, h, cl, n, s, p, na and o are the numbers of carbon, hydrogen, chlorine, nitrogen, sulfur, phosphorous, sodium and oxygen atoms present, and MW is the molecular weight of the substance.

$$ThOD = \frac{16}{MW}\left[2c + \frac{1}{2}(h - cl - 3n) + 3s + \frac{5}{2}p + \frac{1}{2}na + o\right] \quad \text{Eq 1}$$

The ecotoxicity was determined by a marine phytoplankton test, conducted according to ISO/DIS guideline 10253, using the alga *Skeletonema Costatum* [6]. The algae were inoculated in a concentration series of the chemicals, with triplicates of each concentration. The choice of test concentrations was based on a preliminary test with ten-fold dilutions of the test substance. As controls, algae were also inoculated to algal medium without test substance. All test tubes were incubated with agitation under constant light intensity (60-120 μE/sec/m2) and a temperature of 20°C for 72 hours. *In vivo* chlorophyll fluorescence was measured daily in a fluorometer, as the algae performs photosynthesis and contains chlorophyll. Growth rate were calculated by linear regression analyses of the logarithmic of algal growth curves for each concentration of chemical and for controls.

From these parameters the effective concentrations (mg/l) of test substance inhibiting algal growth by 50 % (EC50) relative to the control cultures, were calculated by 95 % confidence interval using the program TOXEDO [7]. Based on the Convention for the Protection of the Marine Environment of the North-East Atlantic (OSPAR Convention), the Norwegian Pollution Control Authority (SFT) has a minimum recommended value for the marine biodegradation test of 20%, while for the marine phytoplankton test the minimum value is 10 mg/l [8].

EPI Suite™ is an environmental fate estimation model developed by the EPA's Office of Pollution Prevention Toxics and Syracuse Research Corporation (SRC)[9]. In Epi suite™ the aerobic linear prediction model in BIOWIN was used to estimate the probability of a chemical biodegrading in the environment, while the EC50 value for the 96 hour duration test with green algae in ECOSAR was used for predictions on ecotoxicity. BIOWIN uses a group-contribution model to asess the biodegradability of a compound; the methodology and description of the groups are discussed by Howard *et al.*[10]. In BIOWIN a chemical is classified as biodegrading fast, if the value calculated for the compound by the linear model is above 0,5. ECOSAR is based upon the octanol/water partition coefficient (Kow) of the compound, used together with constants based on the class of the compound (including aliphatic amine).

3. Results and discussion

Experimental results are shown in figure 1 for the biodegradation and figure 2 for ecotoxiciy. The results varied considerably in both biodegradation and ecotoxicity tests, even though almost all of the species tested are amines and alkanolamines. In the biodegradation test the results varied from < 1% to 100 % biodegradability (median 21.3 % biodegradability), while the *Skeletonema* test showed an EC_{50} range from 1.84 to > 10000 mg/L (median 198 mg/L). Risk is a combination of persistence and toxicity after discharge of a chemical to the environment. In terms of environmental risks, low biodegradation (persistance) contributed more than ecotoxicity. The ecotoxicity was mostly above the preferred value of 10 mg/l. From figure 1 it can be seen that some of the amines commonly used for capture (MDEA, AMP, Piperazine), would have long persistence in the marine environment due to their low degradability.

To check which functional groups of the chemical that influence the results, the chemicals were sorted according to amine type, and also according to the number of hydroxyl groups. Results are shown in figure 3 and 4, with biodegradability plotted against ecotoxicity, and significant variations in results were determined. All amino acids tested had a high biodegradability in combination with low toxicity, showing good environmental characteristics, and suggesting that this class of compounds were interesting from an environmental point of view. However, it should be taken into consideration that the tested amino acids are all known to be abundant in nature. Most of the tertiary amines tested had a low biodegradability, with the exception of N,N-dimethylethanolamine (DMMEA). Alkanolamines were shown to have toxicity between 10 and 1000 mg/l. It was also observed that all the amines having a quarternary carbon had low biodegradability, while the compounds with carbon chains of four were highly biodegradable. Compounds with ring structures typically showed the highest toxicity of the tested chemicals, with the exception of piperazine and 1-(2-hydroxyethyl)-piperazine.

The group contribution model discussed by Howard *et al.*[10] suggests that compounds containing quarternary carbons and tertiary amines in general are poorly biodegradable, while compounds with carbonchains of four are more easily degradable. It is also expected that primary and secondary amino and hydroxyl groups are more degradable than tertiary amines and compounds containing quarternary carbon. Calamari *et al.* [3] found that the amino group and the linear alkyl chains were usually degraded by bacteria, while branched alkyl chains and etheric linkage gave some resistance to degradation. Aniline, containing a benzene ring with no other substitutes than an amino group was easily degraded. Tert-butylamine, with a branched chain, and morpholine, with an epoxidic group, showed no degradation. Diisopropanolamine, which is less branched than tertbutylamine, was partially degraded by bacteria adapted to the compound. In comparison, tertbutylamine and morpholine were not biodegradable in our study, however, neither was diisopropanolamine. Dimethylamine was found to be biodegradable in our study as well as by Calamari *et al.* [3].

In the study by Price et al. [4] it was found that diethylentriamine was not degradable in freshwater, ethylenediamine was poorly degradable in seawater, while diethanolamine and triethanolamine were degradable both in freshwater and in seawater. With an exception for triethanolamine, which in our study is not found to degrade, the results are consistent with our study.

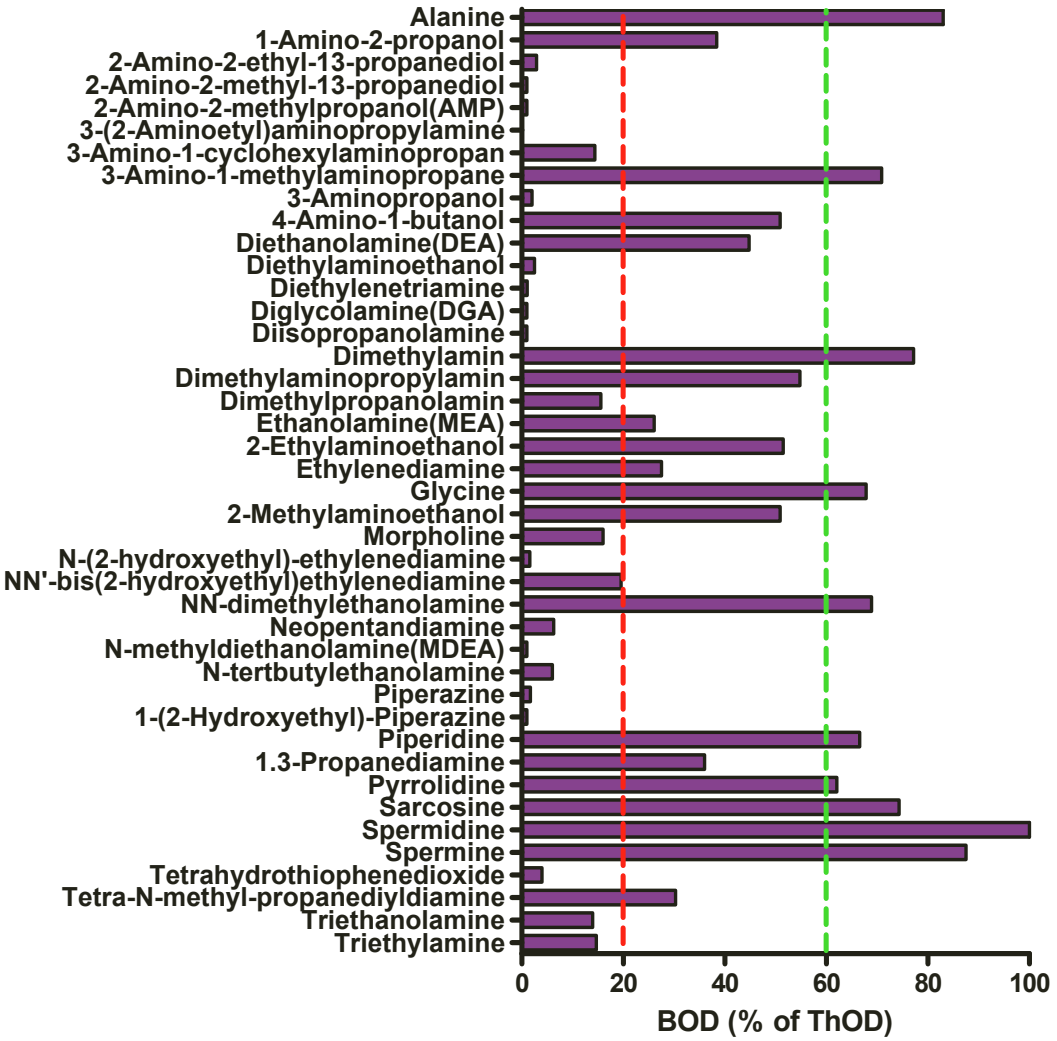


Figure 1: The biodegradability of all the chemicals tested, results shown as percent degraded with regards to the theoretical oxygen demand (ThOD). The red line shows the lowest acceptable value for a chemical to be released in the marine environment, while the green line is the lower limit for a chemical to be released independent of the ecotoxicity [8].

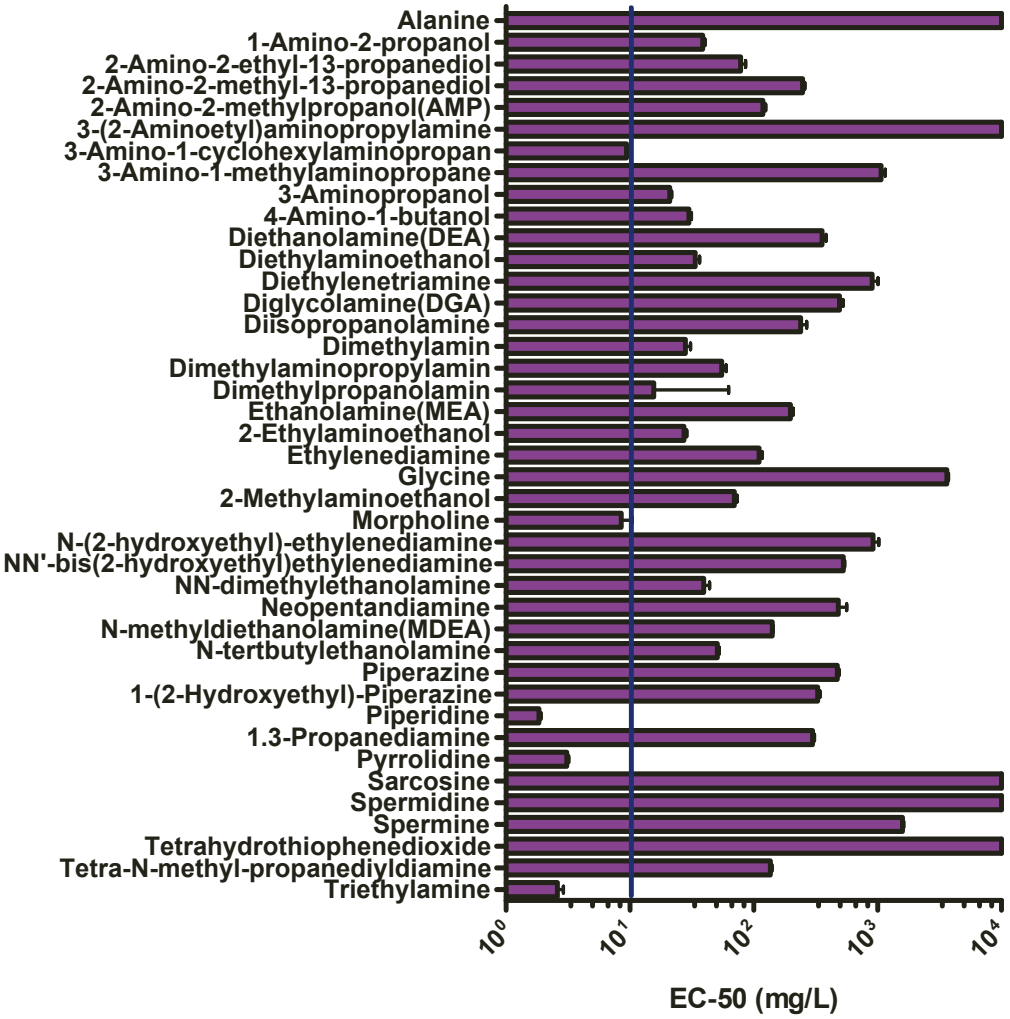


Figure 2: Results of the ecotoxicity testing, shown as concentration where compounds inhibited algal growth by 50% (EC-50). The blue line shows the lowest acceptable value (10 mg/l) for a chemical to be released in the marine environment [8].

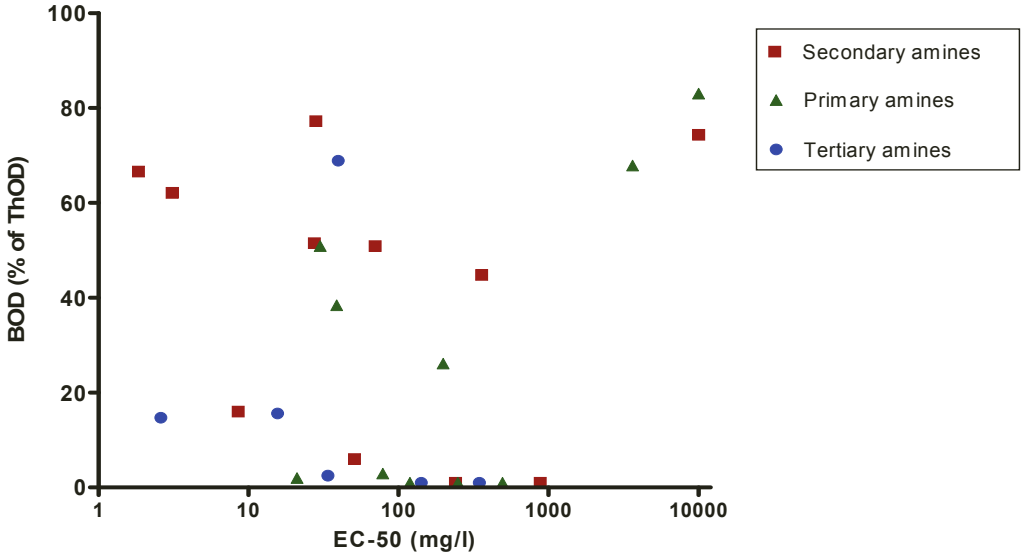


Figure 3: Biodegradability plotted against toxicity for the monoamines, sorted according to amine groups.

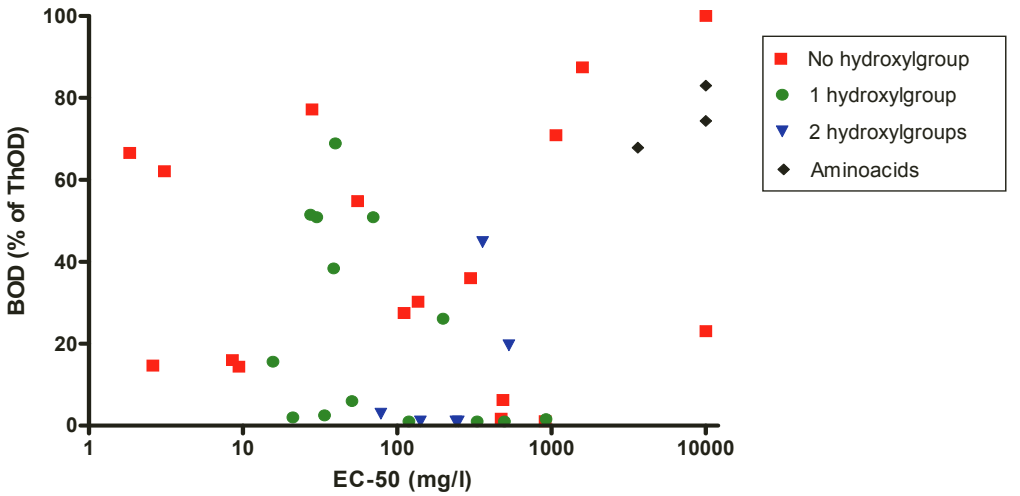


Figure 4: Biodegradability plotted against toxicity, sorted according to amino acid-group and the number of hydroxylgroups.

The predictions from EPI Suite™ compared with the test results are shown in figure 5 and 6. The linear prediction model for aerobic degradability in BIOWIN fails in classifying the non-biodegradable compounds. If the term "biodegrades fast" in BIOWIN is defined as an experimental BOD-value of 20% or more, 20 out of the 42 tested compounds were classified correctly. While the groups employed in the group-contribution model agree with the results obtained in this study, the data base which the model is based on does not include biodegradation tests with pure seawater. As the model is now, it is not suitable as a screening tool for marine biodegradation studies. For ecotoxicity the results were better than for the biodegradation, but there were still large deviations.

The reproducability of the results from the ecotoxicity test was within the limits 0-10, 10-100, 100-1000 and 1000-10000 mg/l. However, ECOSAR only predicted 27 out of 41 tested compound into the right category when using these limits. In general it seemed that the toxicity was over-predicted by the model. ECOSAR uses the octanol-water partition coefficient together with constants based on the class of chemical to calculate the predictions. Altough there seems to be a correlation between the octanol-water coefficient and toxicity, additional descriptors, or a different type of classification of the compounds, are required. It should also be taken into consideration that the test used in our studies are based on 72 hours exposure, not on 96 hours, and the type of algae used is different.

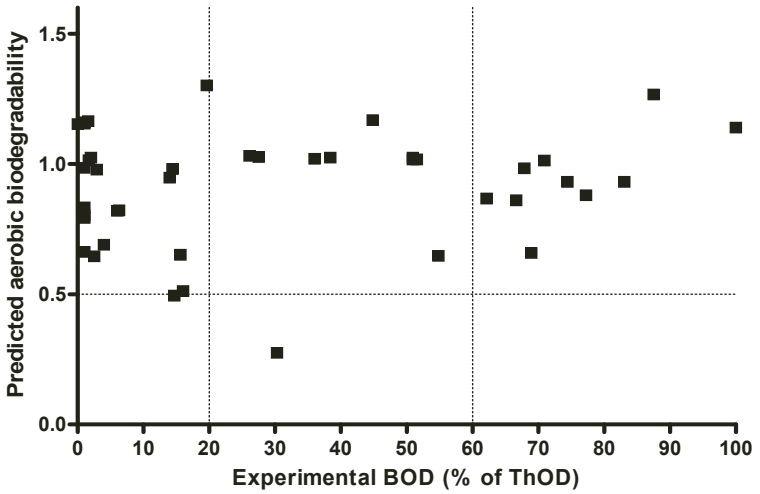


Figure 5: Predicted aerobic biodegradability by the linear model in BIOWIN, as a function of experimentally determined biodegradability (BOD 28). A chemical is classified as "biodegrades fast" by BIOWIN if the predicted value is above 0,5. For experimental BOD, a value above 60% means the chemical is defined as easily degraded, while a value above 20% is the minimum preferred value [8].

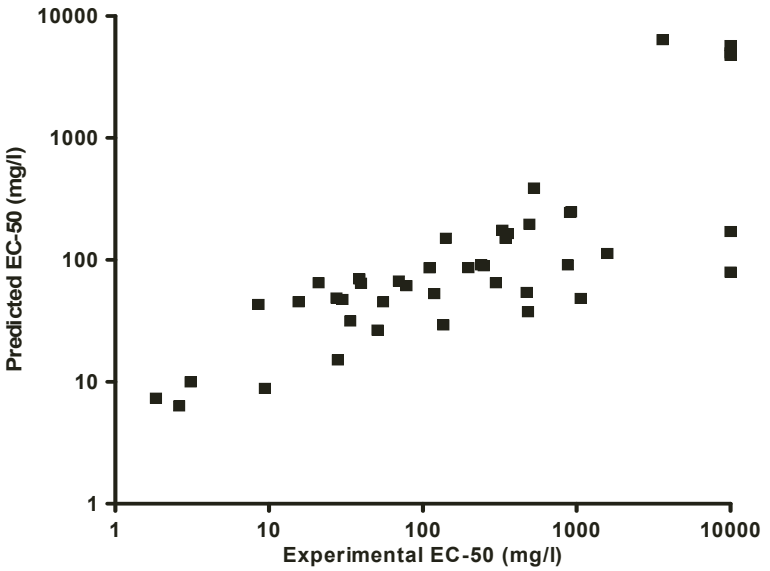


Figure 6: Predictet EC-50 values for green algae by ECOSAR as a function of experimentally determined EC-50 values for the algae *Skeletonema Costatum*.

4. Conclusion

Ecotoxicity and biodegradability in the marine environment have been tested for 41 and 42 compounds respectively. Some of the solvents commonly used for carbon capture, such as N-methyldiethanolamine (MDEA), piperazine and 2-amino-2-methylpropanol (AMP), were shown to have low biodegradability. The tested tertiary amines and compounds containing quarternary carbon did not degrade easily, while the aminoacids both show low toxicity and high biodegradation potential. EPI Suite™ was used to predict the biodegradability and ecotoxicity for the chemicals tested. For biodegradability the predictions only showed agreement with 48 %, while for the ecotoxicity the predictions showed agreement with 66 % of the tested compounds.

Acknowledgement

This publication forms a part of the REACT project, performed under the strategic Norwegian research program Petromaks. The authors acknowledge the partners: StatoilHydro, Shell and the Research Council of Norway (175918/S30) for their support.

References

1. S. Wong and Bioletti, R., *Carbon Dioxide Separation Technologies*. 2002, Carbon & Energy Managment, Alberta Research Council Inc.: Edmonton, Alberta.
2. J.W. Davis and Carpenter, C.L., Rev Environ Contam Toxicol. No.149 (1997) 87-137.
3. D. Calamari, et al., Chemosphere. No.9 (1980) 753-762.
4. K.S. Price, Waggy, G.T., and Conway, R.A., Journal (Water Pollution Control Federation). No.46 (1974) 63-77.
5. OECD guidelines for testing of chemicals no. 306, adopted by the Council 17.7.92: Biodegradability in Seawater.
6. ISO Guideline ISO/DIS 10253 "Water quality - Marine algae growth inhibition test with *Skeletonema costatum* and *Phaeodactylum tricornutum*".
7. VKI., *Program for statistical estimation of EC-values based on experimental data from ecotoxicological assays* in *TOXEDO ver. 1.2* . 1992.
8. Novatech, *Supplementary Guidance for the completing of Harmonised Offshore Chemical Notification Format (HOCNF) 2000 for Norwegian sector. Harmonised Offshore Chemical Notification Format OSPAR Recommendations 2000/5.* 2005.
9. US EPA *Estimation Programs Interface Suite™ for Microsoft® Windows, v[3.20]*. 2008; United States Environmental Protection Agency, Washington DC, USA. Available from: http://www.epa.gov/opptintr/exposure/pubs/episuite.htm [cited 2008 10. march]
10. P.H. Howard, et al., Environmental Toxicology and Chemistry. No.11 (1992) 593-603.

Biological Report 85(1.11)
May 1987

Contaminant Hazard Reviews
Report No. 11

POLYCYCLIC AROMATIC HYDROCARBON HAZARDS TO FISH, WILDLIFE, AND INVERTEBRATES: A SYNOPTIC REVIEW

by

Ronald Eisler

U.S. Fish and Wildlife Service
Patuxent Wildlife Research Center
Laurel, MD 20708

Fish and Wildlife Service
U.S. Department of the Interior

DISCLAIMER

Mention of trade names or commercial products does not constitute U.S. Government endorsement or recommendation for use.

This report should be cited as follows:

Eisler, R. 1987. Polycyclic aromatic hydrocarbon hazards to fish, wildlife, and invertebrates: a synoptic review. U.S. Fish Wildl. Serv. Biol. Rep. 85(1.11). 81 pp.

SUMMARY

This account synthesizes available technical literature on ecological and toxicological aspects of polycyclic aromatic hydrocarbons (PAHs) in the environment, with special reference to natural resources. Subtopics include: chemical properties, sources, and fate; background concentrations in biological and nonbiological samples; toxic and sublethal effects to flora and fauna; and proposed criteria and research needs for the protection of sensitive species.

PAHs consist of hydrogen and carbon arranged in the form of two or more fused benzene rings. There are thousands of PAH compounds, each differing in the number and position of aromatic rings, and in the position of substituents on the basic ring system. Environmental concern has focused on PAHs that range in molecular weight from 128.16 (naphthalene, 2-ring structure) to 300.36 (coronene, 7-ring structure). Unsubstituted lower molecular weight PAH compounds, containing 2 or 3 rings, exhibit significant acute toxicity and other adverse effects to some organisms, but are noncarcinogenic; the higher molecular weight PAHs, containing 4 to 7 rings, are significantly less toxic, but many of the 4- to 7-ring compounds are demonstrably carcinogenic, mutagenic, or teratogenic to a wide variety of organisms, including fish and other aquatic life, amphibians, birds, and mammals. In general, PAHs show little tendency to biomagnify in food chains, despite their high lipid solubility, probably because most PAHs are rapidly metabolized. Inter- and intraspecies responses to individual PAHs are quite variable, and are significantly modified by many inorganic and organic compounds, including other PAHs. Until these interaction effects are clarified, the results of single substance laboratory tests may be extremely difficult to apply to field situations of suspected PAH contamination.

PAHs are ubiquitous in nature--as evidenced by their detection in sediments, soils, air, surface waters, and plant and animal tissues--primarily as a result of natural processes such as forest fires, microbial synthesis, and volcanic activities. Anthropogenic activities associated with significant production of PAHs--leading, in some cases, to localized areas of high contamination--include high-temperature (>700 ^{o}C) pyrolysis of organic materials typical of some processes used in the iron and steel industry, heating and power generation, and petroleum refining. Aquatic environments may receive PAHs from accidental releases of petroleum and its products, from sewage effluents, and from other sources. Sediments heavily contaminated

with industrial PAH wastes have directly caused elevated PAH body burdens and increased frequency of liver neoplasia in fishes.

At present, no criteria or standards have been promulgated for PAHs by any regulatory agency for the protection of sensitive species of aquatic organisms or wildlife. This observation was not unexpected in view of the paucity of data on PAH background concentrations in wildlife and other natural resources, the absence of information on results of chronic oral feeding studies of PAH mixtures, the lack of a representative PAH mixture for test purposes, and the demonstrable--and, as yet, poorly understood--effects of biological and nonbiological modifiers on PAH toxicity and metabolism. By contrast, criteria for human health protection and total PAHs, carcinogenic PAHs, and benzo(a)pyrene have been proposed for drinking water and air, and for total PAHs and benzo(a)pyrene in food: drinking water, 0.01 to <0.2 ug total PAHs/l, <0.002 ug carcinogenic PAHs/l, and <0.0006 ug benzo(a)pyrene/l; air, <0.01 ug total PAHs/m^3, <0.002 ug carcinogenic PAHs/m^3, and <0.0005 ug benzo(a)pyrene/m^3; food, 1.6 to <16.0 ug total PAHs daily, and 0.16 to <1.6 ug benzo(a)pyrene daily. In view of the carcinogenic characteristics of many PAH compounds and their increasing concentrations in the environment, it now seems prudent to reduce or eliminate them wherever possible, pending acquisition of more definitive ecotoxicological data.

CONTENTS

TABLES

FIGURES

ACKNOWLEDGMENTS

I thank Jean Higgis and Julia Armstrong for secretarial help; Lynda J. Garrett, Nancy A. Bushby, and Joyce E. Haber for literature retrieval; Matthew C. Perry, Martha L. Gay, David J. Hoffman, and Paul C. Baumann for constructively reviewing the manuscript; and Curtis H. Halvorson and James R. Zuboy for editorial services.

INTRODUCTION

Several polycyclic aromatic hydrocarbons (PAHs) are among the most potent carcinogens known to exist, producing tumors in some organisms through single exposures to microgram quantities. PAHs act at both the site of application and at organs distant to the site of absorption; their effects have been demonstrated in nearly every tissue and species tested, regardless of the route of administration (Lee and Grant 1981). The evidence implicating PAHs as an inducer of cancerous and precancerous lesions is becoming overwhelming, and this class of substances is probably a major contributor to the recent increase in cancer rates reported for industrialized nations (Cooke and Dennis 1984). PAHs were the first compounds known to be associated with carcinogenesis (Lee and Grant 1981). Occupational skin cancer was first documented in London chimney sweeps in 1775 and in German coal tar workers in the late 1800's. By the early 1900's, soot, coal tar, and pitch were all found to be carcinogenic to humans. By 1918, it was shown that topical applications of coal tar produced skin tumors in mice and rabbits; benzo(a)pyrene, a PAH, was identified as one of the most carcinogenic compounds in coal tar (Dipple 1985). The carcinogenic activity to man of soots, tars, and oils is beyond dispute. In addition to the skin cancers noted initially, higher incidences of respiratory tract and upper gastrointestinal tract tumors were associated with occupational exposures to these carcinogens (Dipple 1985). PAH-induced cancers in laboratory animals is well documented. Benzo(a)pyrene, for example, has produced tumors in mice, rats, hamsters, guinea pigs, rabbits, ducks, and monkeys following administration by oral, dermal, and intraperitoneal routes (Pucknat 1981). Teratogenic or carcinogenic responses have been induced in sponges, planarians, echinoderm larvae, teleosts, amphibians, and plants by exposure to carcinogenic PAHs (Neff 1979, 1982b). An unusually high prevalence of oral, dermal, and hepatic neoplasms have been observed in bottom-dwelling fish from polluted sediments containing grossly-elevated PAH levels (Couch and Harshbarger 1985). PAH compounds have damaged chromosomes in cytogenetic tests, have produced mutations in mammalian cell culture systems, and have induced DNA repair synthesis in human fibroblast cultures (EPA 1980). While some PAHs are potent mutagens and carcinogens, others are less active or suspected carcinogens. Some, especially those of biological origin, are probably not carcinogens (Jackim and Lake 1978). Certain lower molecular weight, noncarcinogenic PAHs, at environmentally realistic levels, were acutely toxic to aquatic organisms, or produced deleterious sublethal

responses (Neff 1985). However, few generalizations can be made about the class of PAH compounds because of the extreme variability in toxicity and physicochemical properties of PAHs and their various effects on individual species (Lee and Grant 1981).

PAHs are widely-distributed in the environment, almost ubiquitous, and have been detected in animal and plant tissues, sediments, soils, air, surface water, drinking water, industrial effluents, ambient river water, well water, and groundwater (EPA 1980). Man probably has always been exposed to PAHs from the natural background level in soils and plants (Harrison et al. 1975); avoiding exposure to nanogram quantities of these substances on a daily basis is now considered essentially impossible for all living resources (Dipple 1985). Ever since benzo(a)pyrene was recognized as a carcinogen at the beginning of this century, the presence of it and of other PAHs in the environment has received continuous attention. As one consequence, many reviews have been published on ecological and toxicological aspects of PAH in the environment, with special reference to their carcinogenic properties.[1]

In this report, I summarize selected data on environmental aspects of PAHs, emphasizing PAH effects to aquatic and wildlife resources. This brief review is part of a continuing series prepared in response to informational requests from environmental specialists of the U. S. Fish and Wildlife Service.

---

[1] Harrison et al. (1975); Barnett (1976); Suess (1976); Gelboin and Ts'o (1978a, 1978b, 1981); Jackim and Lake (1978); Jones and Freudenthal (1978); Lo and Sandi (1978); Jones and Leber (1979); Neff (1979, 1982a, 1982b, 1985); Tsang and Griffin (1979); Bjorseth and Dennis (1980); EPA (1980); Cooke and Dennis (1981, 1983, 1984); Futoma et al. (1981); Lee and Grant (1981); Pucknat (1981); Sims and Grover (1981); Stegemen (1981); Cooke et al. (1982); Richards and Jackson (1982); Couch et al. (1983); Edwards (1983); Grimmer (1983); Quaghebeur et al. (1983); Sims and Overcash (1983); Couch and Harshbarger (1985); Harvey (1985); Johnson et al. (1985); Sugimura (1986).

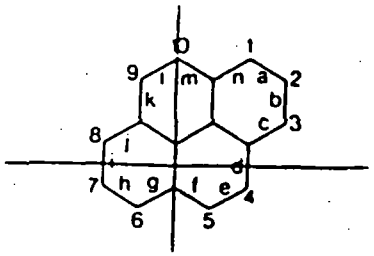
ENVIRONMENTAL CHEMISTRY, SOURCES, AND FATE

PROPERTIES

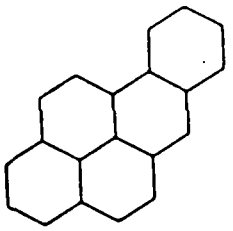
Polycyclic aromatic hydrocarbons (PAHs), also known as polynuclear aromatic hydrocarbons (PNAs) and polycyclic organic matter (POM), are composed of hydrogen and carbon arranged in the form of two or more fused benzene rings in linear, angular, or cluster arrangements, which may or may not have substituted groups attached to one or more rings (Sims and Overcash 1983). In some cases, the newly defined substituted PAH has strikingly greater toxicological effects than does the parent compound (Cooke and Dennis 1984). The nomenclature of PAH compounds has been ambiguous in the past due to different peripheral numbering systems. The currently accepted nomenclature is shown in Figure 1.

Of major environmental concern are mobile PAHs that vary in molecular weight from 128.16 (naphthalene, $C_{10}H_8$) to 300.36 (coronene, $C_{24}H_{12}$). Higher molecular weight PAHs are relatively immobile because of their large molecular volumes and their extremely low volatility and solubility. Among the mobile forms are thousands of compounds that differ in the number and position of aromatic rings, and in the position of substituents on the basic ring system. The lower molecular weight unsubstituted PAH compounds, containing 2 to 3 rings, such as naphthalenes, fluorenes, phenanthrenes, and anthracenes (Figure 2), have significant acute toxicity to some organisms, whereas the higher molecular weight 4- to 7-ring aromatics do not. However, all known PAH carcinogens, cocarcinogens, and tumor producers are in the high molecular weight PAH group (Figure 3).

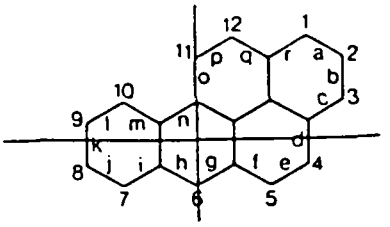
Physical and chemical characteristics of PAHs generally vary with molecular weight. With increasing molecular weight, aqueous solubility decreases, and melting point, boiling point, and the log Kow (octanol/water partition coefficient) increases (Table 1), suggesting increased solubility in fats, a decrease in resistance to oxidation and reduction, and a decrease in vapor pressure. Accordingly, PAHs of different molecular weight vary substantially in their behavior and distribution in the environment and in



1



2



3

Figure 1. Nomenclature of PAHs (modified from Lee and Grant 1981, and Grimmer 1983). The PAH formula is oriented so that the greatest number of rings are in a horizontal row and a maximum number of rings are above and to the right of the horizontal row. The first carbon atom that belongs to the uppermost ring and is not engaged in ring fusion with another ring is given the number C-1; numbering continues in a clockwise direction omitting those carbon atoms which do not carry a hydrogen atom. The bond between C-1 and C-2 is designated as side "a"; other peripheral sides continue in clockwise direction in alphabetical order. Examples are: (1) pyrene (correctly oriented, numbered, and lettered), (2) benzo(a)pyrene (not oriented correctly), (3) benzo(a)pyrene (correctly oriented, numbered, and lettered).

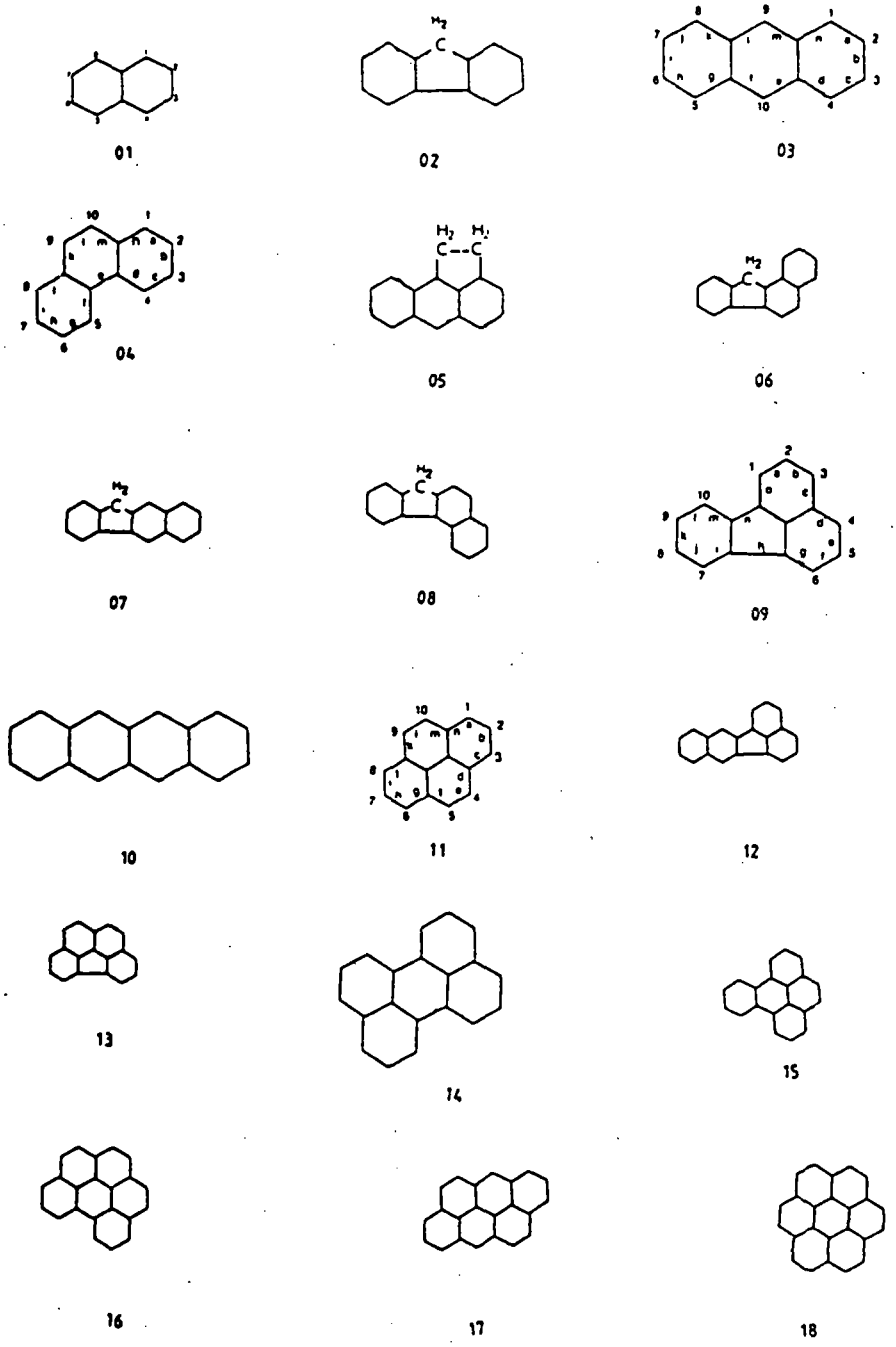


Figure 2. Ring structures of representative noncarcinogenic PAHs (modified from Lee and Grant 1981, and Neff 1985). The numbering and lettering system for several PAHs is also given. Compounds are: (1) naphthalene, (2) fluorene, (3) anthracene, (4) phenanthrene, (5) aceanthrylene, (6) benzo(a)fluorene, (7) benzo(b)fluorene, (8) benzo(c)fluorene, (9) fluoranthene, (10) naphthacene, (11) pyrene, (12) benzo(k)fluoranthene, (13) benzo(g,h,i)fluoranthene, (14) perylene, (15) benzo(e)pyrene, (16) benzo(g,h,i)perylene, (17) anthanthrene, (18) coronene.

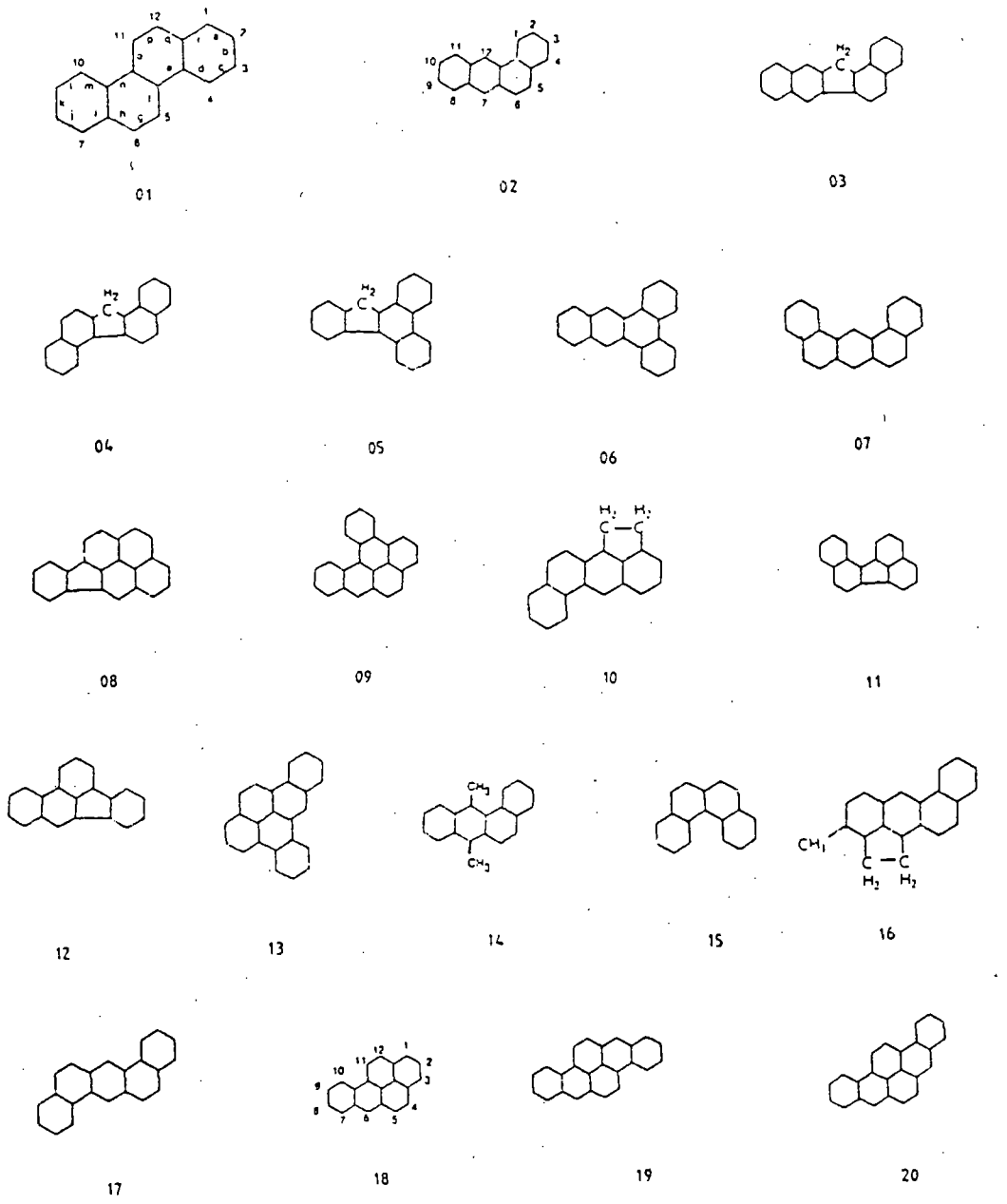


Figure 3. Ring structures of representative tumorogenic, cocarcinogenic, and carcinogenic PAHs (modified from Lee and Grant 1981). The numbering and lettering system for several PAHs is also given. Compounds are: (1) chrysene, (2) benz(a)anthracene, (3) dibenzo(a,h)fluorene, (4) dibenzo(a,g)fluorene, (5) dibenzo(a,c)fluorene, (6) dibenz(a,c)anthracene, (7) dibenz(a,j)anthracene, (8) indeno (1,2,3-cd) pyrene, (9) dibenzo(a,l)pyrene, (10) cholanthrene, (11) benzo(j)fluoranthene, (12) benzo(b)fluoranthene, (13) dibenzo(a,e)pyrene, (14) dimethylbenz(a)anthracene, (15) benzo(c)phenanthrene, (16) 3-methylcholanthrene, (17) dibenz(a,h)anthracene, (18) benzo(a)pyrene, (19) dibenzo(a,h)pyrene, (20) dibenzo(a,i)pyrene. Compounds 1 to 9 are weakly carcinogenic, cocarcinogenic, or tumorogenic; compounds 10 to 13 are carcinogenic; compounds 14 to 20 are strongly carcinogenic.

Table 1. Some physical and chemical properties of selected PAHs.

Compound	Number of rings	Approximate molecular weight	Melting point (°C)	Solubility in water (mg/l)	log Kow
Naphthalene	2	128	80	30.0	3.37
Anthracene	3	178	216	0.07	4.45
Benz(a)anthracene	4	228	158	0.014	5.61
Benzo(a)pyrene	5	252	179	0.0038	6.04
Benzo(g,h,i)perylene	6	276	222	0.00026	7.23

their biological effects. Additional and more comprehensive data on the physical and chemical properties of PAHs are given in Barnett (1976), Lo and Sandi (1978), Neff (1979, 1985), EPA (1980), Futoma et al. (1981), Lee and Grant (1981), Pucknat (1981), Edwards (1983), Grimmer (1983), Sims and Overcash (1983), and Whitehouse (1985).

SOURCES

About 43,000 metric tons of PAHs are discharged into the atmosphere each year, and another 230,000 tons enter aquatic environments (Table 2). PAHs are ubiquitous in nature as a consequence of synthesis in terrestrial vegetation, microbial synthesis, and volcanic activity, but quantities formed by these natural processes are small in comparison with those produced from forest and prairie fires and anthropogenic sources (Barnett 1976; Suess 1976; Lo and Sandi 1978; Neff 1979, 1985; EPA 1980; Lee and Grant 1981; Pucknat 1981; Edwards 1983; Grimmer 1983; Sims and Overcash 1983). Anthropogenic activities associated with significant production of PAHs include: coke production in the iron and steel industry; catalytic cracking in the petroleum industry; the manufacture of carbon black, coal tar pitch, and asphalt; heating and power generation; controlled refuse incineration; open burning; and emissions from internal combustion engines used in transportation. Thus, the formation of PAHs in the environment is due to an endogenous synthesis by microorganisms,

Table 2. Major sources of PAHs in atmospheric and aquatic environments (modified from Lo and Sandi 1978; Neff 1979; Edwards 1983; Sims and Overcash 1983).

Ecosystem and sources	Annual input, in metric tons
ATMOSPHERE	
Total PAHs	
Forest and prairie fires	19,513
Agricultural burning	13,009
Refuse burning	4,769
Enclosed incineration	3,902
Heating and power	2,168
Benzo(a)pyrene	
Heating and power	
Worldwide	2,604
USA only	475
Industrial processes (mostly coke production)	
Worldwide	1,045
USA only	198
Refuse and open burning	
Worldwide	1,350
USA only	588
Motor vehicles	
Worldwide	45
USA only	22
AQUATIC ENVIRONMENTS	
Total PAHs	
Petroleum spillage	170,000
Atmospheric deposition	50,000
Wastewaters	4,400
Surface land runoff	2,940
Biosynthesis	2,700
Total benzo(a)pyrene	700

algae, and macrophytes which provide natural background, and to a second process which is connected to man-controlled high-temperature (>700 °C) pyrolysis of organic materials, to open burning, and to natural volcanic activities. The discovery in fossil fuels of complex mixtures of PAHs spanning a wide range of molecular weights has led to the conclusion that, given sufficient time (i.e., millions of years), pyrolysis of organic materials at temperatures as low as 100 to 150 °C can also lead to production of PAHs (Neff 1985).

Forest and prairie fires release much greater amounts of PAHs to the atmosphere than does fossil fuel burning. Nearly all of the airborne PAHs produced by flame pyrolysis are associated with the particulate fraction produced during combustion, and these are significantly modified by the chemical composition of the fuel, the pyrolysis temperature, the duration of exposure to elevated temperatures, and to other factors (Neff 1979; Edwards 1983). In one study, a PAH profile was established for a series of laboratory fires simulating the prescribed burning of pine needle litter (McMahon and Tsoukalas 1978). Heading fires (moving with wind) produced more total particulate matter than backing fires (moving against wind), but backing fires produced significantly higher amounts of PAHs, with the actual amounts formed dependent on fuel loading and the residence time of combustible gases in the burning zone. Emission factors for benzo(a)pyrene varied from 238 to 3,454 ug/kg in backing fires and 38 to 97 ug/kg in heading fires.

PAHs present in the atmosphere enter rain as a result of in-cloud and below-cloud scavenging (van Noort and Wondergem 1985). Total PAHs deposited on land and water are almost equivalent to PAH content in rainfall; significant quantities of PAHs are found in presumed pollution-free areas, indicating the importance of rain in transport and distribution of PAHs (Quaghebeur et al. 1983).

PAHs may reach aquatic environments in domestic and industrial sewage effluents, in surface runoff from land, from deposition of airborne particulates, and especially from spillage of petroleum and petroleum products into water bodies (Jackim and Lake 1978; Lake et al. 1979; Neff 1979; EPA 1980; Martens 1982; Boehm and Farrington 1984; Hoffman et al. 1984; Prahl et al. 1984). The majority of PAHs entering aquatic environments remains close to sites of deposition, suggesting that lakes, rivers, estuaries, and coastal marine environments near centers of human populations are the primary repositories of aquatic PAHs (Neff 1979). Large variations in aquatic PAH contents were evident due to localized source inputs and physicochemical conditions. For example, urban runoff from stormwater and highways to Narragansett Bay, Rhode Island, accounted for 71% of the total inputs for higher molecular weight PAHs, and 36% of the total PAHs (Hoffman et al. 1984). More than 30% of all combustion-derived PAHs in coastal sediments of Washington State is supplied by riverine transport of suspended particulate materials, while direct atmospheric input accounts for a maximum of 10% (Prahl et al. 1984). In contrast, concentrations of PAHs in sediments from the

vicinity of Georges Bank, off the US northeastern coast, varied from 1 to 100 ug/kg dry weight, and were directly related to total organic carbon, silt, and clay contents in sediments; combustion-derived PAHs dominated at the higher concentrations, while lower levels were often associated with a fossil fuel origin (Boehm and Farrington 1984).

Discharge water from hydrostatic testing of natural gas pipelines is a significant source of PAH loading into aquatic environments, contributing as much as 32,000 ug PAHs/l of discharge water, mostly as naphthalenes (Eiceman et al. 1984). More than 25 PAHs, primarily anthracenes and pyrenes, were detected in pipeline residues on inner walls of natural gas pipelines at concentrations up to 2,400 ug/m^2 of inner surface; the same compounds may be reasonably expected in aqueous wastes from pipeline maintenance (Eiceman et al. 1985). Release of these, or similar, discharge waters directly into aquatic environments will result in contamination similar to that caused by oil spills; however, these sites for pollution may occur in locations far distant from oil production and refinery activities (Eiceman et al. 1984). PAHs are also present in tap water at concentrations of 0.1 to 1.0 ng/l, primarily as mono- and dichlorinated derivitives of naphthalene, phenanthrene, fluorene, and fluoranthene (Shiraishi et al. 1985). The presence of PAHs and chlorinated PAHs in tap water indicates the reaction of PAHs with chlorine; however, their significance to human health and to aquatic biota is unknown.

FATE

Concern about PAHs in the environment is due to their persistence and to the fact that some are known to be potent mammalian carcinogens, although environmental effects of most noncarcinogenic PAHs are poorly understood (Neff 1985). Prior to 1900, a natural balance existed between the production and the degradation of PAHs. Synthesis of PAHs by microorganisms and volcanic activity and production by man-made high temperature pyrolytic reactions and open burning seemed to be balanced by PAH destruction via photodegradation and microbial transformation. With increased industrial development and increased emphasis of fossil fuels as energy sources, the balance has been disturbed to the extent that PAH production and introduction into the environment greatly exceeds known PAH removal processes (Suess 1976; Sims and Overcash 1983).

When released into the atmosphere, PAH compounds will become associated with particulate materials. Their residence time in the atmosphere and transport to different geographic locations are governed by particle size, meteorological conditions, and atmospheric physics. The highly reactive PAHs photodecompose readily in the atmosphere by reaction with ozone and various oxidants; degradation times range from several days to six weeks for PAHs adsorbed onto particulates <1 um in diameter (in the absence of rainfall) to <1 day to several days for those adsorbed to larger particles (Suess 1976).

Smaller atmospheric particulates containing PAHs are easily inhaled (Lee and Grant 1981), and may pose special problems, as yet unevaluated, for airborne organisms such as birds, insects, and bats. Photooxidation, one of the most important processes in the removal of PAHs from the atmosphere, can also produce reaction products that are carcinogenic or mutagenic, although little is known of their persistence (Edwards 1983). One of the more common photooxidation reactions of PAHs is the formation of endoperoxides that ultimately undergo a series of reactions to form quinones (Edwards 1983). Various parameters may modify chemical and photochemical transformation of PAHs in the atmosphere, including light intensity, concentration of gaseous pollutants (O_3, NO_x, SO_x), and chemicophysical characteristics of particulates or substrates into which the PAHs are adsorbed; depending on these variables, the half-life of benzo(a)pyrene in the atmosphere varies from 10 minutes to 72 days (Valerio et al. 1984). Atmospheric PAHs are transported over relatively long distances from industrial areas and from natural forest and prairie fires (Edwards 1983); however, sites nearer urban centers have much higher PAH deposition rates than more rural areas (Hites and Gschwend 1982).

Much of the PAHs released into the atmosphere eventually reaches the soil by direct deposition or by deposition on vegetation. The PAHs may be adsorbed or assimilated by plant leaves before entering the animal food chain, although some adsorbed PAHs may be washed off by rain, chemically oxidized to other products, or returned to the soil as the plants decay. PAHs assimilated by vegetation may be translocated, metabolized, and possibly photodegraded within the plant. In some plants growing in highly contaminated areas, assimilation may exceed metabolism and degradation, resulting in an accumulation in plant tissues (Edwards 1983).

In water, PAHs may either evaporate, disperse into the water column, become incorporated into bottom sediments, concentrate in aquatic biota, or experience chemical oxidation and biodegradation (Suess 1976). The most important degradative processes for PAHs in aquatic systems are photooxidation, chemical oxidation, and biological transformation by bacteria and animals (Neff 1979). Most PAHs in aquatic environments are associated with particulate materials; only about 33% are present in dissolved form (Lee and Grant 1981). PAHs dissolved in the water column will probably degrade rapidly through photooxidation (EPA 1980), and degrade most rapidly at higher concentrations, at elevated temperatures, at elevated oxygen levels, and at higher incidences of solar radiation (McGinnis and Snoeyink 1974; Suess 1976; Bauer and Capone 1985). The ultimate fate of those PAHs that accumulate in sediments is believed to be biotransformation and biodegradation by benthic organisms (EPA 1980). PAHs in aquatic sediments, however, degrade very slowly in the absence of penetrating radiation and oxygen (Suess 1976), and may persist indefinitely in oxygen-poor basins or in anoxic sediments (Neff 1979). PAH degradation in aquatic environments occurs at a slower rate than that in the atmosphere (Suess 1976), and the cycling of PAHs in aquatic environments, as is true for other ecological systems, is poorly understood (Neff 1979).

Animals and microorganisms can metabolize PAHs to products that may ultimately experience complete degradation. The degradation of most PAHs is not completely understood. Those in the soil may be assimilated by plants, degraded by soil microorganisms, or accumulated to relatively high levels in the soil. High PAH concentrations in soil can lead to increased populations of microorganisms capable of degrading the compounds. Of equal importance to PAH cycling dynamics is the physical state of the PAH, i.e., whether in vapor phase or associated with particles such as flyash. Particles may increase or decrease the susceptibility of PAHs to degradation, depending on the PAH and particles involved (Edwards 1983).

PAHs can be taken into the mammalian body by inhalation, skin contact, or ingestion, although they are poorly absorbed from the gastrointestinal tract. The main routes of elimination of PAHs and their metabolites include the hepatobiliary system and the gastrointestinal tract (Sims and Overcash 1983). In mammals, an enzyme system variously known as the cytochrome P-450-dependent mixed-function oxidase, mixed-function oxidase, mixed-function oxygenase, aryl hydrocarbon hydroxylase, or drug metabolizing system, is responsible for initiating the metabolism of various lipophilic organic compounds, including PAHs. The primary function of this system is to render poorly water soluble lipophilic materials more water soluble, and therefore more available for excretion. Some PAHs are transformed to intermediates, which are highly toxic, mutagenic, or carcinogenic to the host. Oxidative metabolism of PAHs in this system proceeds via high electrophilic intermediate arene oxides, some of which bind covalently to cellular macromolecules such as DNA, RNA, and protein. Most authorities agree that metabolic activation by the mixed-function oxidase system is a necessary prerequisite for PAH-induced carcinogenesis and mutagenesis (Neff 1979). This enzyme system is known to be present in rodent tissues, and human liver, skin, placenta, fetal liver, macrophages, lymphocytes, and monocytes (Lo and Sandi 1978). Studies with rodents have shown that the mixed-function oxidase system can convert PAHs to various hydroxylated derivatives including phenols, quinones, and epoxides, and can also activate PAHs to produce carcinogenic metabolites (Lo and Sandi 1978). Fish and most crustaceans tested to date possess the enzymes necessary for activation (Statham et al. 1976; Varanasi et al. 1980; Fabacher and Baumann 1985), but some molluscs and other invertebrates are unable to efficiently metabolize PAHs (Jackim and Lake 1978; Varanasi et al. 1985). Although many aquatic organisms possess the requisite enzyme systems for metabolic activation of PAHs, it is not certain in most cases whether these enzymes produce the same metabolites as those produced by mammalian enzymes (Neff 1979).

PAHs are metabolized by liver mixed-function oxidases to epoxides, dihydrodiols, phenols, and quinones. The intermediate metabolites have been identified as the mutagenic, carcinogenic, and teratogenic agents (Sims and Overcash 1983). The activation mechanisms occur by hydroxylation or

production of unstable epoxides of PAHs which damage DNA, initiating the carcinogenic process (Jackim and Lake 1978). Metabolic formation of bay region diol epoxides represents an important pathway by which PAHs are activated to carcinogens (Figure 4). Such metabolic activation proceeds via initial formation of the dihydrodiol with the bay region double bond, followed by subsequent oxidation of the dihydrodiol to the bay region diol epoxide (Sims and Overcash 1983). Active epoxides may be converted to less toxic products by various enzymatic and other reactions (Neff 1979). In the case of benzo(a)pyrene, the "ultimate carcinogen" (7 beta, 8 alpha-dihydroxy-,7,8,9,10 tetrahydrobenzo(a)pyrene- 9 alpha, 10 alpha-epoxide) reacts with the guanine of RNA and DNA, the linkage taking place between the C-10 atom of benzo(a)pyrene and the C-2 amino group of guanine (Grimmer 1983; Dipple 1985; Figure 4). Additional information on actual and theoretical mechanisms involved in the metabolic activation of PAHs are given in Cavalieri et al. (1978, 1980), Bjorseth and Dennis (1980), Herd and Greene (1980), Cooke and Dennis (1981), Sims and Grover (1981), Grimmer (1983), Szentpaly (1984), Harvey (1985), and Yan (1985).

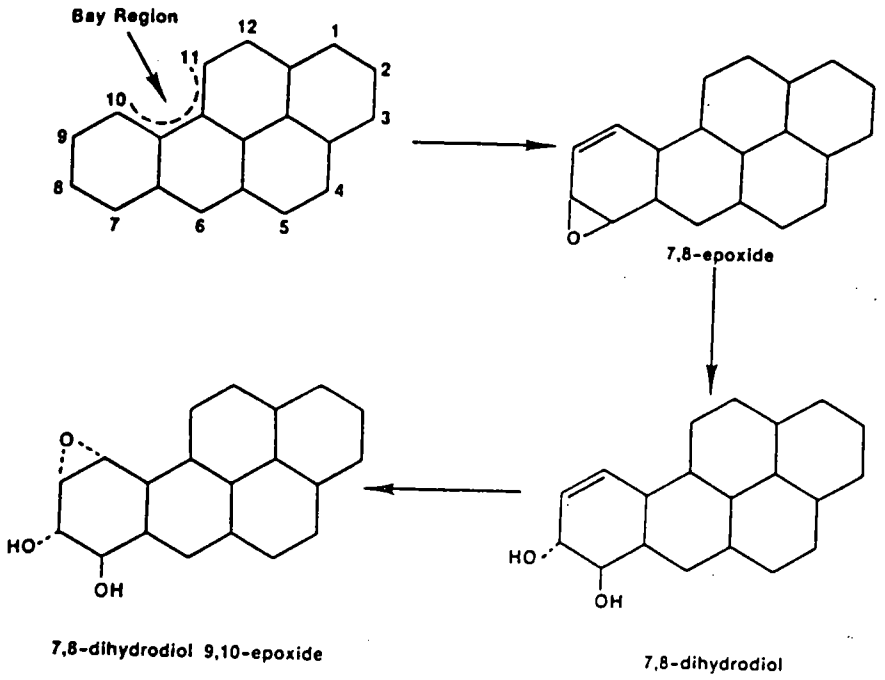


Figure 4. The bay region dihydrodiol epoxide route of benzo(a)pyrene (modified from Dipple 1985).

BACKGROUND CONCENTRATIONS

GENERAL

PAHs are ubiquitous in the environment. In nonbiological materials, concentrations are elevated in the vicinity of urban industrialized locales, and from areas of significant wood burning activities such as forest fires and residential home heating. Terrestrial vegetation and aquatic invertebrates can accumulate significant concentrations of PAHs, possibly due to inefficient or missing mixed-function oxidase systems. Fish do not appear to contain grossly elevated PAH residues; this may be related to their efficient degradation system. At present, data are lacking or unavailable on PAH background concentrations in natural populations of birds and other wildlife --although it seems unlikely that significant accumulations will occur. Some investigators have shown that aquatic invertebrates, fish, and amphibians collected from areas of high sediment PAH content show elevated frequencies of hyperplasia and neoplasia (Rose 1977; Mix 1982; Black 1983; Malins et al. 1984, 1985a, 1985b; Black et al. 1985; Baumann et al., in press), and, recently, that hepatic carcinoma has been induced in rainbow trout (Salmo gairdneri) by benzo(a)pyrene through dietary and intraperitoneal injection routes (Hendricks et al. 1985).

More comprehensive information on PAH background levels in various biological and nonbiological compartments is given in Lo and Sandi (1978), Neff (1979, 1985), Pucknat (1981), Edwards (1983), Grimmer (1983), and Sims and Overcash (1983).

NONBIOLOGICAL SAMPLES

Total PAH levels in air are usually much higher in winter than in summer, higher in urban communities than in rural areas (Table 3; Grimmer 1983), and appear to be related primarily to the weight of total suspended particulates in the atmosphere (Hites and Gschwend 1982; Greenberg et al. 1985; Srivastava et al. 1985; Ang et al. 1986). PAH levels in precipitation are significantly higher in winter than in summer, primarily due to emissions from household heating (Quaghebeur et al. 1983; van Noort and Wondergem 1985). Among

Table 3. PAH concentrations in selected nonbiological materials.

Material (units), and other variables	Concentration	Reference[a]
AIR (ng/m^3)		
USA cities, 1959, total PAHs		
Detroit	95.1	EPA 1980
Birmingham	63.4	
Nashville	60.6	
New Orleans	33.6	
Los Angeles	31.8	
Atlanta	26.3	
San Francisco	13.7	
Sydney, Australia		
Winter	8.2	Barnett 1976
Summer	0.6	
USA cities, 1971-1977		
Benzo(a)perylene = BaPER	0.2-9.2	EPA 1980
Benzo(e)pyrene = BeP	0.9-4.6	
Benzo(k)fluoranthene = BkFL	0.03-1.3	
Pyrene = PYR	0.18-5.2	
Coronene = COR	0.2-6.4	
Perylene = PER	0.01-1.2	
Anthracene = A	0.07-0.3	
Naphthalene = NA	Max. 0.4	
Benz(a)anthracene = BaA	Max. 4.6	
Indeno(1,2,3-cd)pyrene = IP	Max. 1.3	
Steel mill, Ontario, Canada, 1971-1979		
Station 0.8 km distant		
Benzo(a)pyrene = BaP	9.4 (Max. 110.0)	Potvin
BkFL	8.9 (Max. 142.0)	et al. 1981
Fluoranthene = FL	7.0 (Max. 43.3)	
PER	9.1 (Max. 106.0)	
Benzo(g,h,i)perylene = BghiPER	13.7 (Max. 90.0)	
Station 2.8 km distant		
BaP	0.4 (Max. 7.9)	
BkFL	0.7 (Max. 5.1)	
FL	1.1 (Max. 4.8)	
PER	0.7 (Max. 9.1)	
BghiPER	1.4 (Max. 8.5)	
Benzo(a)pyrene = BaP		
Urban areas	0.1-61.0	Edwards 1983
Downwind from coal gasification plant, Yugoslavia	Max. 80.0	

Table 3. (Continued)

Material (units), and other variables	Concentration	Reference[a]
Urban areas		
1966	3.2	EPA 1980
1970	2.1	
1976	0.5	
Rural areas	0.01-1.9	Edwards 1983
Rural areas		
1966	0.4	EPA 1980
1976	0.1	
SOILS		
Near M6 Motorway, Lancaster, UK (maximum deposition rate, ng/m^2/week)		
Distance from roadway		
3.8 meters		
A	2,300	Johnston and
FL	15,200	Harrison 1984
BaA	5,800	
Benzo(b)fluoranthene = BbFL	7,300	
BkFL	2,800	
BaP	4,900	
9.0 - 47 meters		
A	420	
FL	1,700	
BaA	260	
BbFL	690	
BkFL	470	
BaP	290	
Vicinity slash burn site, Oregon (g/ha)		
0-2 cm depth		
Preburn		
Phenanthrene = PHEN	0.5	Sullivan
FL	0.6	and Mix 1985
103 days postburn		
PHEN	9.8	
FL	3.6	
365 days postburn		
PHEN	ND	
FL	0.8	
2-5 cm depth		

Table 3. (Continued)

Material (units), and other variables	Concentration	Reference[a]
105 days postburn		
PHEN	1.3	
FL	0.3	
365 days postburn		
PHEN	ND	
FL	ND	
BaP (ug/kg)		
Rural areas	0.4	Barnett 1976
Industrial areas	400.0	
Nonpolluted areas	up to 1,000	Edwards 1983
Near known sources	>100,000	
Near coal-tar pitch disposal site, Germany	650,000	Lee and Grant 1981
Near recreation area, USSR	0.4	Harrison
Forest soil	1.5-4.0	et al. 1975
LITTER		
Forest, Oregon (g/ha)		
3 days postburn		
PHEN	603	Sullivan and
FL	245	Mix 1985
32 days postburn		
PHEN	ND	
FL	ND	
Coniferous trees (ug/kg)		
BghiPER	42	Thomas
BaP	51	et al. 1984
IP	47	
FL	164	
SEDIMENTS (ug/kg)		
Buffalo River, near Buffalo, NY		
Sediments		
BaA	7,300	Black 1983
Chrysene = CHRY	4,300	
BbFL	3,500	

Table 3. (Continued)

Material (units), and other variables	Concentration	Reference[a]
BaP	4,500	
Dibenz(a,h)anthracene = DBA	1,000	
IP	4,400	
Sediment extracts		
BaA	16,000	
CHRY	14,000	
BbFL	13,900	
BaP	15,400	
DBA	3,300	
IP	12,300	
Cayuga Lake, Ithaca, NY, 1978		
Total PAHs		
Within marinas	4,600-13,900	Heit 1985
Deepwater	1,260-2,500	
Near power plant	104-6,800	
FL		
Within marinas	1,700	
Deepwater	285	
Near power plant	8-1,000	
Penobscot Bay, Maine		
Total PAHs	286-8,794	Johnson
PHEN	17-252	et al. 1985
A	ND-49	
FL	156-3,700	
Pyrene = PYR	16-539	
BaA	14-540	
CHRY	9-578	
BbFL	17-1,000	
BkFL	14-696	
BaP	10-540	
DBA	2-120	
BghiPER	23-641	
IP	9-228	
Casco Bay, Maine, total PAHs	215-14,425	
Charles River, Mass., total PAHs	87,000-120,000	
Boston Harbor, Mass., total PAHs	8,500	
New Bedford Harbor, Mass., total PAHs	63,000	
Lake Erie, total PAHs	530-3,750	
Adirondack Lakes, total PAHs	4,070-12,807	

Table 3. (Continued)

Material (units), and other variables	Concentration	Reference[a]
Alaska, total PAHs	5-113	
Tamar estuary, UK, total PAHs	4,900	
Southampton estuary, UK, total PAHs	91,000-1,791,000	
Severn estuary, UK, total PAHs	1,600-25,700	
Monaco, total PAHs	5,200-12,100	
Gulf of Finland, total PAHs	437	
Norway, total PAHs	284-99,452	
Walvis Bay, Africa, total PAHs	68	
Amazon River system, total PAHs	ND-544	
SEWAGE		
Waters, worldwide, total PAHs(ug/l)	100-500	Lee and Grant 1981
Sludge, total PAHs		
United Kingdom, 12 sites, (ug/kg)		
Fresh weight	80-1,760	McIntyre et al. 1981
Dry weight	200-50,300	
Texas, Reese Air Force Base		
Effluent lagoon (ug/kg fresh weight)		
PER	300.0	Rose 1977
PYR	5.8	
FL	5.7	
BaA	1.4	
CHRY	1.3	
BaP	0.5	
BeP	0.2	
A	0.2	
MOTOR OILS (ug/l)		
Unused		
BaP	115	Pasquini and Monarca 1983
CHRY	56	
PER	11	
Used		
BaP	1,382	
CHRY	10,170	
PER	1,024	

Table 3. (Continued)

Material (units), and other variables	Concentration	Reference[a]
GROUNDWATER (ug/l)		
Worldwide		
Total PAHs	0.01-0.05	Lee and Grant 1981
Total PAHs	0.045-0.51	Harrison et al. 1975
Carcinogenic PAHs	0.00-0.081	
Germany		
Total PAHs	0.04	EPA 1980
Carcinogenic PAHs	0.003	
Champaign, Illinois		
Total PAHs	0.007	
Carcinogenic PAHs	0.003	
Elkhart, Indiana		
Total PAHs	0.02	
Carcinogenic PAHs	0.004	
DRINKING WATER (ug/l)		
USA, total PAHs	0.015	Lee and Grant 1981
Europe, total PAHs	0.04-0.06	
Monongehala River, Pittsburgh, PA		
Untreated		
Total PAHs	0.6	EPA 1980
Carcinogenic PAHs	0.14	
Treated		
Total PAHs	0.003	
Carcinogenic PAHs	0.002	
Ohio River, Wheeling, WV		
Untreated		
Total PAHs	1.59	
Carcinogenic PAHs	0.57	
Treated		
Total PAHs	0.14	
Carcinogenic PAHs	0.011	
Lake Winnebago, Appleton, WI		
Untreated		
Total PAHs	0.007	
Carcinogenic PAHs	0.002	

Table 3. (Concluded)

Material (units), and other variables	Concentration	Reference[a]
Treated		
Total PAHs	0.006	
Carcinogenic PAHs	0.002	
SURFACE WATER (ug/l)		
Worldwide		
Low level contamination	0.05-0.25	Lee and
Medium polluted	0.2-1.0	Grant 1981
Germany, Rhine River		
Total PAHs	1.12	EPA 1980
Carcinogenic PAHs	0.49	
Thames River, UK		
Total PAHs	0.5-1.33	
Carcinogenic PAHs	0.18-0.56	

[a]Each reference applies to data in the same row and in the rows that immediately follow for which no reference is indicated.

industrial sources, the production of metallurgical coke is the single most significant source of atmospheric PAHs in Ontario, Canada. Coke production in 1977 represented about 52% of all PAH emissions from Ontario sources versus about 46% formed as a result of forest fires (Potvin et al. 1981). Beyond 2 km distant from the coke point source, PAH concentrations in air were typical of those measured in major urban nonindustrialized areas (Table 3; Potvin et al. 1981). A variety of PAHs have been detected in ambient air in the USA and elsewhere. Benzo(a)pyrene, because of its carcinogenic properties, has been monitored extensively, and has frequently been used as an indicator of PAHs (EPA 1980). In general, total PAHs in air is about 10X higher than benzo(a)pyrene levels, although this relation is extremely variable (Lee and Grant 1981). Benzo(a)pyrene levels, like total PAHs, were higher in winter than summer, probably due to residential and industrial heating; air levels in urban areas with coke ovens were 40% to 70% higher than in cities without coke ovens, but this may be related to higher industrial emissions in those cities (Lee and Grant 1981). In one case, benzo(a)pyrene levels in air from the center of a remote mountain community in Colorado were several times higher than what is usually found in U.S. metropolitan areas, and was attributed to extensive residential wood burning (Murphy et al. 1982). Average concentrations of benzo(a)pyrene in urban air Nationwide declined from 3.2 ng/m^3 in 1966 to 0.5 ng/m^3 in 1978, an 80% decrease (Lee and Grant 1981). These decreases are believed to be due primarily to decreases in coal consumption for commercial and residential heating, improved disposal of solid wastes, and restrictions on open burning (EPA 1980).

A major source of PAHs in soils and soil litter is from emissions and deposition from forest fires. In a controlled burn study, Sullivan and Mix (1985) showed that lower molecular weight PAHs, such as phenanthrene and fluorene, which had been deposited in soil litter, degraded to nondetectable levels within 2 years after burning. Higher molecular weight PAHs such as benzo(k)fluorene, benzo(a)pyrene, benzo(g,h,i)perylene, perylene, and indeno(1,2,3-cd)pyrene, were more persistent in litter, decreasing after 5 years to about 20% of initial deposition. Although movement into the top 2 cm of the soil profile was initially more pronounced for lower molecular weight PAHs, all compounds appeared to reach equilibrium between litter and soil on the basis of organic content within one year postburn. Differential persistence and fate of PAHs on slash burn sites is explained by solubility, Kow, and other physicochemical properties (Sullivan and Mix 1985). PAHs from vehicle emissions constitute a minor, but measurable, source of soil PAHs (Table 3). The majority of highway-derived PAHs appears to be deposited within 3.8 m of the road, but the influence of the highway may extend to nearly 70 m (Johnston and Harrison 1984). The use of composted municipal wastes for conditioning of agricultural soils is not recommended, as these contain at least nine identified carcinogenic PAHs (Martens 1982).

Some sediments were found to be highly contaminated with PAHs. Sediments and sediment extracts from the Buffalo River, New York, contained elevated levels of carcinogenic PAHs (1,000-16,000 ug/kg). Brown bullheads (Ictalurus

nebulosus), in response to repeated applications of Buffalo River sediment extracts, showed epidermal hyperplasia and neoplasia when compared to controls (Black 1983). PAH concentrations in sediments from the Great Barrier Reef, Australia, were always <0.8 ug/kg dry weight, except in small areas close to sites frequently visited by power boats; in those instances, total PAH levels exceeded 13,400 ug/kg (Smith et al. 1985). Highest PAH levels measured in sediments of Cayuga Lake, New York, were found in marinas or areas of the lake receiving urban runoff, and were apparently not related to stack emissions from a nearby coal-fired power plant; Heit (1985) believed that stack emissions were either masked by other sources or were atmospherically transported and deposited elsewhere. Coastal and offshore sediments are subject to highly elevated PAH levels from a variety of sources, mostly unknown, relative to preindustrial times (Johnson et al. 1985). For example, PAH levels in sediments of Penobscot Bay, Maine, fell within the range found in sediments near industrialized regions, and were significantly higher than expected for an area previously considered to be uncontaminated (Table 3; Johnson et al. 1985).

Sewage effluents usually contained measurable levels of PAHs, although extreme variability between and among sites is common. For example, during a heavy storm, individual PAH levels in a sewage works may increase more than 100X over a dry weather period (Harrison et al. 1975). Conventional sewage treatment plant processes remove up to 90% of carcinogenic PAHs, and this may be increased to 99% using percolating filters and activated sludge processes (Harrison et al. 1975). Tiger salamanders (Ambystoma tigrinum), collected in 1975 from a 13-ha sewage effluent lagoon at Reese Air Force Base, Texas, showed a remarkably high incidence (53%) of neoplastic and other lesions (Rose 1977). Analysis of sludge composites showed elevated PAH levels, especially perylene; levels of organochlorine and organophosphorus pesticides, nitrosamines, and heavy metals were judged to be nonelevated (Rose 1977).

Careful disposal of used motor oils is warranted, as these contain high quantities of mutagenic and carcinogenic PAHs (Table 3; Pasquini and Monarca 1983).

All but the most heavily contaminated fresh and marine waters contain total PAH concentrations in the part-per-trillion or low part-per-billion range (Table 3; Neff 1982b). A large proportion of the PAH content in water is probably adsorbed onto suspended solids (Harrison et al. 1975). In Lake Michigan, concentrations of total PAHs in the surface microlayer varied from 0.15 to 0.45 ug/l, representing on a relative scale 10^6 times the concentration in air, suggesting that aerosols are a major source of these compounds and that the microlayer is a repository until the PAHs are removed by adsorption and sedimentation (Strand and Andren 1980).

BIOLOGICAL SAMPLES

Carcinogenic PAHs have been extracted from a large variety of fresh plants, including root and leaf vegetables, fruits, grains, and edible mushrooms, as well as from various marine bacteria and phytoplankton under circumstances suggesting that PAHs were present due to local biosynthesis (Suess 1976). Vegetation and soil near known PAH sources are more highly contaminated with PAHs than those collected at greater distances (Edwards 1983). PAH levels in lettuce (Lactuca sativa) grown in Sweden seemed to be directly related to its proximity to local recognized point sources of PAH emitters (Table 4; Larsson and Sahlberg 1982). Washing lettuce with water had little effect on phenanthrene levels, but significantly reduced other PAHs, such as benzo(a)pyrene, benz(a)anthracene, and benzo(g,h,i)perylene by 68% to 87% (Larsson and Sahlberg 1982). Fruits and vegetables grown in polluted atmospheres may contain up to 100X higher levels of total PAHs than those grown in unpolluted environments (EPA 1980; Lee and Grant 1981). PAH concentrations for plants are generally greater on plant surfaces than internal tissues, greater in above ground plant parts than those below ground, and greater in plants with broad leaves (greater surface area) than those with narrow leaves (Edwards 1983). Plants can become contaminated with PAHs through environmental pollution, particularly through deposition from the atmosphere, and also through food processing. For example, the bran portion of milled wheat, as well as finished bran cereal, had a considerably higher PAH content than other fractions or finished products (Lawrence and Weber 1984b). Enrichment of PAHs in plants is associated with deposition of atmospheric particulate matter with relatively small particle sizes; thus, PAH content is usually in the order of humus > mosses > lichens (Thomas et al. 1984). Mosses appear to be good indicators of regional PAH air pollution and have been recommended for this purpose (Herrmann and Hubner 1984). Concentrations of total PAHs in soils, usually the sum of 5 to 20 PAHs, typically exceeded benzo(a)pyrene levels by at least one order of magnitude; however, concentrations of benzo(a)pyrene in vegetation were generally less than those in soil where plants were growing (Edwards 1983).

PAH accumulations in marine molluscs have been reported (Table 4); however, some of these data may be misleadingly low. For example, lengthy cold storage of 10 months can result in loss of volatile PAHs, such as anthracene, in tissues of mussels (Smith et al. 1984); accordingly, background concentrations in these organisms may be underreported. Bivalve molluscs tend to accumulate high PAH levels due to their inability to metabolize and excrete them (Lawrence and Weber 1984a), presumably due to inefficient or missing mixed-function oxidase systems (Sirota and Uthe 1981). Cellular proliferative disorders, resembling neoplastic conditions in vertebrates, were found in mussels with the greatest PAH concentrations: 9.5% vs. 0.7% in control site (Mix 1982). Baseline levels of PAHs in indigenous bivalve molluscs reflected the degree of human onshore activity at the various sample sites, and presumably the level of water contamination; however, little relation was

Table 4. PAH concentrations in field collections of selected biota. Values are shown in ug/kg (ppb) fresh weight (FW), or dry weight (DW).

Taxonomic group, compound, and other variables	Concentration	Reference[a]
ALGAE AND OTHER PLANTS		
Marine algae, Greenland		
Total PAHs	60 FW	Harrison et al. 1975
Marine algae, Benzo(a)pyrene=BaP	Up to 60 DW	Lee and Grant 1981
Freshwater alga,		
Chlorella vulgaris, BaP	10-50 DW	Suess 1976
Bacteria, BaP	2-6 DW	
Moss, Hypnum cupressiforme		
Southern Finland, 1982		
Near center of industrial town		
BaP	110 DW	Herrmann and Hubner 1984
Fluoranthene=FL	250 DW	
Benzo(g,h,i)perylene=BghiPER	90 DW	
Indeno(1,2,3 cd)pyrene=IP	41 DW	
Vegetation		
Total PAHs		
Nonpolluted areas	20-1,000 DW	Edwards 1983
Near known source	25,000 DW	
BaP	0.1-150.0 DW	
Lettuce,		
Lactuca sativa, total PAHs		
Sweden, summer 1980		
Grown near highway		
8-15 m distant	50 FW	Larsson and Sahlberg 1982
15-50 m distant	26 FW	
Near airport, 150-800 m	24 FW	
Aluminum smelter		
0.5-1.5 km distant	654 FW	
2.0-6.5 km	128 FW	
Industrial areas	13 FW	
Residential areas		
Urban	13 FW	
Rural	12 FW	
Seedlings, wheat and rye, BaP	10-20 DW	Suess 1976

Table 4. (Continued)

Taxonomic group, compound, and other variables	Concentration	Reference[a]
INVERTEBRATES		
Rock crab, Cancer irroratus		
Edible portions, 1980		
New York Bight		
Total PAHs	1,600 FW	Humason and
BaP	1 FW	Gadbois 1982
Long Island Sound		
Total PAHs	1,290 FW	
BaP	ND	
American oyster, Crassostrea virginica, soft parts		
South Carolina, 1983, residential resorts		
Total PAHs		
Spring months		
Palmetto Bay	520 FW	Marcus and Stokes 1985
Outdoor Resorts	247 FW	
Fripp Island	55 FW	
Summer months		
Palmetto Bay	269 FW	
Outdoor Resorts	134 FW	
Fripp Island	21 FW	
American lobster, Homarus americanus		
Edible portions, 1980		
New York Bight		
Total PAHs	367 FW	Humason and
BaP	15 FW	Gadbois 1982
Long Island Sound		
Total PAHs	328 FW	
BaP	15 FW	
Softshell clam, Mya arenaria		
Coos Bay, Oregon, 1978-1979		
Soft parts		
Contaminated site		
Total PAHs	555 FW	Mix 1982
Phenanthrene = PHEN	155 FW	
FL	111 FW	
Pyrene = PYR	62 FW	
BaP	55 FW	
Benz(a)anthracene = BaA	42 FW	

Table 4. (Continued)

Taxonomic group, compound, and other variables	Concentration	Reference[a]
Chrysene = CHRY	27 FW	
Benzo(b)fluoranthene = BbFL	12 FW	
Others	<10 FW	
Uncontaminated site		
Total PAHs	76 FW	
PHEN	12 FW	
FL	10 FW	
Others	<10 FW	
Bay mussel, Mytilus edulis		
Oregon, 1979-1980		
Soft parts, total PAHs		
Near industrialized area	106-986 FW	Mix and Schaffer 1983b
Remote site	27-274 FW	
Sea scallop, Placopectin magellanicus		
Baltimore Canyon, east coast USA		
Muscle		
BaA	1 FW	Brown and
BaP	<1 FW	Pancirov 1979
PYR	4 FW	
New York Bight, 1980		
Edible portions		
Total PAHs	127 FW	Humason and
BaP	3 FW	Gadbois 1982
Clam, Tridacna maxima		
Australia, 1980-1982, Great Barrier Reef		
Soft parts, total PAHs		
Pristine areas	<0.07 FW	Smith et al. 1984
Power boat areas	Up to 5 FW	
BaP		
Marine plankton		
Greenland	5 FW	Harrison et al. 1975
Italy	6-21 FW	
France	400 FW	
Worldwide	Up to 400 DW	Lee and Grant 1981
Mussel, Mytilus sp.		
Greenland		
Shell	60 FW	Harrison et al. 1975
Soft parts	18 FW	

Table 4. (Continued)

Taxonomic group, compound, and other variables	Concentration	Reference[a]
Italy		
Shell	11 FW	
Soft parts	130-540 FW	
Bivalve molluscs, 5 spp.		
Edible portions	6 (Max. 36) FW	Stegeman 1981
Decapod crustaceans, 4 spp.		
Edible portions	2 (Max. 8) FW	
Softshell clam, Mya arenaria, soft parts		
Coos Bay, Oregon		
1976-1978		
Near industrialized areas	6-20 FW	Mix and Schaffer 1983a
Remote areas	1-2 FW	
1978-1979		
Near industrialized areas	9 FW	
Remote areas	4 FW	
VERTEBRATES		
Fish, muscle		
Lake Ontario, 6 spp., total PAH	3-8 FW	Lawrence and Weber 1984a
Baltimore Canyon, east coast, USA, 5 spp.		
BaA	Max. 0.3 FW	Brown and Pancirov 1979
BaP	Max. <5 FW	
PYR	Max. <5 FW	
Smoked		
FL	3 FW	EPA 1980
PYR	2 FW	
Nonsmoked		
FL	Max. 1.8 FW	
PYR	Max. 1.4 FW	
Winter flounder, Pseudopleuronectes americanus		
Edible portions, 1980		
New York Bight		
Total PAHs	315 FW	Humason and Gadbois 1982
BaP	21 FW	
Long Island Sound		
Total PAHs	103 FW	
BaP	ND	

Table 4. (Continued)

Taxonomic group, compound, and other variables	Concentration	Reference[a]
Windowpane, Scopthalmus aquosus		
Edible portions, 1980		
New York Bight		
Total PAHs	536 FW	
BaP	4 FW	
Long Island Sound		
Total PAHs	86 FW	
BaP	ND	
Red hake, Urophycus chuss		
Edible portions, 1980		
New York Bight		
Total PAHs	412 FW	
BaP	22 FW	
Long Island Sound		
Total PAHs	124 FW	
BaP	5 FW	
BaP		
Fish		
Marine, edible portions		
9 spp.	Max. 3 FW	Stegeman 1981
Greenland	15 FW	Harrison et al. 1975
Italy	65 FW	
Steak, charcoal broiled	5-8 DW	Barnett 1976
Ribs, barbecued	11 DW	
INTEGRATED STUDIES		
Michigan, 1978, Hersey River		
Near wastewater treatment plant		
PHEN		
Sediments	4,097 FW	Black et al. 1981
Insects, whole	5,488 FW	
Crustaceans, muscle	447 FW	
Fish, muscle	28-15,313 FW	
BaA		
Sediments	3,504 FW	
Insects	2,893 FW	
Crustaceans	40 FW	
Fish	0.2-19 FW	

Table 4. (Concluded)

Taxonomic group, compound, and other variables	Concentration	Reference[a]
BaP		
Sediments	1,194 FW	
Insects	725 FW	
Crustaceans	8 FW	
Fish	0.07-1 FW	
Control location		
Sediments and biota		
PHEN	2-42 FW	
BaA	ND-6.7 FW	
BaP	0.04-1.2 FW	
Nova Scotia, 1980, total PAHs		
Near coking facility		
Sediments	2,830,000 DW	Sirota et al. 1983
American lobster, *Homarus americanus*		
Hepatopancreas	57,300-88,100 FW	
Tail muscle	1,910-2,670 FW	
Control area		
Sediments	<8,220 DW	
American lobster		
Hepatopancreas	1,185 FW	
Tail muscle	216 FW	
Black River, Ohio, contaminated area, total PAHs		
Sediments	6,700 DW	West et al. 1984
Brown bullhead, *Ictalurus nebulosus*	660 FW	
Water	153 FW	

[a]Each reference applies to data in the same row and in the rows that immediately follow for which no reference is indicated.

evident between accumulated levels of individual PAHs and total PAHs (Mix 1982). Elevated PAH concentrations, especially benz(a)anthracene, chrysene, fluorene, phenanthraene, and pyrene in oyster tissues and sediments were measured in samples from the vicinity of marinas, and were higher in oysters in cooler months, when lipids and glycogen were being stored preparatory to spawning (Marcus and Stokes 1985). In general, PAH concentrations in marine clams were highest in areas adjacent to industrialized bayfronts and lowest in clams inhabiting more remote areas; concentrations were lowest in autumn-winter, and highest during spring-summer (Mix and Schaffer 1983a). A similar pattern was observed in mussels, Mytilus edulis, with the more water soluble, lower molecular weight, PAHs bioconcentrated 10X to 100X above that of the higher molecular weight, less water soluble PAHs (Mix and Schaffer 1983b); PAH levels in mussels seemed to be independent of water salinity (Mix and Schaffer 1979). Clams contaminated with PAHs and removed to clean seawater for 24 hours showed significant depuration of unsubstituted 3- and 4-ring PAHs; in contrast, concentrations of all 5-, 6-, and 7-ring compounds, which includes most of the carcinogenic PAHs, were not significantly depurated (Mix 1982). A positive relation exists between PAH isomers in sediments, soft tissues of the mussel Mytilus edulis, and a seaweed (Fucus sp.) collected at Vancouver, British Columbia (Dunn 1980). For mussels, the general trend towards lower levels of higher molecular weight PAHs relative to levels in sediments suggests an uptake mechanism which involves the solution of PAHs in water; superimposed on this pattern is the more rapid turnover and shorter half-life of lower molecular weight PAHs in mussels (Dunn 1980).

PAH residues were higher than expected in American lobsters (Homarus americanus) collected offshore (mean weight 3.6 kg) when compared to smaller (0.6 kg) lobsters collected inshore (Sirota and Uthe 1981), suggesting that age or body size are important modifiers in PAH accumulation dynamics. PAH concentrations in sediments collected near a coking facility in Nova Scotia in 1980 contained up to 2,830 mg/kg dry weight, or more than 20X the levels recorded in Boston (Mass.) Harbor; concentrations in excess of 100 mg/kg dry weight sediment were recorded for phenanthrene, fluorene, pyrene, benz(a)anthracene, chrysene, benzo(e)pyrene, benzo(b)fluoranthene, and benzo(a)pyrene, and these seemed to reflect the elevated tissue levels in American lobsters collected from that locale (Sirota et al. 1983). PAH residues in digestive glands of American lobsters collected in 1979 in Nova Scotia from the vicinity of a major oil spill were higher than those from coastal control sites; however, PAH contents of edible muscle from control and oiled lobsters were similar (Sirota and Uthe 1981).

PAH levels in fish are usually low because this group rapidly metabolizes PAHs (Lawrence and Weber 1984a); furthermore, higher molecular weight PAHs, which include the largest class of chemical carcinogens, do not seem to accumulate in fish (West et al. 1984). Raw fish from unpolluted waters usually do not contain detectable amounts of PAHs, but smoked or cooked fish contain varying levels. The concentration of benzo(a)pyrene in skin of cooked

fish was much higher than in other tissues, suggesting that skin may serve as a barrier to the migration of PAHs in body tissues (EPA 1980).

Sediments and biota collected from the Hersey River, Michigan, in 1978, were heavily contaminated with phenanthrene, benz(a)anthracene, and benzo(a)pyrene when compared to a control site. Elevated PAH concentrations were recorded in sediments, whole insect larvae, crayfish muscle, and flesh of lampreys (family Petromyzontidae), brown trout (Salmo trutta), and white suckers (Catostomus commersoni), in that general order (Black et al. 1981). The polluted collection locale was the former site of a creosote wood preservation facility between 1902 and 1949, and, at the time of the study, received Reed City wastewater treatment plant effluent, described as an oily material with a naphthalene-like odor (Black et al. 1981). In many cases, aquatic organisms from PAH-contaminated environments have a higher incidence of tumors and hyperplastic diseases than those from nonpolluted environments. Carcinogenic PAHs have not been unequivocally identified as the causative agent for an increased incidence of cancer in any natural population of aquatic organisms, according to Neff (1982b). However, a growing body of evidence, mostly circumstantial, links PAHs to cancer in feral fish populations, especially bottom dwelling fish from areas with sediments heavily contaminated with PAHs (Baumann and Lech, in press).

TOXIC AND SUBLETHAL EFFECTS

GENERAL

A wide variety of PAH-caused adverse biological effects have been reported in numerous species of organisms under laboratory conditions, including effects on survival, growth, metabolism, and especially tumor formation. Inter- and intraspecies responses to carcinogenic PAHs were quite variable, and were significantly modified by many chemicals including other PAHs that are weakly carcinogenic or noncarcinogenic. Until these interaction effects are clarified, the results of single substance laboratory tests may be extremely difficult to apply to field situations of suspected PAH contamination.

FUNGI

Fungal degradation of PAHs may be important in the detoxification and elimination of PAHs in the environment. The fungus Cunninghamella elegans, for example, inhibited the mutagenic activity of benzo(a)pyrene, 3-methyl cholanthrene, benz(a)anthracene, and 7,12-dimethylbenz(a)anthracene, as judged by results of the Ames test using Salmonella typhimurium (Cerniglia et al. 1985). The rate of decrease in mutagenic activity in bacterial cultures incubated with PAHs was coincident with the rate of increase in fungal metabolism. C.elegans metabolized PAHs to dihydrodiols, phenols, quinones, and dihydrodiol epoxides, and to sulfate, glucuronide, and glucoside conjugates of these primary metabolites in a manner similar to that reported for mammalian enzyme systems, suggesting that this organism (and perhaps other fungi) is important in PAH metabolism and inactivation (Cerniglia et al. 1985).

TERRESTRIAL PLANTS

Biological effects of PAHs on terrestrial vegetation have been reviewed by EPA (1980), Lee and Grant (1981), Wang and Meresz (1982), Edwards (1983), and Sims and Overcash (1983). In general, these authorities agreed on several points. First, plants and vegetables can absorb PAHs from soils through their roots, and translocate them to other plant parts such as developing shoots. Uptake rates were governed, in part, by PAH concentration, PAH water solubility, soil type, and PAH physicochemical state (vapor or particulate). Lower molecular weight PAHs were absorbed by plants more readily than higher molecular weight PAHs. Under laboratory conditions, some plants concentrated selected PAHs above that of their immediate geophysical surroundings, but this has not been conclusively demonstrated in field-grown cultivated crops or other vegetation. Second, above-ground parts of vegetables, especially the outer shell or skin, contained more PAHs than underground parts, and this was attributed to airborne deposition and subsequent adsorption. Externally deposited PAHs in vegetables were difficult to remove with cold water washings; not more than 25% were removed from lettuce, kale, spinach, leeks, and tomatoes using these procedures. Third, PAH-induced phytotoxic effects were rare; however, the data base on this subject is small. Fourth, most higher plants can catabolize benzo(a)pyrene, and possibly other PAHs, but metabolic pathways have not been clearly defined. Finally, the biomagnification potential of vegetation in terrestrial and aquatic food chains needs to be measured; this work should be conducted with a variety of PAHs in both field and laboratory experiments.

Some plants contain chemicals known to protect against PAH effects. Certain green plants contain ellagic acid, a substance that can destroy the diol epoxide form of benzo(a)pyrene, inactivating its carcinogenic and mutagenic potential (Edwards 1983). PAHs synthesized by plants may act as plant growth hormones (Edwards 1983). Some vegetables, such as cabbage, brussel sprouts, and cauliflower, contain naturally occurring antineoplastic compounds including benzyl isothiocyanate and phenethyl isothiocyanate; these compounds are known to inhibit mammary cancers, stomach tumors, and pulmonary edemas induced in rats by benzo(a)pyrene and 7,12-dimethylbenz(a)anthracene (EPA 1980). Decreased activation of carcinogens has also been demonstrated in animals fed diets that were high in protein, low in carbohydrate, and containing adequate choline; the reverse was observed in diets high in carbohydrate, low in protein, or containing certain organophosphorus insecticides, piperonyl butoxide, carbon tetrachloride, nickel carbonyl, or tin (EPA 1980). In cases where dietary constituents can alter the metabolism of foreign agents, such as PAHs, the anticarcinogenic effect may result from an alteration of steady state levels of activated versus detoxified metabolites (EPA 1980). The implications of these observations to herbivorous wildlife are unknown at present.

AQUATIC BIOTA

PAHs vary substantially in their toxicity to aquatic organisms (Table 5). In general, toxicity increases as molecular weight increases (although high molecular weight PAHs have low acute toxicity, perhaps due to their low solubility in water) and with increasing alkyl substitution on the aromatic ring. Toxicity is most pronounced among crustaceans and least among teleosts (Neff 1979; Table 5). In all but a few cases, PAH concentrations that are acutely toxic to aquatic organisms are several orders of magnitude higher than concentrations found in even the most heavily polluted waters (Neff 1979). Sediments from polluted regions, however, may contain PAH concentrations similar to those which are acutely toxic, but their limited bioavailability would probably render them substantially less toxic than PAHs in solution (Neff 1979).

A growing literature exists on uptake, retention, and translocation of PAHs by aquatic plants and animals. Authorities generally agree that: most species of aquatic organisms studied to date rapidly accumulate (i.e., bioconcentrate) PAHs from low concentrations in the ambient medium; uptake of PAHs is highly species specific, being higher in algae, molluscs, and other species which are incapable of metabolizing PAHs; bioconcentration factors (BCF) tend to increase as the molecular weight of the PAH increases, with increasing octanol/water partition coefficient values, with time until approaching an apparent equilibrium level (sometimes within 24 hours), and with increases in dissolved organic matter in the medium, lipid content of organism, and a variety of endogenous and exogenous factors (Jackim and Lake 1978; Southworth et al. 1978; Lee and Grant 1981; Neff 1982a). BCF values have been determined for selected PAHs and aquatic organisms (Table 6); additional BCF data for aquatic biota are available for plants (Dobroski and Epifanio 1980; Boyle et al. 1984), crustaceans (Southworth 1979; Sirota and Uthe 1981; Fox and Rao 1982; Neff 1982a; Williams et al. 1985), tunicates (Baird et al. 1982), molluscs (Jackim and Wilson 1979; Dobroski and Epifanio 1980; Neff 1982a), and fishes (Southworth 1979; Neff 1982a; Stoker et al. 1984). Algal accumulation of benzo(a)pyrene increased linearly in a 24-hour exposure period, and correlated positively with surface area (Leversee et al. 1981), suggesting adsorption rather than absorption. Algae readily transform benzo(a)pyrene to oxides, peroxides (Kirso et al. 1983), and dihydrodiols (Warshawsky et al. 1983). Photosynthetic rates of algae, and presumably PAH accumulations, were significantly modified by light regimens. For reasons still unexplained, algae grown in "white" light (major energy in blue-green portion of the spectrum) were more sensitive to benzo(a)pyrene than were cultures grown in "gold" light (Warshawsky et al. 1983; Schoeny et al. 1984). Accumulation by oysters (Crassostrea virginica) and clams (Rangia cuneata) of naphthalene, phenanthrene, fluorene, and their methylated derivatives increased with increasing methylation and PAH molecular weight; uptake was more rapid under conditions of aontinuous flow than in static tests (Neff et al. 1976). When returned to PAH-free seawater, molluscs released PAHs to non-

Table 5. Toxicities of selected PAHs to aquatic organisms.

PAH compound, organism, and other variables	Concentration in medium (ug/l)	Effect[a]	Reference[b]
BENZ(a)ANTHRACENE			
Bluegill, *Lepomis macrochirus*	1,000	LC-87 (6 m)	EPA 1980
BENZO(a)PYRENE			
Sandworm, *Neanthes arenceodentata*	>1,000	LC-50 (96 h)	Neff 1979
CHRYSENE			
Sandworm	>1,000	LC-50 (96 h)	
7,12-DIMETHYLBENZ(a)ANTHRACENE			
Minnows, *Poeiciliopsis* spp.			
Juveniles	250	LC-0 (20 h)	Schultz
Juveniles	500	LC-100 (20 h)	and Schultz 1982
DIBENZ(a,h)ANTHRACENE			
Sandworm	>1,000	LC-50 (96 h)	Neff 1979
FLUORANTHENE			
Sandworm	500	LC-50 (96 h)	
FLUORENE			
Grass shrimp, *Palaemonetes pugio*	320	LC-50 (96 h)	
Bluegill	500	LC-12 (30 d)	Finger et al. 1985
Amphipod, *Gammarus pseudoliminaeus*	600	LC-50 (96 h)	
Rainbow trout, *Salmo gairdneri*	820	LC-50 (96 h)	
Bluegill	910	LC-50 (96 h)	
Sandworm	1,000	LC-50 (96 h)	Neff 1979

Table 5. (Continued)

PAH compound, organism, and other variables	Concentration in medium (ug/l)	Effect[a]	Reference[b]
Sheepshead minnow, *Cyprinodon variegatus*	1,680	LC-50 (96 h)	
Snail, *Mudalia potosensis*	5,600	LC-50 (96 h)	Finger et al. 1985
Mayfly, *Hexagenia bilineata*	5,800	LC-50 (120 h)	
Fathead minnow, *Pimephales promelas*	>100,000	LC-0 (96 h)	
NAPHTHALENE			
Copepod, *Eurytemora affinis*	50	LC-30 (10 d)	Neff 1979
Pink salmon, *Oncorhynchus gorbuscha*, fry	920	LC-50 (24 h)	
Dungeness crab, *Cancer magister*	2,000	LC-50 (96 h)	Neff 1985
Grass shrimp	2,400	LC-50 (96 h)	Neff 1979
Sheepshead minnow	2,400	LC-50 (24 h)	
Brown shrimp, *Penaeus aztecus*	2,500	LC-50 (24 h)	
Amphipod, *Elasmopus pectenicrus*	2,680	LC-50 (96 h)	
Coho salmon, *Oncorhyncus kisutch*, fry	3,200	LC-50 (96 h)	Neff 1985
Sandworm	3,800	LC-50 (96 h)	Neff 1979
Mosquitofish, *Gambusia affinis*	150,000	LC-50 (96 h)	
1-METHYLNAPHTHALENE			
Dungeness crab, *Cancer magister*	1,900	LC-50 (96 h)	
Sheepshead minnow	3,400	LC-50 (24 h)	
2-METHYLNAPHTHALENE			
Grass shrimp	1,100	LC-50 (96 h)	Neff 1985
Dungeness crab	1,300	LC-50 (96 h)	
Sheepshead minnow	2,000	LC-50 (24 h)	Neff 1979

Table 5. (Concluded)

PAH compound, organism, and other variables	Concentration in medium (ug/l)	Effect[a]	Reference[b]
TRIMETHYLNAPHTHALENES			
Copepod, Eurytemora affinis	320	LC-50 (24 h)	
Sandworm	2,000	LC-50 (96 h)	
PHENANTHRENE			
Grass shrimp	370	LC-50 (24 h)	
Sandworm	600	LC-50 (96 h)	EPA 1980
1-METHYLPHENANTHRENE			
Sandworm	300	LC-50 (96 h)	

[a] m = months, d = days, h = hours.

[b] Each reference applies to data in the same row and in the rows that immediately follow for which no reference is indicated.

detectable levels in about 60 days, with high molecular weight PAHs depurated more slowly than low molecular weight compounds; brown shrimp (Penaeus aztecus) and longnose killifish (Fundulus similis), which can metabolize PAHs, lost PAHs more quickly than clams and oysters, which apparently lack the detoxifying enzymes (Neff et al. 1976). Pink shrimp (Penaeus duorarum) exposed to 1.0 ug chrysene/l for 28 days and then transferred to unpolluted seawater for an additional 28 days contained concentrations of chrysene (91 ug/kg fresh weight in abdomen, 48 ug/kg in cephalothorax) that were considered potentially hazardous to human consumers over extended periods (Miller et al. 1982). Eggs of the sand sole (Psettichthys melanostictus) exposed to 0.1 ug benzo(a)pyrene /l for 5 days showed reduced and delayed hatch and, when compared to controls, produced larvae with high accumulations (2.1 mg/kg fresh weight) and gross abnormalities, such as twinning and tissue overgrowths, in 50% of the test larvae (Hose et al. 1982). Naphthalene and benzo(a)pyrene were rapidly accumulated from the medium by three species of California marine teleosts; loss was rapid, being >90% for naphthalene in 24 hours, and 20% (muscle) to 90% (gill) for benzo(a)pyrene in a similar period (Lee et al. 1972). Phenanthrene is metabolized by many species of aquatic organisms, including fish. A marine flounder, Platichthys flesus, given a single oral dose of 0.7 mg phenanthrene/kg body weight, contained elevated phenanthrene concentrations in lipids, melanin-rich tissues (such as skin), and the eye lens; most was eliminated within 2 weeks (Solbakken et al. 1984). Different rates of accumulation and depuration of benzo(a)pyrene and naphthalene in bluegill (Lepomis macrochirus) and Daphnia magna have been documented by McCarthy and Jimenez (1985) and McCarthy et al. (1985). Benzo(a)pyrene accumulations in bluegill, for example, were 10X greater than naphthalene, but benzo(a)pyrene is extensively metabolized, whereas naphthalene is not. Consequently, postexposure accumulations of naphthalene greatly exceeded that of the parent benzo(a)pyrene. Because the more hydrophobic PAHs, such as benzo(a)pyrene, show a high affinity for binding to dissolved humic materials and have comparatively rapid biotransformation rates, these interactions may lessen or negate bioaccumulation and food chain transfer of hydrophobic PAHs (McCarthy and Jimenez 1985; McCarthy et al. 1985).

Time to depurate or biotransform 50% of accumulated PAHs (Tb 1/2) varied widely. Tb 1/2 values for Daphnia pulex and all PAH compounds studied ranged between 0.4 and 0.5 hours (Southworth et al. 1978). For marine copepods and naphthalene, a Tb 1/2 of about 36 hours was recorded (Neff 1982a). For most marine bivalve molluscs, Tb 1/2 values ranged from 2 to 16 days. Some species, such as the hardshell clam (Mercenaria mercenaria), showed little or no depuration, while others, such as oysters, eliminated up to 90% of accumulated PAHs in 2 weeks--although the remaining 10% was released slowly, and traces may remain indefinitely (Jackim and Lake 1978). Percent loss of various PAHs in oysters (Crassostrea virginica), 7 days postexposure, ranged from no loss for benzo(a)pyrene to 98% for methylnaphthalene; intermediate were benz(a)anthracene (32%), fluoranthene (66%), anthracene (79%), dimethylnaphthalene (90%), and naphthalene (97%) (Neff 1982a). Teleosts and

Table 6. PAH bioconcentration factors (BCF) for selected species of aquatic organisms.

PAH compound, organism, and other variables	Exposure period[a]	BCF	Reference[b]
ANTHRACENE			
Cladoceran, Daphnia magna	60 m	200	EPA 1980
Fathead minnow, Pimephales promelas	2 to 3 d	485	Southworth 1979
Cladoceran, Daphnia pulex	24 h	760 to 1200	Southworth et al. 1978; Southworth 1979; EPA 1980; Neff 1985
Mayfly, Hexagenia sp.	28 h	3,500	EPA 1980
Rainbow trout, Salmo gairdneri	72 h	4,400 to 9,200	Linder et al. 1985
9-METHYLANTHRACENE			
Cladoceran, Daphnia pulex	24 h	4,583	Neff 1985
BENZ(a)ANTHRACENE			
Cladoceran, Daphnia pulex	24 h	10,109	Southworth et al. 1978
BENZO(a)PYRENE			
Teleosts, 3 spp., Muscle	1 h to 96 h	0.02 to 0.1	EPA 1980
Clam, Rangia cuneata	24 h	9 to 236	Neff 1979; EPA 1980
Bluegill, Lepomis macrochirus	4 h	12	Leversee et al. 1981
Atlantic salmon, Salmo salar Egg	168 h	71	Kuhnhold and Busch 1978
Midge, Chironomus riparius, larvae	8 h	166	Leversee et al. 1981
Rainbow trout, liver	10 d	182 to 920	Gerhart and Carlson 1978

Table 6. (Continued)

PAH compound, organism, and other variables	Exposure period[a]	BCF	Reference[b]
Oyster, Crassostrea virginica	14 d	242	EPA 1980
Northern pike Esox lucius			
Bile and gallbladder	3.3 h	3,974	Balk et al. 1984
"	19.2 h	36,656	
"	8.5 d	82,916	
"	23 d	53,014	
Liver	3.3 h	259	
"	19.2 h	578	
"	8.5 d	1,376	
"	23 d	619	
Gills	3.3 h	283	
"	19.2 h	382	
"	8.5 d	372	
"	23 d	213	
Kidney	3.3 h	192	
"	19.2 h	872	
"	8.5 d	1,603	
Other tissues	3.3 h to 23 d	<55	
Mosquitofish, Gambusia affinis	3 d	930	Lu et al. 1977
Bluegill			
No dissolved humic material (DHM)	48 h	2,657	McCarthy and Jimenez 1985
20 mg/l DHM	48 h	225	
Cladoceran, Daphnia magna	6 h	2,837	Leversee et al. 1981
Alga, Oedogonium cardiacum	3 d	5,258	Lu et al. 1977
Periphyton, mostly diatoms	24 h	9,600	Leversee et al. 1981
Mosquito, Culex pipiens quinquefasciatus	3 d	11,536	Lu et al. 1977
Sand sole, Psettichthys melanostictus			
Egg	6 d	21,000	Hose et al. 1982
Snail, Physa sp.	3 d	82,231	Lu et al. 1977
Cladoceran, Daphnia pulex	3 d	134,248	

Table 6. (Continued)

PAH compound, organism, and other variables	Exposure period[a]	BCF	Reference[b]
CHRYSENE			
Clam, *Rangia cuneata*	24 h	8	Neff 1979
Mangrove snapper, *Lutjanus griseus*			
Liver	4 d	83 to 104	Miller et al. 1982
Liver	20 d	258 to 367	
Pink shrimp, *Penaeus duorarum*			
Cephalothorax	28 d	248 to 361	
Cephalothorax	28 d + 28 d postexposure	21 to 48	
Abdomen	28 d	84 to 199	
Abdomen	28 d + 28 d postexposure	22 to 91	
FLUORANTHENE			
Rainbow trout, liver	21 d	379	Gerhart and Carlson 1978
FLUORENE			
Bluegill	30 d	200 to 1,800	Finger et al. 1985
NAPHTHALENE			
Clam, *Rangia cuneata*	24 h	6	Neff 1979
Sandworm, *Neanthes arenaceodenta*	3 to 24 h	40	Neff 1982a
Sandworm	24 h + 300 h post-treatment	not detectable	
Atlantic salmon, egg	168 h	44 to 83	Kuhnhold and Busch 1978
Cladoceran, *Daphnia pulex*	24 h	131	Neff 1985
Crustaceans, 3 spp.	72 h	195 to 404	Neff 1979
Bluegill, whole	24 h	310	McCarthy and Jimenez 1985

Table 6. (Concluded)

PAH compound, organism, and other variables	Exposure period[a]	BCF	Reference[b]
DIMETHYLNAPHTHALENES			
Crustaceans, 3 spp.	72 h	967 to 1,625	Neff 1979
PERYLENE			
Cladoceran, Daphnia pulex	24 h	7,191	Neff 1985
PHENANTHRENE			
Clam, Rangia cuneata	24 h	32	Neff 1979
Cladoceran, Daphnia pulex	24 h	325	Neff 1985
PYRENE			
Cladoceran Daphnia pulex	24 h	2,702	
Rainbow trout, liver	21 d	69	Gerhart and Carlson 1978

[a] m = minutes, h = hours, d = days.

[b] Each reference applies to the values in the same row and in the rows that follow for which no other reference is indicated.

arthropods usually had low Tb 1/2 values. In bluegill, 89% loss of benzo(a)pyrene was recorded 4 hours postexposure; for midge larvae it was 72% in 8 hours, and for daphnids it was 21% in 18 hours (Leversee et al. 1981).

The role of sediments in PAH uptake kinetics should not be discounted. Sediment-associated anthracene contributed about 77% of the steady state body burden of this compound in the amphipod Hyalella azteca (Landrum and Scavia 1983). For benzo(a)pyrene and the amphipod Pontoporeia hoyi, the sediment source (including interstitial water) accounted for 53% in amphipods collected at 60 m, but only 9% at 23 to 45 m (Landrum et al. 1984). Benthos from the Great Lakes, such as oligochaete worms (Limnodrilus sp., Stylodrilus sp.) and amphipods (Pontoporeia hoyi), obtain a substantial fraction of their PAH body content from the water when sediment PAH concentrations are low. However, when sediment PAH concentrations are elevated, benthos obtain a majority of their PAHs from that source through their ability to mobilize PAHs from the sediment/pore water matrix; the high concentrations of phenanthrene, fluorene, benzo(a)pyrene, and other PAHs measured in these organisms could provide a significant source of PAHs to predator fish (Eadie et al. 1983). Great Lakes benthos appear to contain as much PAHs as the fine grain fraction of the sediment which serves as their food, although overlying water or pore water appears to contribute a larger proportion of PAHs to the organism's body burden than does sediments (Eadie et al. 1984). Marine mussels (Mytilus edulis) and polychaete annelid worms (Nereis virens) exposed for 28 days to sediments heavily contaminated with various PAH compounds accumulated significant concentrations (up to 1,000X control levels) during the first 14 days of exposure, and little thereafter; during a 5-week postexposure period, depuration was rapid, with the more water soluble PAHs excreted most rapidly; PAH levels usually remained above control values to the end of the postexposure period (Lake et al. 1985). English sole (Parophrys vetulus), during exposure for 11 to 51 days to PAH-contaminated sediments, showed significant accumulations of naphthalenes in liver (up to 3.1 mg/kg dry weight) after 11 days, with concentrations declining markedly thereafter; uptake of phenanthrene, chrysene, and benzo(a)pyrene was negligible during the first 7 days (Neff 1982a).

Fluorene effects in freshwater pond ecosystems have recently been evaluated (Boyle et al. 1984, 1985; Finger et al. 1985). In ponds exposed to initial fluorene concentrations of 0.12 to 2.0 mg/l, Tb 1/2 values in water ranged from 6 to 11 days. Ten weeks after fluorene introduction, little degradation had occurred in the organic bottom sediments; fluorene residues were present in fish, invertebrates, and rooted submerged macrophytes. Studies with fingerling bluegills showed that 0.062 mg fluorene/l adversely affected their ability to capture chironomid prey, 0.12 mg/l reduced growth, and 1.0 mg fluorene/l increased their vulnerability to predation by largemouth bass (Micropterus salmoides). The authors concluded that fluorene, at concentrations well below its solubility and at levels that could realistically occur in the environment, represents a potential hazard to aquatic organisms.

Large interspecies differences in ability to absorb and assimilate PAHs from food have been reported. For example, crustaceans (Neff 1982a) and fish (Maccubbin et al. 1985; Malins et al. 1985a, 1985b) readily assimilated PAHs from contaminated food, whereas molluscs and polychaete annelids were limited (Neff 1982a). In all cases where assimilation of ingested PAHs was demonstrated, metabolism and excretion of PAHs were rapid (Neff 1982a). Thus, little potential exists for food chain biomagnification of PAHs (Southworth 1979; Dobroski and Epifanio 1980; Neff 1982a). In laboratory aquatic ecosystem studies, Lu et al. (1977) found that benzo(a)pyrene can be accumulated to high, and potentially hazardous, levels in fish and invertebrates. In the case of mosquitofish (Gambusia affinis), almost all of the accumulated benzo(a)pyrene was from its diet, with negligible accumulations from the medium. However, mosquitofish degraded benzo(a)pyrene about as rapidly as it was absorbed, in contrast to organisms such as snails (Physa sp.) which retained most (88%) of the accumulated benzo(a)pyrene for at least 3 days postexposure, presumably due to deficiencies in their mixed-function oxidase detoxication system (Lu et al. 1977). Benzo(a)pyrene, when administered to northern pike (Esox lucius) through the diet or the medium, followed similar pathways: entry via the gills or gastrointestinal system, metabolism in the liver, and excretion in the urine and bile (Balk et al. 1984). Benthic marine fishes exposed to naphthalene or benzo(a)pyrene, either in diet or through contaminated sediments, accumulated substantial concentrations in tissues and body fluids (Varanasi and Gmur 1981). The tendency of fish to metabolize PAHs extensively and rapidly may explain why benzo(a)pyrene, for example, is frequently undetected, or only detected in low concentrations in livers of fish from environments heavily contaminated with PAHs (Varanasi and Gmur 1980, 1981). Extensive metabolism of benzo(a)pyrene plus the presence of large proportions of polyhydroxy metabolites in liver of English sole indicates the formation of reactive intermediates such as diol epoxides and phenol epoxides of benzo(a)pyrene, both of which are implicated in mammalian mutagenesis and carcinogenesis (Varanasi and Gmur 1981).

Cytotoxic, mutagenic, and carcinogenic effects of many PAHs are generally believed to be mediated through active epoxides formed by interaction with microsomal monooxygenases. These highly active arene oxides can interact with macromolecular tissue components and can further be metabolized or rearranged to phenols or various conjugates. They can also be affected by epoxide hydrolase to form dihydrodiols, which are precursors of biologically active diol epoxides--a group that has been implicated as ultimate carcinogens. Investigators generally agree that marine and freshwater fishes are as well equipped as mammals with liver PAH-metabolizing enzymes; rapidly metabolize PAHs by liver mixed-function oxidases, with little evidence of accumulation; translocate conjugated PAH metabolites to the gall bladder prior to excretion in feces and urine; and have mixed-function oxidase degradation rates that are significantly modified by sex, age, diet, water temperature, dose-time relationships, and other variables. In addition, many species of fishes can convert PAHs, benzo(a)pyrene for example, to potent mutagenic metabolites, but because detection of the 7,8-dihydrodiol, 9,10-epoxide by analytical

methods is extremely difficult, most investigators must use biological assays, such as the Ames test, to detect mutagenic agents. At present, the interaction effects of PAHs with inorganic and other organic compounds are poorly understood. Specific examples of the above listed phenomena for PAH compounds and teleosts are documented for benzo(a)pyrene (Ahokas et al. 1975; Lu et al. 1977; Gerhart and Carlson 1978; Melius et al. 1980; Varanasi et al. 1980, 1984; Stegeman et al. 1982; Couch et al. 1983; Hendricks 1984; Melius 1984; Schoor 1984; Schoor and Srivastava 1984; Hendricks et al. 1985; Neff 1985; Fair 1986; von Hofe and Puffer 1986), 3-methylcholanthrene (Gerhart and Carlson 1978; Melius et al. 1980; Melius and Elam 1983; Schoor and Srivastava 1984; Neff 1985), benz(a)anthracene, chrysene, and pyrene (Gerhard and Carlson 1978), and 7,12-dimethylbenz(a)anthracene (Stegeman et al. 1982).

Baumann et al. (1982) summarized reports on increasing frequencies of liver tumors in wild populations of fish during the past decade, especially in brown bullhead (Ictalurus nebulosus) from the Fox River, Illinois (12% tumor frequency), in Atlantic hagfish (Myxine glutinosa) from Swedish estuaries (6%), in English sole from the Duwamish estuary, Washington (32%), and in tomcod (Microgadus tomcod) from the Hudson River, New York (25%). In all of these instances, significant levels of contaminants were present in the sediments, including PAHs. PAHs have been identified as genotoxic pollutants in sediments from the Black River, Ohio, where a high incidence of hepatoma and other tumors has been observed in ictalurid fishes (West et al. 1984, 1986). Reports of tumors in Great Lakes fish populations have been increasing. Tumors of thyroid, gonad, skin, and liver are reported, with tumor frequency greatest near areas contaminated by industrial effluents such as PAHs; liver tumors were common among brown bullhead populations at sites with large amounts of PAHs in sediments (Baumann 1984). A positive relationship was finally established between sediment PAH levels and prevalence of liver lesions in English sole in Puget Sound, Washington (Malins et al. 1984; Varanasi et al. 1984), and sediment levels and liver tumor frequency in brown bullheads from the Black River, Ohio (Baumann and Harshbarger 1985; Black et al. 1985). Sediment PAH levels in the Black River, Ohio, from the vicinity of a coke plant outfall, were up to 10,000 times greater than those from a control location: concentrations were greater than 100 mg/kg for pyrene, fluoranthene, and phenanthrene; between 50 and 100 mg/kg for benz(a)anthracene, chrysene, and benzofluoranthenes; and between 10 and 50 mg/kg for individual naphthalenes, benzo(e)pyrene, benzo(a)pyrene, perylene, indeno(1,2,3-cd)pyrene, benzo(g,h,i)perylene, and anthanthrene (Baumann et al. 1982). Brown bullheads from this location contained >1.0 mg/kg of acenapthalene (2.4), phenanthrene (5.7), fluoranthene (1.9), and pyrene (1.1), and lower concentrations of heavier molecular weight PAHs; bullheads also exhibited a high (33%) liver tumor frequency, which seemed to correspond to their PAH body burdens. Investigators concluded that the elevated frequency of liver neoplasia in Black River bullheads was chemically induced, and was the result of exposure to PAHs (Baumann et al. 1982; Baumann and Harshbarger 1985).

Neoplasms in several species of fishes have been produced experimentally with 3-methylcholanthrene, acetylaminofluorene, benzo(a)pyrene, and 7,12-dimethylbenz(a)anthracene, with tumors evident 3 to 12 months postexposure (Couch and Harshbarger 1985; Hendricks et al. 1985). Under laboratory conditions, liver neoplasms were induced in two species of minnows (Poeciliopsis spp.) by repeated short-term exposures (6 hours once a week, for 5 weeks) to an aqueous suspension of 5 mg/l of 7,12-dimethyl-benz(a)anthracene. About 44% of the fish surviving this treatment developed hepatocellular neoplasms 6 to 9 months postexposure (Schultz and Schultz 1982). Eastern mudminnows (Umbra pygmaea) kept in water containing up to 700 ug PAHs/l for 11 days showed increased frequencies of chromosomal aberrations in gills: 30% vs. 8% in controls (Prein et al. 1978). High dietary benzo(a)pyrene levels of 500 mg/kg produced significant elevations in hepatic mixed-function oxidase levels in rainbow trout after 9 weeks (Hendricks et al. 1985). Rainbow trout fed diets containing 1,000 mg benzo(a)pyrene/kg for 12 months developed liver tumors (Couch et al. 1983). About 25% of rainbow trout kept on diets containing 1,000 mg benzo(a)pyrene/kg for 18 months had histologically confirmed liver neoplasms as compared to 15% after 12 months, with no evidence of neoplasia in controls (Hendricks et al. 1985). Young English sole may activate and degrade carcinogenic PAHs, such as benzo(a)pyrene, to a greater extent than adults, but additional research is needed to determine if younger fish are at greater risk than older sole to PAH-induced toxicity (Varanasi et al. 1984). In English sole, a high significant positive correlation between PAH metabolites (1-and 3-hydroxy benzo(a)pyrene, hydroxy and dihydrodiol metabolites of pyrene and fluoranthene) in bile, and idiopathic liver lesions, prevalance of neoplasms, megalocytic hepatosis, and total number of hepatic lesions (Krahn et al. 1986) suggests that selected PAH metabolites and key organs or tissues may be the most effective monitors of PAH contamination in aquatic organisms.

In addition to those effects of PAHs emphasizing survival, uptake, depuration, and carcinogenesis previously listed, a wide variety of additional effects have been documented for aquatic organisms. These include: inhibited reproduction of daphnids and delayed emergence of larval midges by fluorene (Finger et al. 1985); decreased respiration and heart rate in mussels (Mytilus californianus) by benzo(a)pyrene (Sabourin and Tullis 1981); increased weight of liver, kidney, gall bladder, and spleen of sea catfish (Arius felis) by 3-methylcholanthrene, which was dose-related (Melius and Elam 1983); photosynthetic inhibition of algae and macrophytes by anthracene, naphthalene, phenanthrene, pyrene (Neff 1985), and fluorene (Finger et al. 1985); immobilization of the protozoan, Paramecium caudatum, by anthracene, with an EC-50 (60 min) of 0.1 ug/l (EPA 1980); perylene accumulation by algae (Stegeman 1981); accumulation without activation of benzo(a)pyrene and benzo(a)anthracene by a marine protozoan (Parauronema acutum), and biotransformation of various fluorenes by P. acutum to mutagenic metabolites (Lindmark 1981); interference by toluene and anthracene with benzo(a)pyrene uptake by freshwater amphipods (Landrum 1983); abnormal blood chemistry in oysters (Crassostrea virginica) exposed for one year to 5 ug 3-methyl-

cholanthrene/l (Couch et al. 1983); and enlarged livers in brown bullheads from a PAH-contaminated river (Fabacher and Baumann 1985).

AMPHIBIANS AND REPTILES

Limited data were available on biological effects of benzo(a)pyrene, 3-methylcholanthrene, and perylene to reptiles and amphibians (Balls 1964; Stegeman 1981; Anderson et al. 1982; Schwen and Mannering 1982a, 1982b; Couch et al. 1983).

Implantation of 1.5 mg of benzo(a)pyrene crystals into the abdominal cavity of adult South African clawed toads (Xenopus laevis) produced lymphosarcomas in 11 of the 13 toads (85%) after 86 to 288 days (Balls 1964). Immature toads were more resistant, with only 45% bearing lymphoid tumors of liver, kidney, spleen, or abdominal muscle 272 to 310 days after implantation of 1.5 mg of benzo(a)pyrene crystals in the dorsal lymph sac or abdominal cavity. Implantation of 3-methylcholanthrene crystals into X. laevis provokes development of lymphoid tumors similar to those occurring naturally in this species; moreover, these tumors are readily transplantable into other Xenopus or into the urodele species Triturus cristatus (Balls 1964). Intraperitoneal injection of perylene into tiger salamanders can result in hepatic tumors (Couch et al. 1983).

A critical point of interaction between PAHs and reptiles/amphibians involves the transformation of these compounds by cytochrome P-450-dependent monooxygenase systems (Stegeman 1981); in general, reaction rates in this group are considerably slower than those observed in hepatic microsomes from mammals (Schwen and Mannering 1982a). Mixed-function oxidation systems can be induced in liver and skin of tiger salamanders by perylene (Couch et al. 1983) and 3-methylcholanthrene (Anderson et al. 1982), and in liver of the leopard frog (Rana pipiens) and garter snake (Thamnophis sp.) by benzo(a)pyrene and 3-methylcholanthrene (Stegeman 1981; Schwen and Mannering 1982a, 1982b). A single dose of 40 mg/kg body weight of 3-methylcholanthrene was sufficient to induce mixed-function oxidase activity for several weeks in the leopard frog (Schwen and Mannering 1982b). Amphibians, including tiger salamanders, are quite resistant to PAH carcinogenesis when compared to mammals, according to Anderson et al. (1982). This conclusion was based on studies with Ambystoma hepatic microsomes and their inability to produce mutagenic metabolites of benzo(a)pyrene and perylene (as measured by bacterial Salmonella typhimurium strains used in the Ames test); however, rat liver preparations did produce mutagenic metabolites under these procedures (Anderson et al. 1982).

BIRDS

Only two articles were available on PAHs and avian wildlife, and both concerned mallards (Anas platyrhynchos). In one study, Patton and Dieter (1980) fed mallards diets that contained 4,000 mg PAHs/kg (mostly as naphthalenes, naphthenes, and phenanthrene) for a period of 7 months. No mortality or visible signs of toxicity were evident during exposure; however, liver weight increased 25% and blood flow to liver increased 30%, when compared to controls. In the second study, Hoffman and Gay (1981) measured embryotoxicity of various PAHs applied externally, in a comparatively innocuous synthetic petroleum mixture, to the surface of mallard eggs. The most embryotoxic PAH tested was 7,12-dimethylbenz(a)anthracene: approximately 0.002 ug/egg (equivalent to about 0.036 ug/kg fresh weight, based on an average weight of 55 g per egg) caused 26% mortality in 18 days, and, among survivors, produced significant reduction in embryonic growth and a significant increase in the percent of anomalies, e.g., incomplete skeletal ossification, defects in eye, brain, liver, feathers, and bill. At 0.01 ug 7,12-dimethylbenz(a)anthracene/egg, only 10% survived to day 18. Similar results were obtained with 0.015 ug (and higher) chrysene/egg. For benzo(a)pyrene, 0.002 ug/egg did not affect mallard survival, but did cause embryonic growth reduction and an increased incidence of abnormal survivors. At 0.01 ug benzo(a)pyrene/egg, 60% died in 18 days; at 0.05 ug/egg, 75% were dead within 3 days of treatment. Embryos may contain microsomal enzymes that can metabolize PAHs to more highly toxic intermediates than can adults, and avian embryos may have a greater capacity to metabolize PAHs in this manner than do mammalian embryos and fetuses (as quoted in Hoffman and Gay 1981); this observation warrants additional research. Several investigators have suggested that the presence of PAHs in petroleum, including benzo(a)pyrene, chrysene, and 7,12-dimethylbenz(a)anthracene, significantly enhances the overall embryotoxicity in avian species, and that the relatively small percent of the aromatic hydrocarbons contributed by PAHs in petroleum may confer much of the adverse biological effects reported after eggs have been exposed to microliter quantities of polluting oils (Hoffman and Gay 1981; Albers 1983).

MAMMALS

Numerous PAH compounds are distinct in their ability to produce tumors in skin and in most epithelial tissues of practically all animal species tested; malignancies were often induced by acute exposures to microgram quantities. In some cases, the latency period can be as short as 4 to 8 weeks, with the tumors resembling human carcinomas (EPA 1980). Certain carcinogenic PAHs are capable of passage across skin, lungs, and intestine, and can enter the rat fetus, for example, following intragastric or intravenous administration to pregnant dams (EPA 1980). In most cases, the process of carcinogenesis occurs

over a period of many months in experimental animals, and many years in man. The tissue affected is determined by the route of administration and species under investigation. Thus, 7,12-dimethylbenz(a)anthracene is a potent carcinogen for the mammary gland of young female rats after oral or intravenous administration; dietary benzo(a)pyrene leads to leukemia, lung adenoma, and stomach tumors in mice; and both PAH compounds can induce hepatomas in skin of male mice when injected shortly after birth (Dipple 1985). Acute and chronic exposure to various carcinogenic PAHs have resulted in destruction of hematopoietic and lymphoid tissues, ovotoxicity, antispermatogonic effects, adrenal necrosis, changes in the intestinal and respiratory epithelia, and other effects (Table 7; EPA 1980; Lee and Grant 1981). For the most part, however, tissue damage occurs at dose levels that would also be expected to induce carcinomas, and thus the threat of malignancy predominates in evaluating PAH toxicity. There is a scarcity of data available on the toxicological properties of PAHs which are not demonstrably carcinogenic to mammals (EPA 1980; Lee and Grant 1981).

Target organs for PAH toxic action are diverse, due partly to extensive distribution in the body and also to selective attack by these chemicals on proliferating cells (EPA 1980). Damage to the hematopoietic and lymphoid system in experimental animals is a particularly common observation (EPA 1980). In rats, the target organs for 7,12-dimethylbenz(a)anthracene are skin, small intestine, kidney, and mammary gland, whereas in fish the primary target organ is liver (Schultz and Schultz 1982). Application of carcinogenic PAHs to mouse skin leads to destruction of sebaceous glands and to hyperplasia, hyperkeratosis, and ulceration (EPA 1980). Tumors are induced in mouse skin by the repeated application of small doses of PAHs, by a single application of a large dose, or by the single application of a subcarcinogenic dose (initiation) followed by repeated application of certain noncarcinogenic agents (promotion) (Dipple 1985). Newborn mice were highly susceptible to 3-methylcholanthrene, with many mice dying from acute or chronic wasting disease following treatment; some strains of mice eventually developed thynomas, but other strains showed no evidence despite serious damage to the thymus (EPA 1980).

In general, PAH carcinogens transform cells through genetic injury involving metabolism of the parent compound to a reactive diol epoxide. This, in turn, can then form adducts with cellular molecules, such as DNA, RNA, and proteins, resulting in cell transformation (Dipple 1985; Ward et al. 1985). In the case of benzo(a)pyrene, one isomer of the 7,8-diol, 9,10-epoxide is an exceptionally potent carcinogen to newborn mice and is believed to be the ultimate carcinogenic metabolite of this PAH (Slaga et al. 1978). One of the most toxicologically significant processes involved in the response to PAH absorption is the interaction with drug metabolizing enzyme systems (Lee and Grant 1981). Increased production of mixed-function oxidase enzymes in various small mammals has been induced by halogenated napthalenes (Campbell et al. 1983), 3-methylcholanthrene (Miranda and Chhabra 1980), and numerous other PAHs (EPA 1980). PAH metabolites produced by microsomal enzymes in mammals

Table 7. Some effects of PAHs on selected laboratory animals.

Effect (units), organism, PAH compound	Concentration	Reference[a]
LD-50, ACUTE ORAL (mg/kg body weight)		
Rodents (*Rattus* spp., *Mus* spp.)		
Benzo(a)pyrene	50	Sims and Overcash 1978
Phenanthrene	700	
Naphthalene	1,780	
Fluoranthene	2,000	
CARCINOGENICITY, CHRONIC ORAL (mg/kg body weight)		
Rodents		
7,12-dimethylbenz(a)anthracene	0.00004-0.00025	Lo and Sandi 1978
Benzo(a)pyrene	0.002	Sims and Overcash 1983
Dibenz(a,h)anthracene	0.006	
Benz(a)anthracene	2.0	
Benzo(b)fluoranthene	40.0	
Benzo(k)fluoranthene	72.0	
Indeno(1,2,3-cd)pyrene	72.0	
Chrysene	99.0	
Anthracene	3,300.0	
CARCINOGENICITY, APPLIED EXTERNALLY AS TOPICAL (mg)		
Mice, *Mus* spp.		
Benzo(a)pyrene	0.001	Lo and Sandi 1978
Dibenz(a,c)anthracene	0.001	
7,12-dimethylbenz(a)anthracene	0.02	
Dibenz(a,j)anthracene	0.039	
Anthracene	0.08	
Benzo(g,h,i)perylene	0.8	
Benz(a)anthracene	1.0	
CARCINOGENICITY, SUBCUTANEOUS (mg)		
Mice		
Dibenz(a,h)anthracene		
Adults	>0.0002	

Table 7. (Continued)

Effect (units), organism, PAH compound	Concentration	Reference[a]
Newborn	>0.00008	
Dibenzo(a,i)pyrene		
In sesame oil	0.05	
In peanut oil	0.6	
Benzo(a)pyrene	0.06	
Dibenzo(a,e)pyrene	>0.6	
Benzo(b)fluoranthene	1.8	
Benz(a)anthracene	5.0	
Dibenzo(a,h)pyrene	6.0	
TESTICULAR DAMAGE (mg)		
Rat, Rattus spp.		
Benzo(a)pyrene, oral	100.0 (no effect)	EPA 1980
7,12-dimethylbenz(a)anthracene		
Intravenous		
Young rats	0.5 - 2.0	
Older rats	5.0	
Oral	20.0	
OOCYTE AND FOLLICLE DESTRUCTION, SINGLE INTRAPERITONEAL INJECTION (mg/kg body weight)		
Mice		
Benzo(a)pyrene	80.0	Mattison 1980
3-methylcholanthrene	80.0	
7,12-dimethylbenz(a)anthracene	80.0	
ALTERED BLOOD SERUM CHEMISTRY AND NEPHROTOXICITY, SINGLE INTRAPERITONEAL INJECTION (mg/kg body weight)		
Rat		
Phenanthrene	150.0	Yoshikawa et al. 1985
Pyrene	150.0	

Table 7. (Concluded)

Effect (units), organism, PAH compound	Concentration	Reference[a]
FOOD CONSUMPTION, DAILY FOR 5 DAYS (mg/kg body weight)		
Deer mice, Peromyscus maniculatus		
2-methoxynaphthalene		
30% reduction	825	Schafer and Bowles 1985
2-ethoxynaphthalene		
3% reduction	1,213	
House mice, Mus musculus		
2-methoxynaphthalene		
50% reduction	825	
2-ethyoxynaphthalene		
50% reduction	1,213	

[a]Each reference applies to the values in the same row, and in the rows that follow for which no other reference is indicated.

can be arbitrarily divided into water soluble groups, and organosoluble groups such as phenols, dihydrodiols, hydroxymethyl derivatives, quinones, and epoxides (EPA 1980). In the case of benzo(a)pyrene, the diol epoxides are usually considered as the ultimate carcinogens. Other microsomal enzymes convert epoxide metabolites to easily excretable water soluble compounds, with excretion primarily through feces and the hepatobiliary system (EPA 1980). Interspecies differences in sensivity to PAH-induced carcinogenesis are due largely to differences in levels of mixed function oxidase activities, and these will directly affect rates at which active metabolites are converted to less active products (Neff 1979).

Investigators agree that unsubstituted aromatic PAHs with less than 4 condensed rings have not shown tumorgenic activity; that many, but not all, 4-, 5-, and 6-ring PAH compounds are carcinogenic; and that only a few unsubstituted hydrocarbons with 7 rings or greater are tumorogenic or carcinogenic (Neff 1979; EPA 1980; Dipple 1985). Many PAH compounds containing 4 and 5 rings, and some containing 6 or more rings, provoke local tumors after repeated application to the dorsal skin of mice; the tumor incidence exhibited a significant dose-reponse relationship (Grimmer et al. 1985). Among unsubstituted PAHs containing a nonaromatic ring, e.g., cholanthrene and acenapthanthracene, all active carcinogens retained an intact phenanthrene segment (EPA 1980). The addition of alkyl substitutents in certain positions in the ring system of a fully aromatic PAH will often confer carcinogenic activity or dramatically enhance existing carcinogenic potency. For example, monomethyl substitution of benz(a)anthracene can lead to strong carcinogenicity in mice, with potency depending on the position of substitution in the order 7 > 6 > 8 = 12 > 9; a further enhancement of carcinogenic activity is produced by appropriate dimethyl substitution, with 7,12-dimethylbenz(a)anthracene among the most potent PAH carcinogens known. Alkyl substitution of partially aromatic condensed ring systems may also add considerable carcinogenic activity, as is the case with 3-methylcholanthrene. With alkyl substitutes longer than methyl, carcinogenicity levels decrease, possibly due to a decrease in transport through cell membranes (EPA 1980).

A good correlation exists between skin tumor initiating activities of various benzo(a)pyrene metabolites and their mutagenic activity in mammalian cell mutagenesis systems (Slaga et al. 1978), although variations in chromosome number and structure may accompany tumors induced by various carcinogenic PAHs in rats, mice, and hamsters (Bayer 1978; EPA 1980). Active PAH metabolites, e.g., dihydrodiols or diol epoxides, can produce sister chromatid exchanges in Chinese hamster ovary cell (Bayer 1978; EPA 1980; Pal 1984). When exchanges were induced by the diol epoxide, a close relationship exists between the frequency of sister chromatid exchanges and the levels of deoxyribnucleoside-diol-epoxide adduct formation (Pal 1984). In general, noncarcinogenic PAHs were not mutagenic (EPA 1980).

Laboratory studies with mice have shown that many carcinogenic PAHs adversely affect the immune system, thus directly impacting an organism's

general health, although noncarcinogenic analogues had no immunosuppressive effect; further, the more carcinogenic the PAH, the greater the immunosuppression (Ward et al. 1985).

Destruction of oocytes and follicles in mice ovary is documented following intraperitoneal injection of benzo(a)pyrene; 3-methylcholanthrene, and 7,12-dimethylbenz(a)anthracene; the rate of destruction was proportional to the activity of the ovarian cytochrome P-450 dependent monooxygenase, as well as the carcinogenicity of the PAH (Mattison 1980). However, no information is presently available to indicate whether PAHs present a hazard to reproductive success. In those cases where teratogenic effects are clearly evident, e.g., 7, 12-dimethylbenz(a)anthracene, the required doses were far in excess of realistic environmental exposures (Lee and Grant 1981).

Numerous studies show that unsubstituted PAHs do not accumulate in mammalian adipose tissues despite their high lipid solubility, probably because they tend to be rapidly and extensively metabolized (EPA 1980; Lee and Grant 1981).

Biological half-life (Tb 1/2) of PAHs is limited, as judged by rodent studies. In the case of benzo(a)pyrene and rat blood and liver, Tb 1/2 values of 5 to 10 minutes were recorded; the initial rapid elimination phase was followed by a slower disappearance phase lasting 6 hours or more (EPA 1980). Tb 1/2 values from the site of subcutaneous injection in mice were 1.75 weeks for benzo(a)pyrene, 3.5 weeks for 3-methylcholanthrene, and 12 weeks for dibenz(a,h)anthracene; the relative carcinogenicity of each compound was directly proportional to the time of retention at the injection site (Pucknat 1981).

Many chemicals are known to modify the action of carcinogenic PAHs in experimental animals, including other PAHs that are weakly carcinogenic or noncarcinogenic. The effects of these modifiers on PAH metabolism appear to fall into three major categories: those which alter the metabolism of the carcinogen, causing decreased activation or increased detoxification; those which scavenge active molecular species of carcinogens to prevent their reaching critical target sites in the cell; and those which exhibit competitive antagonism (DiGiovanni and Slaga 1981b). For example, benz(a)anthracene, a weak carcinogen, when applied simultaneously with dibenz(a,h)anthracene, inhibited the carcinogenic action of the latter in mouse skin; a similar case is made for benzo(e)pyrene or dibenz(a,c)anthracene applied to mouse skin shortly prior to initiation with 7,12-dimethylbenz(a)anthracene, or 3-methylcholanthrene (DiGiovanni and Slaga 1981a). Benzo(a)pyrene, a known carcinogen, interacts synergistically with cyclopenta(cd)pyrene, a moderately strong carcinogen found in automobile exhausts, according to results of mouse skin carcinogenicity studies (Rogan et al. 1983). Other PAH combinations were cocarcinogenic, such as benzo(e)pyrene, pyrene, and fluoranthene applied repeatedly with benzo(a)pyrene to the skins of mice (DiGiovanni and Slaga 1981a). Effective

inhibitors of PAH-induced tumor development include selenium, vitamin E, ascorbic acid, butylated hydroxytoluene, and hydroxyanisole (EPA 1980). In addition, protective effects against PAH-induced tumor formation have been reported for various naturally occurring compounds such as flavones, retenoids, and vitamin A (EPA 1980). Until these interaction effects are clarified, the results of single substance laboratory studies may be extremely difficult to apply to field situations of suspected PAH contamination. Additional work is also needed on PAH dose-response relationships, testing relevant environmental PAHs for carcinogenicity, and elucidating effects of PAH mixtures on tumor formation (Grimmer 1983).

RECOMMENDATIONS

At present, no criteria or standards have been promulgated for PAHs by any regulatory agency for the protection of sensitive species of aquatic organisms or wildlife. This observation is not unexpected in view of several factors: (1) the paucity of data on PAH background concentrations in wildlife and other natural resources; (2) the absence of information on results of chronic oral feeding studies of PAH mixtures and the lack of a representative PAH mixture for test purposes; and (3) the demonstrable--and as yet, poorly understood--effects of biological modifiers, such as sex, age, and diet, and interaction effects of PAHs with inorganic and other organic compounds, including other PAHs.

Nevertheless, the growing data base for aquatic life indicates a number of generalizations: (1) many PAHs are acutely toxic at concentrations between 50 and 1,000 ug/l; (2) deleterious sublethal responses are sometimes observed at concentrations in the range of 0.1 to 5.0 ug/l; (3) uptake can be substantial, but depuration is usually rapid except in some species of invertebrates; and (4) whole body burdens in excess of 300 ug benzo(a)pyrene/kg (and presumably other PAHs) in certain teleosts would be accompanied by a rise in the activity of detoxifying enzymes.

Current aquatic research has focused on PAHs because of their known relationship with carcinogenesis and mutagenesis. Many reports exist of high incidences of cancer-like growths and developmental anomalies in natural populations of aquatic animals and plants, but none conclusively demonstrate the induction of cancer by exposure of aquatic animals to environmentally realistic levels of carcinogenic PAHs in the water column, diet, or sediments (Neff 1982b, 1985). However, recent studies by Baumann, Malins, Black, Varanasi and their coworkers, among others, have now established that sediments heavily contaminated with PAHs from industrial sources were the direct cause of elevated PAH body burdens and elevated frequencies of liver neoplasia in fishes from these locales. At present, only a few sites containing high PAH concentrations in sediments have been identified (Couch and Harshbarger 1985), suggesting an urgent need to identify and to evaluate other PAH-contaminated aquatic sites. Most fishery products consumed by upper trophic levels, including man, contain PAH concentrations similar to those in green vegetables and smoked and charcoal-broiled meats, and would probably represent a minor source of PAH toxicity; however, consumption of aquatic

organisms, especially filter-feeding bivalve molluscs, from regions severely contaminated with petroleum or PAH-containing industrial wastes, should be avoided (Jackim and Lake 1978; Neff 1982b). Neff (1982b) suggested that repeated consumption of PAH-contaminated shellfish may pose a cancer risk to humans. If true, this needs to be evaluated using seabirds, pinnipeds, and other wildlife groups which feed extensively on molluscs that are capable of accumulating high burdens of carcinogenic PAHs, in order to determine if similar risks exist.

For avian wildlife, data are missing on PAH background concentrations and on acute and chronic toxicity; these data should be collected posthaste. Studies with mallard embryos and PAHs applied to the egg surface showed toxic and adverse sublethal effects at concentrations between 0.036 and 0.18 ug PAH/kg whole egg (Hoffman and Gay 1981). Additional research is needed on petroleum-derived PAHs and their effects on developing embryos of seabirds and other waterfowl.

PAH criteria for human health protection (Table 8) were derived from tests with small laboratory mammals, primarily rodents. Accordingly, these proposed criteria should become interim guidelines for protection of nonhuman mammalian resources pending acquisition of more definitive data. The proposed PAH criteria are controversial. Pucknat (1981) states that there is no way at present to quantify the potential human health risks incurred by the interaction of any PAH with other PAHs or with other agents in the environment, including tumor initiators, promotors, and inhibitors. The problem arises primarily from the diversity of test systems and bioassay conditions used for determining carcinogenic potential of individual PAHs in experimental animals, and is confounded by the lack of a representative PAH mixture for test purposes, the absence of data for animal and human chronic oral exposures to PAH mixtures, and the reliance on data derived from studies with benzo(a)pyrene to produce generalizations concerning environmental effects of PAHs--generalizations which may not be scientifically sound--according to Pucknat (1981). EPA (1980) emphasizes that only a small percentage of PAH compounds are known to be carcinogenic, and that measurements of total PAHs (i.e., the sum of all multiple fused-ring hydrocarbons having no heteroatoms) can not be equated with carcinogenic potential; furthermore, when the term "total PAHs" is used, the compounds being considered should be specified for each case. Lee and Grant (1981) state that an analysis of dose-response relationships for PAH-induced tumors in animals shows, in some cases, deviation from linearity in dose-response curves, especially at low doses, suggesting a two-stage model consistent with a linear nonthreshold pattern. Because overt tumor induction follows a dose-response relationship consistent with a multihit promotion process, the multihit component of carcinogenesis may be supplied by environmental stimuli not necessarily linked or related to PAH exposure.

The well-documented existence of carcinogenic and anticarcinogenic agents strongly suggests that a time assessment of carcinogenic risk for a particular

Table 8. Proposed PAH criteria for human health protection (modified from EPA 1980; Lee and Grant 1981; Pucknat 1981).

Criterion, PAH group, and units	Concentration
DRINKING WATER	
Total PAHs	
ug/l[a]	0.0135-0.2
Daily intake, ug[a]	0.027-0.4
Yearly intake, ug	4.0
Benzo(a)pyrene	
ug/l	0.00055
Daily intake, ug	0.0011
Carcinogenic PAHs	
ug/l[b]	0.0021
Daily intake, ug[b]	0.0042
ug/l[c]	
Cancer risk 10^{-5}	0.028
Cancer risk 10^{-6}	0.0028
Cancer risk 10^{-7}	0.00028
Daily intake, ug[c]	
Cancer risk 10^{-5}	0.056
Cancer risk 10^{-6}	0.0056
Cancer risk 10^{-7}	0.00056
FOOD	
Total PAHs	
Daily intake, ug[d]	1.6-16.0
Yearly intake, ug	4,150.0
Benzo(a)pyrene	
Daily intake, ug[e]	0.16-1.6
AIR	
Total PAHs	
ug/m^3	0.0109
Daily intake, ug[f]	0.164-0.251
Cyclohexane extractable fractions	
Coke oven emissions, coal tar products, ug/m^3, 8 to 10 hour-weighted average	100.0-150.0

Table 8. (Concluded)

Criterion, PAH group, and units	Concentration
Benzene soluble fractions	
Coal tar pitch volatiles, ug/m^3, 8-hour, time-weighted average	200.0
Benzo(a)pyrene	
ug/m^3	0.0005
Daily intake, ug[f]	0.005-0.0115
Carcinogenic PAHs	
ug/m^3	0.002
Daily intake, ug[f]	0.03-0.046
ALL SOURCES	
Total PAHs	
Daily, ug	1.79-16.6
Benzo(a)pyrene	
Daily intake, ug	0.166-1.61
Daily allowable limit, ug[g]	0.048
Carcinogenic PAHs (except diet)	
Daily intake, ug[b]	0.086-0.102

[a]Total of 6 PAHs: fluoranthene, benzo(a)pyrene, benzo(g,h,i)perylene, benzo(b)fluoranthene, benzo(k)fluoranthene, and indeno(1,2,3-cd)pyrene.

[b]Total of 3 PAHs: benzo(a)pyrene, benzo(j)fluoranthene, and indeno(1,2,3-cd)pyrene.

[c]Based on all carcinogenic PAHs.

[d]Assuming 1,600 g food daily, 70 kg adult, 1 to 10 ug total PAHs/diet.

[e]As above, except 0.1 to 1.0 ug benzo(a)pyrene/diet.

[f]Assuming average of 15 to 23 m^3 of air inhaled daily.

[g]From Wang and Meresz (1982).

PAH can be evaluated only through a multifactorial analysis (Lee and Grant 1981). One of the most toxicologically significant processes involved in the response to PAH absorption is the interaction with drug metabolizing enzyme systems. The induction of this enzyme activity in various body tissues by PAHs and other xenobiotics is probably critical to the generation of reactive PAH metabolites at the target site for tumor induction. At present, wide variations occur in human and animal carcinogen-metabolizing capacity. Moreover, it has not yet been possible to definitely correlate enzyme activity with susceptibility to carcinogenesis. The obligatory coupling of metabolic activation with PAH-induced neoplasia in animals indicates that the modulation of drug metabolizing enzymes is central to carcinogenesis (Lee and Grant 1981).

PAHs from drinking water contribute only a small proportion of the average total human intake (Harrison et al. 1975). The drinking water quality criterion for carcinogenic PAH compounds is based on the assumption that each compound is as potent as benzo(a)pyrene, and that the carcinogenic effect of the compounds is proportional to the sum of their concentrations (EPA 1980). Based on an oral feeding study of benzo(a)pyrene in mice, the concentration of this compound estimated to result in additional risk of one additional case for every 100,000 individuals exposed (i.e, 10^{-5}) is 0.028 ug/l. Therefore, with this assumption, the sum of the concentrations of all carcinogenic PAH compounds should be less than 0.028 ug/l in order to keep the lifetime cancer risk below 10^{-5} (EPA 1980). The corresponding recommended criteria which may result in an incremental cancer risk of 10^{-6} and 10^{-7} over the lifetime are 0.0028 and 0.00028 ug/l, respectively (Table 8). If the above estimates are made for consumption of aquatic organisms only, the levels are 0.311 (10^{-5}), 0.031(10^{-6}), and 0.003(10^{-7}) ug/kg, respectively (EPA 1980). The use of contaminated water for irrigation can also spread PAHs into other vegetable foodstuffs (EPA 1980). When vegetables grown in a PAH-polluted area are thoroughly washed and peeled, their contribution to total PAH intake in humans is not significant (Wang and Meresz 1982). Herbivorous wildlife, however, may ingest significant quantities of various PAHs from contaminated vegetables--but no data were available on this subject.

PAHs are widely distributed in the environment as evidenced by their detection in sediments, soils, air, surface waters, and plant and animal tissues. However, the ecological impact of PAHs is uncertain. PAHs show little tendency for bioconcentration, despite their high lipid solubility (Pucknat 1981), probably because most PAHs are rapidly metabolized. Sims and Overcash (1983) list a variety of research needs regarding PAHs in soil-plant systems. Specifically, research is needed to establish: the rates of PAH decomposition in soils; the soil PAH levels above which PAH constituents adversely affects the food chain; and enhancement factors that increase degradation rates of PAHs, especially PAHs with more than 3 rings. Once these factors have been determined, PAH disposal into soils may become feasible at environmentally nonhazardous levels.

Diet is the major source of PAHs to humans. Authorities agree that most foods contain 1 to 10 ug total PAHs/kg fresh weight, that smoking or barbecuing fish and meats increases total PAH content up to 100X, that contaminated molluscs and crustaceans may contribute significantly to PAH intake, and that PAH carcinogenic risk to humans has existed at least since man began to cook his food (Barnett 1976; EPA 1980; Lee and Grant 1981; Lawrence and Weber 1984a). A total of 22 PAHs has been identified in foods, of which 11 have been found to be carcinogenic in experimental animals. Of these, only 5 (benzo(a)pyrene, benz(a)anthracene, 3-methylcholanthrene, dibenz(a,h)anthracene, and 7,12-dimethylbenz(a)anthracene) have been demonstrated to induce tumors following oral administration to rats and mice, and only 3 of the 11 exhibited positive dose-response relationships in chronic studies with mice (Lo and Sandi 1978). At the present time, there is no evidence that any of the 11 known carcinogenic PAHs or their combinations can cause cancer in human beings via the oral route, especially in quantities likely to be present in foods (Lo and Sandi 1978).

In view of the carcinogenic characteristics of many PAH compounds, their increasing concentrations in the environment should be considered alarming, and efforts should be made to reduce or eliminate them wherever possible (Suess 1976).

LITERATURE CITED

Ahokas, J.T., O. Pelkonen, and N.T. Karki. 1975. Metabolism of polycyclic hydrocarbons by a highly active aryl hydrocarbon hydroxylase system in the liver of a trout species. Biochem. Biophys. Res. Comm. 63:635-641.

Albers, P.H. 1983. Effects of oil on avian reproduction: a review and discussion. Pages 78-96 in The effects of oil on birds. A multidiscipline symposium. Tri-State Bird Rescue and Research, Inc., Wilmington, Delaware.

Anderson, R.S., J.E. Doos, and F.L. Rose. 1982. Differential ability of Ambystoma tigrinum hepatic microsomes to produce mutagenic metabolites from polycyclic aromatic hydrocarbons and aromatic amines. Cancer Lett. 16:33-41.

Ang, K.P., H. Gunasingham, and B.T. Tay. 1986. The distribution of polynuclear aromatic hydrocarbons in ambient air particulates in Singapore. Environ. Monitor. Assess. 6:171-180.

Baird, W.M., R.A. Chemerys, L. Diamond, T.H. Meedel, and J.R. Whittaker. 1982. Metabolism of benzo(a)pyrene by Ciona intestinalis. Pages 191-200 in N.L. Richards and B.L. Jackson (eds.). Symposium: carcinogenic polynuclear aromatic hydrocarbons in the marine environment. U.S. Environ. Protection Agency Rep. 600/9-82-013.

Balk, L., J. Meijer, J.W. DePierre, and L.E. Appelgren. 1984. The uptake and distribution of (^{3}H) benzo(a)pyrene in the northern pike (Esox lucius). Examination by whole-body autoradiography and scintillation counting. Toxicol. Appl. Pharmacol. 74:430-449.

Balls, M. 1964. Benzpyrene-induced tumours in the clawed toad, Xenopus laevis. Experientia 20:143-145.

Barnett, D. 1976. Polycyclic aromatic hydrocarbons and foods. CSIRO Food Res. Quart. 36:8-12.

Bauer, J.E., and D.G. Capone. 1985. Degradation and mineralization of the polycylic aromatic hydrocarbons anthracene and naphthalene in intertidal marine sediments. Appl. Environ. Microbiol. 50:81-90.

Baumann, P.C. 1984. Cancer in wild freshwater fish populations with emphasis on the Great Lakes. J. Great Lakes Res. 10:251-253.

Baumann, P.C., and J.C. Harshbarger. 1985. Frequencies of liver neoplasia in a feral fish population and associated carcinogens. Mar. Environ. Res. 17:324-327.

Baumann, P.C., and J.J. Lech. In press. PAH's, metabolites, and neoplasia in feral fish populations. in U. Varanasi (ed.). PAH metabolism in the aquatic environment. CRC Press, Boca Raton, Florida.

Baumann, P.C., W.D. Smith, and W.K. Parland. In press. Contaminant concentrations and tumor frequencies in brown bullhead from an industrialized river and a recreational lake. Trans. Am. Fish. Soc.

Baumann, P.C., W.D. Smith, and M. Ribick. 1982. Hepatic tumor rates and polynuclear aromatic hydrocarbon levels in two populations of brown bullhead (Ictalurus nebulosus). Pages 93-102 in M.W. Cooke, A.J. Dennis, and G.L. Fisher (eds.). Polynuclear aromatic hydrocarbons: physical and biological chemistry. Battelle Press, Columbus, Ohio.

Bayer, U. 1978. In vivo induction of sister chromatid exchanges by three polyaromatic hydrocarbons. Pages 423-428 in P.W. Jones and R.I. Freudenthal (eds.). Carcinogenesis - a comprehensive survey. Vol. 3. Polynuclear aromatic hydrocarbons: second international symposium on analysis, chemistry, and biology. Raven Press, New York.

Bjorseth, A., and A.J. Dennis (eds.). 1980. Polynuclear aromatic hydrocarbons: chemistry and biological effects. Battelle Press, Columbus, Ohio. 1,097 pp.

Black, J., H. Fox, P. Black, and F. Bock. 1985. Carcinogenic effects of river sediment extracts in fish and mice. Pages 415-427 in R.L. Jolley, R.J. Bull, W.P. Davis, S. Katz, M.H. Roberts, Jr., and V.A. Jacobs (eds.). Water chlorination. Volume 5. Lewis Publ., Chelsea, Michigan.

Black, J.J. 1983. Epidermal hyperplasia and neoplasia in brown bullheads (Ictalurus nebulosus) in response to repeated applications of a PAH containing extract of polluted river sediment. Pages 99-111 in M. Cooke and A.J. Dennis (eds.). Polynuclear aromatic hydrocarbons: formation, metabolism and measurement. Battelle Press, Columbus, Ohio.

Black, J.J., T.F. Hart, Jr., and E. Evans. 1981. HPLC studies of PAH pollution in a Michigan trout stream. Pages 343-355 in M. Cooke and A.J. Dennis (eds.). Chemical analysis and biological fate: polynuclear aromatic hydrocarbons. Fifth international symposium. Battelle Press, Columbus, Ohio.

Boehm, P.D., and J.W. Farrington. 1984. Aspects of the polycyclic aromatic hydrocarbon geochemistry of recent sediments in the Georges Bank region. Environ. Sci. Technol. 18:840-845.

Boyle, T.P., S.E. Finger, R.L. Paulson, and C.F. Rabeni. 1985. Comparison of laboratory and field assessment of fluorene - Part II: effects on the ecological structure and function of experimental pond ecosystems. Pages 134-151 in T.P. Boyle (ed.). Validation and predictability of laboratory methods for assessing the fate and effects of contaminants in aquatic ecosystems. ASTM STP 865. American Society for Testing and Materials, Philadelphia, Pennsylvania.

Boyle, T.P., S.E. Finger, J.D. Petty, L.M. Smith, and J.N. Huckins. 1984. Distrubution and rate of disappearance of fluorene in pond ecosystems. Chemosphere 13:997-1008.

Brown, R.A., and R.J. Pancirov. 1979. Polynuclear aromatic hydrocarbons in Baltimore Canyon fish. Environ. Sci. Technol. 13:878-879.

Campbell, M.A., S. Bandiera, L. Robertson, A. Parkinson, and S. Safe. 1983. Hepta-, hexa-, tetra- and dichloronaphthalene congeners as inducers of hepatic microsomal drug-metabolizing enzymes. Toxicology 26:193-205.

Cavalieri, E., R. Roth, E. Rogan, C. Grandjean, and J. Althoff. 1978. Mechanisms of tumor initiation by polycyclic aromatic hydrocarbons. Pages 273-284 in P.W. Jones and R.I. Freudenthal (eds.). Carcinogenesis - a comprehensive survey. Vol. 3. Polynuclear aromatic hydrocarbons: second international symposium on analysis, chemistry, and biology. Raven Press, New York.

Cavalieri, E., D. Sinha, and E. Rogan. 1980. Rat mammary gland versus mouse skin: different mechanisms of activation of aromatic hydrocarbons. Pages 215-231 in A. Bjorseth and A.J. Dennis (eds.). Polynuclear aromatic hydrocarbons: chemistry and biological effects. Battelle Press, Columbus, Ohio.

Cerniglia, C.E., G.L. White, and R.H. Heflich. 1985. Fungal metabolism and detoxification of polycyclic aromatic hydrocarbons. Arch. Microbiol. 143:105-110.

Cooke, M., and A.J. Dennis (eds.). 1981. Chemical analysis and biological fate: polynuclear aromatic hydrocarbons. Fifth international symposium. Battelle Press, Columbus, Ohio. 770 pp.

Cooke, M., and A.J. Dennis (eds.). 1983. Polynuclear aromatic hydrocarbons: formation, metabolism and measurement. Battelle Press, Columbus, Ohio. 1,301 pp.

Cooke, M., and A.J. Dennis (eds.). 1984. Polynuclear aromatic hydrocarbons: mechanisms, methods and metabolism. Battelle Press, Columbus, Ohio. 1,464 pp.

Cooke, M., A.J. Dennis, and G.L. Fisher (eds.). 1982. Polynuclear aromatic hydrocarbons: physical and biological chemistry. Battelle Press, Columbus, Ohio. 947 pp.

Couch, J.A., and J.C. Harshbarger. 1985. Effects of carcinogenic agents on aquatic animals: an environmental and experimental overview. J. Environ. Sci. Health, Part C, Environ. Carcin. Rev. 3:63-105.

Couch, J.A., W.P. Schoor, W. Davis, and L. Courtney. 1983. Effects of carcinogens, mutagens, and teratogens on nonhuman species (aquatic animals). U.S. Environ. Protection Agency Rep. 600/9-83-005. 46 pp.

DiGiovanni, J., and T.J. Slaga. 1981a. Effects of benzo(e)pyrene [B(e)P] and dibenz(a,c)anthracene [DB(a,c)A] on the skin tumor-initiating activity of polycyclic aromatic hydrocarbons. Pages 17-31 in M. Cooke and A.J. Dennis (eds.). Chemical analysis and biological fate: polynuclear aromatic hydrocarbons. Fifth international symposium. Battelle Press, Columbus, Ohio.

DiGiovanni, J., and T.J. Slaga. 1981b. Modification of polycyclic aromatic hydrocarbon carcinogensis. Pages 259-292 in H.V. Gelboin and P.O. Ts'o (eds.). Polycyclic hydrocarbons and cancer. Vol. 3. Academic Press, New York.

Dipple, A. 1985. Polycyclic aromatic hydrocarbon carcinogensis: an introduction. Pages 1-17 in R.D. Harvey (ed.). Polycyclic hydrocarbons and carcinogensis. ACS Symp. Ser. 283. Amer. Chem. Soc., Washington, D.C.

Dobroski, C.J., Jr., and C.E. Epifanio. 1980. Accumulation of benzo(a)pyrene in a larval bivalve via trophic transfer. Canad. J. Fish. Aquatic Sci. 37:2318-2322.

Dunn, B.P. 1980. Polycyclic aromatic hydrocarbons in marine sediments, bivalves, and seaweeds: analysis by high-pressure liquid chromatography. Pages 367-377 in A. Bjorseth and A.J. Dennis (eds.). Polynuclear aromatic hydrocarbons: chemistry and biological effects. Battelle Press, Columbus, Ohio.

Eadie, B.J., W.R. Faust, P.F. Landrum, and N.R. Morehead. 1984. Factors affecting bioconcentration of PAH by the dominant benthic organisms of the Great Lakes. Pages 363-377 in M. Cooke and A.J. Dennis (eds.). Polynuclear aromatic hydrocarbons: mechanisms, methods and metabolism.

Battelle Press, Columbus, Ohio.

Eadie, B.J., W.R. Faust, P.F. Landrum, N.R. Morehead, W.S. Gardner, and T. Nalepa. 1983. Bioconcentrations of PAH by some benthic organisms of the Great Lakes. Pages 437-449 in M. Cooke and A.J. Dennis (eds.). Polynuclear aromatic hydrocarbons: formation, metabolism and measurement. Battelle Press, Columbus, Ohio.

Edwards, N.T. 1983. Polycyclic aromatic hydrocarbons (PAH's) in the terrestrial environment - a review. J. Environ. Qual. 12:427-441.

Eiceman, G.A., B. Davani, and J.A. Dodson. 1984. Discharge water from hydrostatic testing of natural gas pipelines as source of PAH loading into aquatic environments. Int. J. Environ. Anal. Chem. 19:27-39.

Eiceman, G.A., B. Davani, M.E. Wilcox, J.L. Gardea, and J.A. Dodson. 1985. High molecular weight hydrocarbons including polycyclic aromatic hydrocarbons in natural gas from consumer distribution pipelines and in pipeline residue. Environ. Sci. Technol. 19:603-608.

EPA. 1980. Ambient water quality criteria for polynuclear aromatic hydrocarbons. U.S. Environ. Protection Agency. Rep. 440/5-80-069. 193 pp.

Fabacher, D.L., and P.C. Baumann. 1985. Enlarged livers and hepatic microsomal mixed-function oxidase components in tumor-bearing brown bullheads from a chemically contaminated river. Environ. Toxicol. Chem. 4:703-710.

Fair, P.H. 1986. Interaction of benzo(a) pyrene and cadmium on GSH-S-trsnsferase and benzo(a)pyrene hydroxylase in the black sea bass Centropristis striata. Arch. Environ. Contam. Toxicol. 15:257-263.

Finger, S.E., E.F. Little, M.G. Henry, J.F. Fairchild, and T.P. Boyle. 1985. Comparison of laboratory and field assessment of fluorene - Part I: effects of fluorene on the survival, growth, reproduction, and behavior of aquatic organisms in laboratory tests. Pages 120-133 in T.P. Boyle (ed.). Validation and predictability of laboratory methods for assessing the fate and effects of contaminants in aquatic ecosystems. ASTM STP 865. American Society for Testing and Materials, Philadelphia, Pennsylvania.

Fox, F.R., and K.R. Rao. 1982. Accumulation, tissue distribution, and depuration of benzo(a)pyrene and benz(a)anthracene in the grass shrimp, Palaemonetes pugio. Pages 336-349 in N.L. Richards and B.L. Jackson (eds.). Symposium: carcinogenic polynuclear aromatic hydrocarbons in the marine environment. U.S. Environ. Protection Agency Rep. 600/9-82-013.

Futoma, D.J., S.R. Smith, T.E. Smith, and J. Tanaka. 1981. Polycyclic

aromatic hydrocarbons in water systems. CRC Press, Boca Raton, Florida. 190 pp.

Gelboin, H.V., and P.O. Ts'o (eds.). 1978a. Polycyclic hydrocarbons and cancer. Vol. 1. Environment, chemistry, and metabolism. Academic Press, New York. 408 pp.

Gelboin, H.V., and P.O. Ts'o (eds.). 1978b. Polycyclic hydrocarbons and cancer. Vol. 2. Molecular and cell biology. Academic Press, New York. 452 pp.

Gelboin, H.V., and P.O. Ts'o (eds.). 1981. Polycyclic hydrocarbons and cancer. Vol. 3. Academic Press, New York. 351 pp.

Gerhart, E.H., and R.M. Carlson. 1978. Hepatic mixed-function oxidase activity in rainbow trout exposed to several polycyclic aromatic compounds. Environ. Res. 17:284-295.

Greenberg, A., F. Darack, R. Harkov, P. Lioy, and J. Daisey. 1985. Polycyclic aromatic hydrocarbons in New Jersey: a comparison of winter and summer concentrations over a two-year period. Atmospher. Environ. 19:1325-1339.

Grimmer, G. (ed.). 1983. Environmental carcinogens: polycyclic aromatic hydrocarbons. Chemistry, occurrence, biochemistry, carcinogenicity. CRC Press, Boca Raton, Florida. 261 pp.

Grimmer, G., H. Brune, R. Deutsch-Wenzel, G. Dettbarn, J. Misfeld, U. Able, and J. Timm. 1985. The contribution of polycyclic aromatic hydrocarbon fractions with different boiling ranges to the carcinogenic impact of emission condensate from coal fired residential furnaces as evaluated by topical application to the skin of mice. Cancer Lett. 28:203-211.

Harrison, R.M., R. Perry, and R.A. Wellings. 1975. Polynuclear aromatic hydrocarbons in raw, potable and waste waters. Water Res. 9:331-346.

Harvey, R.G. (ed.). 1985. Polycyclic hydrocarbons and carcinogensis. ACS Symp. Ser. 283. Amer. Chem. Soc., Washington, D.C. 406 pp.

Heit, M. 1985. The relationship of a coal fired power plant to the levels of polycyclic aromatic hydrocarbons (PAH) in the sediment of Cayuga Lake. Water, Air, Soil Pollut. 24:41-61.

Hendricks, J.D. 1984. Use of small fish species in carcinogenicity testing. Pages 397-404 in K.L. Hoover (ed.). National Cancer Institute Monograph 65, Washington, D.C.

Hendricks, J.D., T.R. Meyers, D.W. Shelton, J.L. Casteel, and G.S. Bailey. 1985. Hepatocarcinogenicity of benzo(a)pyrene to rainbow trout by dietary

exposure and intraperitoneal injection. J. Natl. Cancer Inst. 74:839-851.

Herd, J.E., and F.E. Greene. 1980. Effects of perinatal exposure to benzo(a)pyrene on the aryl hydrocarbon hydroxylase system of adult rat liver. Biol. Neonate 38:291-299.

Herrmann, R., and D. Hubner. 1984. Concentrations of micropollutants (PAH, chlorinated hydrocarbons and trace metals) in the moss Hypnum cupressiforme in and around a small industrial town in southern Finland. Ann. Bot. Fennici 21:337-342.

Hites, R.A., and P.M. Gschwend. 1982. The ultimate fates of polycyclic aromatic hydrocarbons in marine and lacustrine sediments. Pages 357-365 in M. Cooke, A.J. Dennis, and F.L. Fisher (eds.). Polynuclear aromatic hydrocarbons: physical and biological chemistry. Battelle Press, Columbus, Ohio.

Hoffman, D.J., and M.L. Gay. 1981. Embryotoxic effects of benzo(a)pyrene, chrysene, and 7,12-dimethylbenz(a)anthracene in petroleum hydrocarbon mixtures in mallard ducks. J. Toxicol. Environ. Health 7:775-787.

Hoffman, E.J., G.L. Mills, J.S. Latimer, and J.G. Quinn. 1984. Urban runoff as a source of polycyclic aromatic hydrocarbons to coastal waters. Environ. Sci. Technol. 18:580-587.

Hose, J.E., J.B. Hannah, D. DiJulio, M.L. Landolt, B.S. Miller, W.T. Iwaoka, and S.P. Felton. 1982. Effects of benzo(a)pyrene on early development of flatfish. Arch. Environ. Contam. Toxicol. 11:167-171.

Humason, A.W., and D.F. Gadbois. 1982. Determination of polynuclear aromatic hydrocarbons in the New York Bight area. Mimeo MSG-506. Natl. Mar. Fish. Serv., Gloucester, Massachusetts.

Jackim, E., and C. Lake. 1978. Polynuclear aromatic hydrocarbons in estuarine and nearshore environments. Pages 415-428 in M.L. Wiley (ed.). Estuarine interactions. Academic Press, New York.

Jackim, E., and L. Wilson. 1979. Benzo(a)pyrene accumulation and depuration in the soft-shell clam (Mya arenaria). Pages 91-94 in D.S. Wilt (ed.). Proceedings tenth National Shellfish Sanitation workshop, Hunt Valley, Maryland.

Johnson, A.C., P.F. Larsen, D.F. Gadbois, and A.W. Humason. 1985. The distribution of polycyclic aromatic hydrocarbons in the surficial sediments of Penobscot Bay (Maine, USA) in relation to possible sources and to other sites worldwide. Mar. Environ. Res. 5:1-16.

Johnston, W.R., and R.M. Harrison. 1984. Deposition of metallic and organic pollutants alongside the M6 motorway. Sci. Total Environ. 33:119-127.

Jones, P.W., and R.I. Freudenthal (eds.). 1978. Carcinogensis - a comprehensive survey. Vol. 3. Polynuclear aromatic hydrocarbons: second international symposium on analysis, chemistry, and biology, Raven Press, New York. 487 pp.

Jones, P.W., and P. Leber (eds.). 1979. Polynuclear aromatic hydrocarbons. Third international symposium on chemistry and biology - carcinogenesis and mutagenesis. Ann Arbor Science Publ., Ann Arbor, Michigan. 892 pp.

Kirso, U., L. Belykh, D. Stom, N. Irha, and E. Urbas. 1983. Oxidation of benzo(a)pyrene by plant enzymes. Pages 679-687 in M. Cooke and A.J. Dennis (eds.). Polynuclear aromatic hydrocarbons: formation, metabolism and measurement. Battelle Press, Columbus, Ohio.

Krahn, M.M., L.D. Rhodes, M.S. Myers, L.K. Moore, W.D. MacLeod, Jr., and D.C. Malins. 1986. Associations between metabolites of aromatic compounds in bile and the occurrence of hepatic lesions in English sole (Parophrys vetulus) from Puget Sound, Washington. Arch. Environ. Contam. Toxicol. 15:61-67

Kuhnhold, W.W., and F. Busch. 1978. On the uptake of three different types of hydrocarbons by salmon eggs (Salmo salar L.). Meeresforsch. 26:50-59.

Lake, J., G.L. Hoffman, and S.C. Schimmel. 1985. Bioaccumulation of contaminants from Black Rock Harbor dredged material by mussels and polychaetes. U.S. Environ. Protection Agency Tech. Rep. D-85-2. 150 pp. Avail. from Natl. Tech. Inform. Serv., Springfield, Virginia.

Lake, J.L., C. Norwood, C. Dimock, and R. Bowen. 1979. Origins of polycyclic aromatic hydrocarbons in estuarine sediments. Geochim. Cosmochim. Acta 43:1847-1854.

Landrum, P.F. 1983. The effect of co-contaminants on the bioavailability of polycyclic aromatic hydrocarbons to Pontoporeia hoyi. Pages 731-743 in M. Cooke and A.J. Dennis (eds.). Polynuclear aromatic hydrocarbons: formation, metabolism and measurement. Battelle Press, Columbus, Ohio.

Landrum, P.F., B.J. Eadie, W.R. Faust, N.R. Morehead, and M.J. McCormick. 1984. Role of sediment in the bioaccumulation of benzo(a)pyrene by the amphipod, Pontoporeia hoyi. Pages 799-812 in M. Cooke and A.J. Dennis (eds.). Polynuclear aromatic hydrocarbons: mechanisms, methods and metabolism. Battelle Press, Columbus, Ohio.

Landrum, P.F., and D. Scavia. 1983. Influence of sediment on anthracene uptake, depuration, and biotransformation by the amphipod Hyalella azteca.

Canad. J. Fish. Aquatic Sci. 40:298-305.

Larsson, B., and G. Sahlberg. 1982. Polycyclic aromatic hydrocarbons in lettuce. Influence of a highway and an aluminium smelter. Pages 417-426 in M. Cooke, A.J. Dennis, and G.L. Fisher (eds.). Polynuclear aromatic hydrocarbons: physical and biological chemistry. Battelle Press, Columbus, Ohio.

Lawrence J.F., and D.F. Weber. 1984a. Determination of polycyclic aromatic hydrocarbons in some Canadian commercial fish, shellfish, and meat products by liquid chromatography with confirmation by capillary gas chromatography-mass spectrometry. J. Agric. Food Chem. 32:789-794.

Lawrence, J.F., and D.F. Weber. 1984b. Determination of polycyclic aromatic hydrocarbons in Canadian samples of processed vegetable and dairy products by liquid chromatography with fluorescence detection. J. Agric. Food Chem. 32:794-797.

Lee, R.F., R. Sauerheber, and G.D. Dobbs. 1972. Uptake, metabolism and discharge of polycyclic aromatic hydrocarbons by marine fish. Mar. Biol. 17:201-208.

Lee, S.D., and L. Grant (eds.). 1981. Health and ecological assessment of polynuclear aromatic hydrocarbons. Pathotex Publ., Park Forest South, Illinois. 364 pp.

Leversee, G.J., J.P. Geisy, P.F. Landrum, S. Bartell, S. Gerould, M. Bruno, A. Spacie, J. Bowling, J. Haddock, and T. Fannin. 1981. Disposition of benzo(a)pyrene in aquatic systems components: periphyton, chironomids, daphnia, fish. Pages 357-366 in M. Cooke and A.J. Dennis (eds.). Chemical analysis and biological fate: polynuclear aromatic hydrocarbons. Fifth international symposium. Battelle Press, Columbus, Ohio.

Linder, G., H.L. Bergman, and J.S. Meyer. 1985. Anthracene bioconcentration in rainbow trout during single-compound and complex-mixture exposures. Environ. Toxicol. Chem. 4:549-558.

Lindmark, D.G. 1981. Activation of polynuclear aromatic hydrocarbons to mutagens by the marine ciliate Parauronema acutum. Appl. Environ. Microbiol. 41:1238-1242.

Lo, M-T., and E. Sandi. 1978. Polycyclic aromatic hydrocarbons (polynuclears) in foods. Residue Rev. 69:35-86.

Lu, P-Y., R.L. Metcalf, N. Plummer, and D. Mandrel. 1977. The environmental fate of three carcinogens: benzo-(a)-pyrene, benzidine, and vinyl chloride evaluated in laboratory model ecosystems. Arch. Environ. Contam. Toxicol. 6:129-142.

Maccubbin, A.E., P. Black, L. Trzeciak, and J.J. Black. 1985. Evidence for polynuclear aromatic hydrocarbons in the diet of bottom-feeding fish. Bull. Environ. Contam. Toxicol. 34:876-882.

Malins, D.C., M.M. Krahn, D.W. Brown, L.D. Rhodes, M.S. Myers, B.B. McCain, and S.-L. Chan. 1985a. Toxic chemicals in marine sediment and biota from Mukilteo, Washington: relationships with hepatic neoplasms and other hepatic lesions in English sole (Parophrys vetulus). J. Natl. Cancer Inst. 74:487-494.

Malins, D.C., M.M. Krahn, M.S. Myers, L.D. Rhodes, D.W. Brown, C.A. Krone, B.B. McCain, and S.-L. Chan. 1985b. Toxic chemicals in sediments and biota from a creosote-polluted harbor: relationships with hepatic neoplasms and other hepatic lesions in English sole (Parophrys vetulus). Carcinogenesis 6:1463-1469.

Malins, D.C., B.B. McCain, D.W. Brown, S.-L. Chan, M.S. Myers, J.T. Landahl, P.G. Prohaska, A.J. Friedman, L.D. Rhodes, D.G. Burrows, W.D. Gronlund, and H.O. Hodgins. 1984. Chemical pollutants in sediments and diseases of bottom-dwelling fish in Puget Sound, Washington. Environ. Sci. Technol. 18:705-713.

Marcus, J.M., and T.P. Stokes. 1985. Polynuclear aromatic hydrocarbons in oyster tissue around three coastal marinas. Bull. Environ. Contam. Toxicol. 35:835-844.

Martens, R. 1982. Concentrations and microbial mineralization of four to six ring polycyclic aromatic hydrocarbons in composted municipal waste. Chemosphere 11:761-770.

Mattison, D.R. 1980. Morphology of oocyte and follicle destruction by polycyclic aromatic hydrocarbons in mice. Toxicol. Appl. Pharmacol. 53:249-259.

McCarthy, J.F., and B.D. Jimenez. 1985. Reduction in bioavailability to bluegills of polycyclic aromatic hydrocarbons bound to dissolved humic material. Environ. Toxicol. Chem. 4:511-521.

McCarthy, J.F., B.D. Jimenez, and T. Barbee. 1985. Effect of dissolved humic material on accumulation of polycyclic aromatic hydrocarbons: structure-activity relationships. Aquatic Toxicol. 7:15-24.

McGinnes, P.R., and V.L. Snoeyink. 1974. Determination of the fate of polynuclear aromatic hydrocarbons in natural water systems. Univ. Illinois at Urbana-Champaign, Water Res. Center, UILU-WRC--74-0080, Res. Rep. No. 80. 56 pp.

McIntyre, A.E., R. Perry, and J.N. Lester. 1981. Analysis of polynuclear aromatic hydrocarbons in sewage sludges. Anal. Lett. 14(A4):291-309.

McMahon, C.K., and S.N. Tsoukalas. 1978. Polynuclear aromatic hydrocarbons in forest fire smoke. Pages 61-73 in P.W. Jones and R.I. Freudenthal (eds.). Carcinogenesis - a comprehensive survey. Vol. 3. Polynuclear aromatic hydrocarbons: second international symposium on analysis, chemistry, and biology. Raven Press, New York.

Melius, P. 1984. Comparative benzo(a)pyrene metabolite patterns in fish and rodents. Pages 387-390 in K.L. Hoover (ed.). Use of small fish in carcinogenicity testing. Natl. Cancer Inst. Mono. 65. Bethesda, Maryland.

Melius, P., and D.L. Elam. 1983. Mixed function oxidase in sea catfish. Pages 877-895 in M. Cooke and A.J. Dennis (eds.). Polynuclear aromatic hydrocarbons: formation, metabolism and measurement. Battelle Press, Columbus, Ohio.

Melius, P., D. Elam, M. Kilgore, B. Tan, and W.P. Schoor. 1980. Mixed function oxidase inducibility and polyaromatic hydrocarbon metabolism in the mullet, sea catfish, and Gulf killifish. Pages 1059-1075 in A. Bjorseth and A.J. Dennis (eds.). Polynuclear aromatic hydrocarbons: chemistry and biological effects. Battelle Press, Columbus, Ohio.

Miller, D.L., J.P. Corliss, R.N. Farragut, and H.C. Thompson, Jr. 1982. Some aspects of the uptake and elimination of the polynuclear aromatic hydrocarbon chrysene by mangrove snapper, Lutjanus griseus, and pink shrimp, Penaeus duorarum. Pages 321-335 in N.L. Richards and B.L. Jackson (eds.). Symposium: carcinogenic polynuclear aromatic hydrocarbons in the marine environment. U.S. Environ. Protection Agency Rep. 600/9-82-013.

Miranda, C.L., and R.S. Chhabra. 1980. Species differences in stimulation of intestinal and hepatic microsomal mixed-function oxidase enzymes. Biochem. Pharmacol. 29:1161-1165.

Mix, M.C. 1982. Polynuclear aromatic hydrocarbons and cellular proliferative disorders in bivalve molluscs from Oregon estuaries. U.S. Environ. Protection Agency Rep. 600/4-82-026. 49 pp.

Mix, M.C., and R.L. Schaffer. 1979. Benzo(a)pyrene concentrations in mussels (Mytilus edulis) from Yaquina Bay, Oregon during June 1976 - May 1978. Bull. Environ. Contam. Toxicol. 23:677-684.

Mix, M.C., and R.L. Schaffer. 1983a. Concentrations of unsubstituted polycyclic aromatic hydrocarbons in softshell clams from Coos Bay, Oregon, USA. Mar. Pollut. Bull. 14:94-97.

Mix, M.C., and R.L. Schaffer. 1983b. Concentrations of unsubstituted polynuclear aromatic hydrocarbons in bay mussels (Mytilus edulis) from Oregon, USA. Mar. Environ. Res. 9:193-209.

Murphy, D.J., R.M. Buchan, and D.G. Fox. 1982. Ambient particulate and benzo(a)pyrene concentrations from residential wood combustion, in a mountain community. Pages 567-574 in M. Cooke, A.J. Dennis, and G.L. Fisher (eds.). Polynuclear aromatic hydrocarbons: physical and biological chemistry. Battelle Press, Columbus, Ohio.

Neff, J.M. 1979. Polycyclic aromatic hydrocarbons in the aquatic environment. Applied Science Publ. Ltd., London. 262 pp.

Neff, J.M. 1982a. Accumulation and release of polycyclic aromatic hydrocarbons from water, food, and sediment by marine animals. Pages 282-320 in N.L. Richards and B.L. Jackson (eds.). Symposium: carcinogenic polynuclear aromatic hydrocarbons in the marine environment. U.S. Environ. Protection Agency Rep. 600/9-82-013.

Neff, J.M. 1982b. Polycyclic aromatic hydrocarbons in the aquatic environment and cancer risk to aquatic organisms and man. Pages 385-409 in N.L. Richards and B.L. Jackson (eds.). Symposium: carcinogenic polynuclear aromatic hydrocarbons in the marine environment. U.S. Environ. Protection Agency Rep. 600/9-82-013.

Neff, J.M. 1985. Polycyclic aromatic hydrocarbons. Pages 416-454 in G.M. Rand and S.R. Petrocelli (eds.). Fundamentals of aquatic toxicology. Hemisphere Publ. Corp., New York.

Neff, J.M., B.A. Cox, D. Dixit, and J.W. Anderson. 1976. Accumulation and release of petroleum derived aromatic hydrocarbons by four species of marine animals. Mar. Biol. 38:279-289.

Pal, K. 1984. The relationship between the levels of DNA-hydrocarbon adducts and the formation of sister-chromatid exchanges in Chinese hamster ovary cells treated with derivitives of polycyclic aromatic hydrocarbons. Mutat. Res. 129:365-372.

Pasquini, R., and S. Monarca. 1983. Detection of mutagenic/carcinogenic compounds in unused and used motor oils. Sci. Total Environ. 32:55-64.

Patton, J.F., and M.P. Dieter. 1980. Effects of petroleum hydrocarbons on hepatic function in the duck. Comp. Biochem. Physiol. 65C:33-36.

Potvin, R.R., E.G. Adamek, and D. Balsillie. 1981. Ambient PAH levels near a steel mill in northern Ontario. Pages 741-753 in M. Cooke and A.J. Dennis (eds.). Chemical analysis and biological fate: polynuclear aromatic hydrocarbons. Fifth international symposium. Battelle Press, Columbus, Ohio.

Prahl, F.G., E. Crecellus, and R. Carpenter. 1984. Polycyclic aromatic hydrocarbons in Washington coastal sediments: an evaluation of atmospheric and riverine routes of introduction. Environ. Sci. Technol. 18:687-693.

Prein, A.E., G.M. Thie, G.M. Alink, and J.H. Koeman. 1978. Cytogenetic changes in fish exposed to water of the River Rhine. Sci. Total Environ. 9:287-291.

Pucknat, A.W. (ed.). 1981. Health impacts of polynuclear aromatic hydrocarbons. Environmental Health Review No. 5. Noyes Data Corp., Park Ridge, New Jersey. 271 pp.

Quaghebeur, D, E. DeWulf, M.C. Ravelingien, and G. Janssens. 1983. Polycyclic aromatic hydrocarbons in rainwater. Sci. Total Environ. 32:35-54.

Richards, N.L., and B.L. Jackson (eds.). 1982. Symposium: carcinogenic polynuclear aromatic hydrocarbons in the marine environment. U.S. Environ. Protection Agency Rep. 600/9-82-013. 409 pp.

Rogan, E., E. Cavalieri, A. Munhall, and S. Salmasi. 1983. Synergistic effect of the environmental contaminants cyclopenta(cd)pyrene and benzo(a)pyrene in mouse skin carcinogenicity. Pages 1035-1046 in M. Cooke and A.J. Dennis (eds.). Polynuclear aromatic hydrocarbons: formation, metabolism and measurement. Battelle Press, Columbus, Ohio.

Rose, F.L. 1977. Tissue lesions of tiger salamander (Ambystoma tigrinum): relationship to sewage effluents. Ann. N.Y. Acad. Sci. 298:270-279.

Sabourin, T.D., and R.E. Tullis. 1981. Effect of three aromatic hydrocarbons on respiration and heart rates of the mussel, Mytilus californianus. Bull. Environ. Contam. Toxicol. 26:729-736.

Schafer, E.W., Jr., and W.A. Bowles, Jr. 1985. Acute oral toxicity and repellency of 933 chemicals to house and deer mice. Arch. Environ. Contam. Toxicol. 14:111-129.

Schoeny, R., T. Cody, M. Radike, J. Caruso, J. Rogers, B. Smiddy, M. Trentman, and D. Warshawsky. 1984. Metabolism, growth effects and mutagenicity of plant metabolites of benzo(a)pyrene. Pages 1197-1211 in M. Cooke and A.J. Dennis (eds.). Polynuclear aromatic hydrocarbons: mechanisms, methods and metabolism. Battelle Press, Columbus, Ohio.

Schoor, W.P. 1984. Benzo(a)pyrene metabolism in marine fish and some analytical aspects of its metabolites. Pages 391-396 in K.L. Hoover (ed.). Use of small fish in carcinogenicity testing. Natl. Cancer Inst. Mono. 65. Bethesda, Maryland.

Schoor, W.P., and M. Srivastava. 1984. Position-specific induction of benzo(a)pyrene metabolism by 3-methylcholanthrene and phenobarbital in mullet (Mugil cephalus), a marine fish. Comp. Biochem. Physiol. 73C:391-396.

Schultz, M.E., and J.R. Schultz. 1982. Induction of hepatic tumors with 7,12-dimethylbenz(a)anthracene in two species of viviparous fishes (genus Poeciliopsis). Environ. Res. 27:337-351.

Schwen, R.J., and G.J. Mannering. 1982a. Hepatic cytochrome P-450-dependent monooxygenase systems of the trout, frog and snake - II. Monooxygenase activities. Comp. Biochem. Physiol. 71B:437-443.

Schwen, R.J., and G.J. Mannering. 1982b. Hepatic cytochrome P-450-dependent monooxygenase systems of the trout, frog and snake - III. Induction. Comp. Biochem. Physiol. 71B:445-453.

Shiraishi, H., N.H. Pilkington, A. Otuski, and K. Fuwa. 1985. Occurrence of chlorinated polynuclear aromatic hydrocarbons in tap water. Environ. Sci. Technol. 19:585-590.

Sims, P., and P.L. Grover. 1981. Involvement of dihydrodiols and diol epoxides in the metabolic activation of polycyclic hydrocarbons other than benzo(a)pyrene. Pages 117-181 in H.V. Gelboin and P.O. Ts'o (eds.). Polycyclic hydrocarbons and cancer. Vol. 3. Academic Press, New York.

Sims, R.C., and R. Overcash. 1983. Fate of polynuclear aromatic compounds (PNAs) in soil-plant systems. Residue Rev. 88:1-68

Sirota, G.R., and J.F. Uthe. 1981. Polynuclear aromatic hydrocarbon contamination in marine shellfish. Pages 329-341 in M. Cooke and A.J. Dennis (eds.). Chemical analysis and biological fate: polynuclear aromatic hydrocarbons. Fifth international symposium. Battelle Press, Columbus, Ohio.

Sirota, G.R., J.F. Uthe, A. Sreedharan, R. Matheson, C.J. Musial, and K. Hamilton. 1983. Polynuclear aromatic hydrocarbons in lobster (Homarus americanus) and sediments in the vicinity of a coking facility. Pages 1123-1136 in M. Cooke and A.J. Dennis (eds.). Polynuclear aromatic hydrocarbons: formation, metabolism and measurement. Battelle Press, Columbus, Ohio.

Slaga, T.J., W.M. Bracken, A. Viaje, D.L. Berry, S.M. Fischer, D.R. Miller, W.

Levin, A.H. Conney, H. Yagi, and D.M. Jerina. 1978. Tumor initiating and promoting activities of various benzo(a)pyrene metabolites in mouse skin. Pages 371-382 in P.W. Jones and R.I. Freudenthal (eds.). Carcinogenesis - a comprehensive survey. Vol. 3. Polynuclear aromatic hydrocarbons: second international symposium on analysis, chemistry, and biology. Raven Press, New York.

Smith, J.D., J. Bagg, and B.M. Bycroft. 1984. Polycyclic aromatic hydrocarbons in the clam Tridacna maxima from the Great Barrier Reef, Australia. Environ. Sci. Technol. 18:353-358.

Smith, J.D., J.Y. Hauser, and J. Bagg. 1985. Polycyclic aromatic hydrocarbons in sediments of the Great Barrier Reef region, Australia. Mar. Pollut. Bull. 16:110-114.

Solbakken, J.E., K. Ingebrigtsen, and K.H. Palmork. 1984. Comparative study on the fate of the polychlorinated biphenyl 2, 4, 5, 2′, 4′, 5′ - hexachlorobiphenyl and the polycyclic aromatic hydrocarbon phenanthrene in flounder (Platichthys flesus), determined by liquid scintillation counting and autoradiography. Mar. Biol. 83:239-246.

Southworth, G.R. 1979. Transport and transformation of anthracene in natural waters. Pages 359-380 in L.L Marking and R.A. Kimerle (eds.). Aquatic toxicology. ASTM STP 667. American Society for Testing and Materials, Philadelphia, Pennsylvania.

Southworth, G.R., J.J. Beauchamp, and P.K. Schmeider. 1978. Bioaaccumulation potential of polycyclic aromatic hydrocarbons in Daphnia pulex. Water Res. 12:973-977.

Srivastava, V.K., P.K. Srivastava, and U.K. Misra. 1985. Polycyclic aromatic hydrocarbons of coal fly ash: analysis by gas-liquid chromatography using nematic liquid crystals. J. Toxicol. Environ. Health 15:333-337.

Statham, C.N., M.J. Melancon, Jr., and J.J. Lech. 1976. Bioconcentration of xenobiotics in trout bile: a proposed monitoring aid for some waterborne chemicals. Science 193:680-681.

Stegeman, J. 1981. Polynuclear aromatic hydrocarbons and their metabolism in the marine environment. Pages 1-60 in H.V. Gelboin and P.O. Ts'o (eds.). Polycyclic hydrocarbons and cancer. Vol. 3. Academic Press, New York.

Stegeman, J.J., T.R. Skopek, and W.G. Thilly. 1982. Bioactivation of polynuclear aromatic hydrocarbons to cytotoxic and mutagenic products by marine fish. Pages 201-211 in N.L. Richards and B.L. Jackson (eds.). Symposium: carcinogenic polynuclear aromatic hydrocarbons in the marine environment. U.S. Environ. Protection Agency Rep. 600/9-82-013.

Stoker, P.W., M.R. Plasterer, R.L. McDowell, R. Campbell, S. Fields, R. Price, C. Muehle, W.R. West, G.M. Booth, J.R. Larsen, and M.L. Lee. 1984. Effects of coal-derived tars on selected aquatic and mammalian organisms. Pages 1239-1257 in M. Cooke and A.J. Dennis (eds.). Polynuclear aromatic hydrocarbons: mechanisms, methods and metabolism. Battelle Press, Columbus, Ohio.

Strand, J.W., and A.W. Andren. 1980. Polyaromatic hydrocarbons in aerosols over Lake Michigan, fluxes to the Lake. Pages 127-137 in A. Bjorseth and A.J. Dennis (eds.). Polynuclear aromatic hydrocarbons: chemistry and biological effects. Battelle Press, Columbus, Ohio.

Suess, M.J. 1976. The environmental load and cycle of polycyclic aromatic hydrocarbons. Sci. Total Environ. 6:239-250.

Sugimura, T. 1986. Studies on environmental chemical carcinogenesis in Japan. Science 233:312-318.

Sullivan, T.J., and M.C. Mix. 1985. Persistence and fate of polynuclear aromatic hydrocarbons deposited on slash burn sites in the Cascade Mountains and coast range of Oregon. Arch. Environ. Contam. Toxicol. 14:187-192.

Szentpaly, L.V. 1984. Carcinogenesis by polycyclic aromatic hydrocarbons: a multilinear regression on new type PMO indices. J. Am. Chem. Soc. 106:6021-6028.

Thomas, W., A. Ruhling, and H. Simon. 1984. Accumulation of airborne pollutants (PAH, chlorinated hydrocarbons, heavy metals) in various plant species and humus. Environ. Pollut. 36A:295-310.

Tsang, W-S., and G.W. Griffin. 1979. Metabolic activation of polynuclear aromatic hydrocarbons. Pergamon Press, Oxford, England. 125 pp.

Valerio, F., P. Bottino, D. Ugolini, M.R. Cimberle, G.A. Tozzi, and A. Frigerio. 1984. Chemical and photochemical degradation of polycyclic aromatic hydrocarbons in the atmosphere. Sci. Total Environ. 40:169-188.

van Noort, P.C.M., and E. Wondergem. 1985. Scavenging of airborne polycyclic aromatic hydrocarbons by rain. Environ. Sci. Technol. 19:1044-1048.

Varanasi, U., and D.J. Gmur. 1980. Metabolic activation and covalent binding of benzo(a)pyrene to deoxyribonucleic acid catalyzed by liver enzymes of marine fish. Biochem. Pharmacol. 29:753-762.

Varanasi, U., and D.J. Gmur. 1981. In vivo metabolism of naphthalene and benzo(a)pyrene by flatfish. Pages 367-376 in M. Cooke and A.J. Dennis (eds.). Chemical analysis and biological fate: polynuclear aromatic hydrocarbons. Fifth international symposium. Battelle Press, Columbus, Ohio.

Varanasi, U., D.J. Gmur, and M.M. Krahn. 1980. Metabolism and subsequent binding of benzo(a)pyrene to DNA in pleuronectid and salmonid fish. Pages 455-470 in A. Bjorseth and A.J. Dennis (eds.). Polynuclear aromatic hydrocarbons: chemistry and biological effects. Battelle Press, Columbus, Ohio.

Varanasi, U., M. Nishimoto, and J. Stover. 1984. Analyses of biliary conjugates and hepatic DNA binding in benzo(a)pyrene-exposed English sole. Pages 1315-1328 in M. Cooke and A.J. Dennis (eds.). Polynuclear aromatic hydrocarbons: mechanisms, methods and metabolism. Battelle Press, Columbus, Ohio.

Varanasi, U., W.L. Reichert, J.E. Stein, D.W. Brown, and H.R. Sanborn. 1985. Bioavailability and biotransformation of aromatic hydrocarbons in benthic organisms exposed to sediment from an urban estuary. Environ. Sci. Technol. 19:836-841.

von Hofe, E., and H.W. Puffer. 1986. In vitro metabolism and in vivo binding of benzo(a)pyrene in the California killifish (Fundulus parvipinnis) and speckled sanddab (Citharicthys stigmaeous). Arch. Environ. Contam. Toxicol. 15:251-256.

Wang, D.T., and O. Meresz. 1982. Occurrence and potential uptake of polynuclear aromatic hydrocarbons of highway traffic origin by proximally grown food crops. Pages 885-896 in M. Cooke, A.J. Dennis, and G.L. Fisher (eds.). Polynuclear aromatic hydrocarbons: physical and biological chemistry. Battelle Press, Columbus, Ohio.

Ward, E.C., M.J. Murray, and J.H. Dean. 1985. Immunotoxicity of nonhalogenated polycyclic aromatic hydrocarbons. Pages 291-313 in J.H. Dean, M.I. Luster, A.E. Munson, and H. Amos (eds.). Immunotoxicology and immunopharamacology. Raven Press, New York.

Warshawsky, D., T. Cody, M. Radike, B.A. Smiddy, and B. Nagel. 1983. Toxicity and metabolism of benzo(a)pyrene in the green alga Selenastrum capricornutum. Pages 1235-1245 in M. Cooke and A.J. Dennis (eds.). Polynuclear aromatic hydrocarbons: formation, metabolism and measurement. Battelle Press, Columbus, Ohio.

West, W.R., P.A. Smith, G.M. Booth, S.A. Wise, and M.L. Lee. 1986. Determination of genotoxic polycyclic aromatic hydrocarbons in a sediment from the Black River (Ohio). Arch. Environ. Contam. Toxicol. 15:241-249.

West, W.R., P.A. Smith, P.W. Stoker, G.M. Booth, T. Smith-Oliver, B.E. Butterworth, and M.L. Lee. 1984. Analysis and genotoxicity of a PAC-polluted river sediment. Pages 1395-1411 in M. Cooke and A.J. Dennis (eds.). Polynuclear aromatic hydrocarbons: mechanisms, methods and metabolism. Battelle Press, Columbus, Ohio.

Whitehouse, B. 1985. The effects of dissolved organic matter on the aqueous partitioning of polynuclear aromatic hydrocarbons. Estuar. Coastal Shelf Sci. 20:393-402.

Williams, U.P., J.W. Kiceniuk, and J.R. Botta. 1985. Polycyclic aromatic hydrocarbon accumulation and sensory evaluation of lobsters (Homarus americanus) exposed to diesel oil at Arnold's Cove, Newfoundland. Canad. Tech. Rep. Fish. Aquatic Sci. 1402. 13 pp.

Yan, L.S. 1985. Study of the carcinogenic mechanism for polycyclic aromatic hydrocarbons - extended bay region theory and its quantitative model. Carcinogenesis 6:1-6.

Yoshikawa, T., L.P. Ruhr, W. Flory, D. Giamalva, D.F. Church, and W.A. Pryor. 1985. Toxicity of polycyclic aromatic hydrocarbons. I. Effect of phenanthrene, pyrene, and their ozonized products on blood chemistry in rats. Toxicol. Appl. Pharmacol. 79:218-226.

50272-101

REPORT DOCUMENTATION PAGE	1. REPORT NO. Biological Report 85 (1.11)	2.	3. Recipient's Accession No.

4. Title and Subtitle
Polycyclic aromatic hydrocarbon hazards to fish, wildlife, and invertebrates: a synoptic review

5. Report Date
May 1987

6.

7. Author(s)
Ronald Eisler

8. Performing Organization Rept. No.

9. Performing Organization Name and Address
Patuxent Wildlife Research Center
U.S. Fish and Wildlife Service
Laurel, MD 20708

10. Project/Task/Work Unit No.

11. Contract(C) or Grant(G) No.
(C)
(G)

12. Sponsoring Organization Name and Address
Division of Wildlife and Contaminant Research
Fish and Wildlife Service
U.S. Department of the Interior
Washington, D.C. 20240

13. Type of Report & Period Covered

14.

15. Supplementary Notes

16. Abstract (Limit: 200 words)

This report synthesizes technical literature on ecological and toxicological aspects of polycyclic aromatic hydrocarbons (PAH) in the environment, with special reference to fisheries and wildlife resources. Subtopics include: chemical properties, sources, and fate; background concentrations in biological and nonbiological samples; toxic and sublethal effects of PAH to flora and fauna; and proposed criteria and research needs for the protection of sensitive, nonhuman organisms.

17. Document Analysis a. Descriptors

Contaminants
Toxicity
Fishes
Wildlife
Natural resources
Invertebrates

b. Identifiers/Open-Ended Terms

Polycyclic aromatic hydrocarbons
PAH
Carcinogenesis
Mutagenesis
Teratogenesis

c. COSATI Field/Group

18. Availability Statement
Release unlimited

19. Security Class (This Report) Unclassified

21. No. of Pages 81

20. Security Class (This Page) Unclassified

22. Price

(See ANSI-Z39.18) See Instructions on Reverse

OPTIONAL FORM 272 (4-77)
(Formerly NTIS-35)
Department of Commerce

☆ U.S. GOVERNMENT PRINTING OFFICE: 1987—775-217/65127