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CYTOCHROME P4501A BIOMARKER INDICATION OF OIL EXPOSURE IN HARLEQUIN DUCKS UP TO 20 YEARS AFTER THE *EXXON VALDEZ* OIL SPILL

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Abstract—Hydrocarbon-inducible cytochrome P4501A (CYP1A) expression was measured, as ethoxyresorufin-*O*-deethylase (EROD) activity, in livers of wintering harlequin ducks (*Histrionicus histrionicus*) captured in areas of Prince William Sound, Alaska, USA, oiled by the 1989 *Exxon Valdez* spill and in birds from nearby unoiled areas, during 2005 to 2009 (up to 20 years following the spill). The present work repeated studies conducted in 1998 that demonstrated that in harlequin ducks using areas that received *Exxon Valdez* oil, EROD activity was elevated nearly a decade after the spill. The present findings strongly supported the conclusion that average levels of hepatic EROD activity were higher in ducks from oiled areas than those from unoiled areas during 2005 to 2009. This result was consistent across four sampling periods; furthermore, results generated from two independent laboratories using paired liver samples from one of the sampling periods were similar. The EROD activity did not vary in relation to age, sex, or body mass of individuals, nor did it vary strongly by season in birds collected early and late in the winter of 2006 to 2007, indicating that these factors did not confound inferences about observed differences between oiled and unoiled areas. We interpret these results to indicate that harlequin ducks continued to be exposed to residual *Exxon Valdez* oil up to 20 years after the original spill. This adds to a growing body of literature suggesting that oil spills have the potential to affect wildlife for much longer time frames than previously assumed. Environ. Toxicol. Chem. 2010;29:1138–1145. © 2010 SETAC

Keywords—*Exxon Valdez* Harlequin duck Oil exposure

INTRODUCTION

Effects of the 1989 *Exxon Valdez* oil spill on wildlife populations and communities in Prince William Sound, Alaska, USA, have been intensively studied, and debated, over the two decades since the spill. One of the more remarkable and unanticipated findings from this body of work was the length of time (at least a decade) over which animals were exposed to residual oil and showed depression of various population demographic attributes [1–3]. Peterson et al. [3] considered these results to represent a paradigm shift in the way in which oil contamination is thought to affect the environment; in particular, chronic, delayed, and indirect effects of oil spills appear to have much longer and larger consequences on wildlife populations and communities than previously assumed.

Research has continued in areas of Prince William Sound affected by the *Exxon Valdez* spill, to document the process and timeline of population and ecosystem recovery. Spatial and temporal extents of wildlife exposure to lingering *Exxon Valdez* oil have been inferred from indicators of induction of certain members of the cytochrome P450 1 gene subfamily (CYP1A). Vertebrate CYP1A genes are induced by larger polycyclic aromatic hydrocarbons (PAHs), including those found in crude oil, and halogenated aromatic hydrocarbons, including planar polychlorinated biphenyls (PCBs) and polychlorinated dibenzo-*p*-dioxins and difurans [4,5]. Because CYP1A is strongly induced by a limited number of compounds, it can be a particularly useful biomarker, i.e., a measurable physiological response by an organism, for evaluating exposure to those chemicals. Although CYP1A induction does not necessarily indicate deleterious effects on individuals or populations [6], elevated CYP1A levels indicate exposure to inducing compounds and, hence, at least the potential for associated toxic consequences, including subtle effects that may be difficult to detect in nature [7]. Therefore, indicators of CYP1A have been part of many considerations of environmental effects of contamination, including those associated with the *Exxon Valdez* oil spill.

Indicators of induction of CYP1A have been used routinely to evaluate exposure to PAHs, PCBs, and dioxins in fish [5,7–9]. Although such studies are less common for birds and mammals, indicators of CYP1A levels have been used successfully as biomarkers of exposure of these taxa to inducing compounds, including PAHs [10–13]. In the case of the *Exxon Valdez* oil spill, indicators of CYP1A induction have been used to examine exposure to lingering oil for a number of vertebrates [8,14]. These studies demonstrated that, within Prince William Sound, CYP1A expression levels in many species were higher in areas oiled by the *Exxon Valdez* spill relative to unoiled areas nearly a decade after the spill. The authors of these studies concluded that oil remaining in the environment, particularly in intertidal areas, was encountered and ingested by some near-shore verte-

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brates. This conclusion is consistent with confirmation of the occurrence of residual *Exxon Valdez* oil in intertidal sediments of Prince William Sound during the same period in which elevated CYP1A was indicated [15] as well as calculations that intertidal-foraging vertebrates would be likely to encounter lingering oil repeatedly through the course of a year [16].

Harlequin ducks (*Histrionicus histrionicus*) were one of the species showing indications of elevated CYP1A induction in oiled areas of Prince William Sound relative to unoiled areas [14]. Harlequin ducks are marine birds that spend most of their annual cycle in intertidal and shallow subtidal zones of temperate and subarctic areas of the Pacific coast of North America. They are common in Prince William Sound during the nonbreeding season (average of 14,500 individuals between 1990 and 2005 [17]) and are at higher risk of exposure to residual *Exxon Valdez* oil than many other seabirds, given their exclusive occurrence in near-shore habitats where a disproportionate amount of oil was deposited [18] and where lingering oil has remained [15].

In addition to a higher likelihood of exposure, a number of natural history and life history characteristics make harlequin duck individuals and populations particularly sensitive to oil pollution [2]. These include a diet consisting of invertebrates that live on or in near-shore sediments, a life history strategy predicated on high survival rates, and a small body size, relative to other sea ducks, that may limit their flexibility when faced with increased energetic demands. Consistent with these sensitivities to effects of oil contamination, demographic problems were observed in oiled areas of Prince William Sound during the same period in which elevated CYP1A was indicated, including reductions in population trends [19], densities [20], and female survival [21] relative to unoiled areas. It was concluded that continued exposure to lingering oil was likely a constraint on population recovery [2].

Because of the history of elevated indicators of CYP1A induction [14], continued occurrence of lingering oil in intertidal habitats where harlequin ducks occur [15], and vulnerability of harlequin ducks to effects of oil exposure [2], the present study was conducted to follow up on the original research describing elevated biomarkers of CYP1A in this species. In that study, Trust et al. [14] found that average ($\pm$SE) CYP1A expression levels, measured by hepatic 7-ethoxyresorufin-*O*-deethylase activity, were significantly higher in wintering harlequin ducks captured in areas oiled by the *Exxon Valdez* spill than in those captured in nearby unoiled areas (204.6 ± 20.3 SE and 70.7 ± 21.5 pmol/min/mg protein, respectively). Samples for the Trust et al. [14] study were collected in March and April, 1998, 9 years after the oil spill. Our primary objective for the present study was to repeat the Trust et al. [14] work during 2005 to 2009, 16 to 20 years after the *Exxon Valdez* oil spill, to evaluate whether differences in EROD activity persisted.

In addition to assessment of interannual variation, potential effects of individual attributes (age, sex, and body mass) and season on variation in CYP1A induction also were considered. Age, sex, and season have been shown to affect CYP1A induction in some fish [9], so these factors should be accounted for when evaluating sources of variation in CYP1A induction [6].

MATERIALS AND METHODS

Capture and sample collection

To facilitate comparisons, the present study closely followed the design and procedures of Trust et al. [14]. We captured wintering harlequin ducks using a floating mist net during four capture periods: March, 2005; November, 2006; March and April, 2007; and March, 2009. Birds were captured in a number of areas oiled during the *Exxon Valdez* spill, including Bay of Isles (60° 22′ N, 147° 40′ W), Herring Bay (60° 28′ N, 147° 44′ W), Crafton Island (60° 29′ N, 147° 57′ W), Green Island (60°18′ N, 147° 24′ W), and Foul Pass (60° 29′ N, 147° 38′ W). Also, birds were captured on nearby northwestern Montague Island (60° 15′ N, 147° 12′ W), which was not oiled and thus was considered a reference site. Harlequin ducks in Prince William Sound exhibit high site fidelity during winter, with approximately 95% remaining all winter on the same island or coastline region where they were originally captured [22]. We assume that this level of movement had little influence on our ability to draw inferences about differences in EROD activity between areas. Captured birds were placed in portable pet carriers and transported by skiff to a chartered research vessel for processing. Each individual was marked with a uniquely numbered, U.S. Geological Survey Bird Banding Laboratory metal tarsus band; the band number was used to identify the data and samples for that individual. Sex of each bird was determined by plumage and cloacal characteristics, and age class was determined by the depth of the bursa of Fabricius for females and bursal depth and plumage characteristics for males [23,24]. Age class was summarized as either hatch-year (HY), i.e., hatched the previous breeding season, or after-hatch-year (AHY). Numbers of individuals used in analyses of CYP1A induction are indicated in Table 1, by year, season, age class, sex, and area (oiled vs. unoiled).

Small (<0.5 g) liver biopsies were surgically removed by a veterinarian from each harlequin duck while the ducks were under general anesthesia using vaporized and inhaled Isoflurane. Once removed, liver samples were immediately placed into a labeled cryovial and frozen in liquid nitrogen. All samples were maintained in liquid nitrogen or a −80°C freezer until they were shipped to the laboratory in liquid nitrogen.

During the March, 2005, sampling, two liver biopsies were taken from each individual and sent to different laboratories for EROD analysis. Dual samples were taken to allow interlaboratory comparisons for validation of analytical integrity and inference of exposure between oiled and unoiled areas. One of the paired samples was sent to Woods Hole Oceanographic Institution (WHOI), where all pre-2005 samples were analyzed, and the other to the University of California Davis (UCD), where analyses were conducted in subsequent years. Both laboratories conducted all analyses without a priori knowledge of the areas from which the samples were collected, and neither had access to the data from the other.

Laboratory analyses

The CYP1A induction was determined by measuring hepatic 7-ethoxyresorufin-*O*-deethylase activity, which is a catalytic function principally of hydrocarbon-inducible CYP1A enzymes. Birds possess two CYP1A genes (CYP1A4 and CYP1A5), and the expression of both appears to be inducible by aryl hydrocarbon receptor agonists in some species [12,25]. Both CYP1As also catalyze EROD in some species (A. Kubota, personal communication). In studies of captive harlequin ducks, EROD activity was confirmed to be significantly higher in birds chronically ingesting weathered Prudhoe Bay crude oil compared with controls [26]. Similarly, oil-dosed Steller's eiders (*Polysticta stelleri*), another sea duck, had roughly fourfold increased EROD activity compared with controls [13]. Methods of EROD activity analysis followed standard procedures used in

Table 1. Sample sizes of harlequin ducks captured in Prince William Sound, Alaska, USA, for analyses of cytochrome P4501A induction[a]

	March, 2005		November, 2006		March/April, 2007		March, 2009	
Cohort[b]	Oiled	Unoiled	Oiled	Unoiled	Oiled	Unoiled	Oiled	Unoiled
AHY M	13	11	14	9	11	10	14	13
HY M	1	1	0	2	2	0	0	2
AHY F	5	3	9	8	7	9	4	4
HY F	1	1	2	1	5	1	1	1
Total	20	20	25	20	25	20	19	20

[a] Numbers are listed by sampling period, sex and age class cohort, and capture area (oiled during *Exxon Valdez* oil spill vs. unoiled).
[b] Cohort consists of an age class designation (HY = hatch-year, i.e., within 1 y of hatching; AHY = after-hatch-year) and sex (M = male; F = female).

previous studies, described in detail by Trust et al. [14] for analyses at WHOI and Miles et al. [13] for those at UCD. The measure of EROD activity is expressed in picomoles per minute per milligram of protein.

Statistical analyses

Four analyses were conducted to evaluate variation in EROD activity. First, results from the WHOI and UCD laboratories were compared for paired samples collected during March, 2005, to determine whether consistent results were obtained. Next, EROD activity was evaluated in relation to capture location (oiled or unoiled area) and individual attributes (age, sex, and body mass) for the March, 2005, samples using data from both WHOI and UCD laboratories. Third, EROD activity was evaluated in relation to location of capture, individual attributes, and season for samples obtained during November, 2006, and March/April, 2007, and analyzed at the UCD laboratory. Finally, variation in EROD activity was analyzed in relation to capture location and individual attributes for birds captures during March, 2009. Separate analyses were run for data from each winter because we wished to compare results from both laboratories in 2005, so analyzing 2005 separately facilitated direct comparison of inferences from each data set. Also, considerable variation can occur between laboratory runs [26]; this does not affect contrasts between treatments within runs but could complicate interpretation across runs. Finally, different sets of explanatory variables were used among winters, because seasonal effects could be evaluated only using data from winter, 2006 to 2007. For consistency, results from the UCD laboratory were used as the primary data, although results and inferences were contrasted across both laboratories for the 2005 data.

Laboratory comparison

Using the paired samples collected from 40 individuals, EROD activity of liver samples collected during March, 2005, was compared between laboratories. Laboratory results were contrasted using a simple linear regression, with the expectation that there would be low unexplained variation around this relationship if the two laboratories were giving similar results. We recognize that different analytical runs, even within a laboratory, may generate different absolute values of EROD activity [26], but one would still expect the different laboratories to have a strong correlation, even if the slope differs from 1.

EROD activity March, 2005

For the 2005 data, our primary interest was to determine whether area (oiled vs. unoiled) explained variation in EROD activity, after accounting for any effects of age class, sex, and body mass. Least squares general linear models (GLM) were used to estimate variation explained by each of a candidate set of models that included different combinations of variables of interest, and an information-theoretic approach was used for model selection and inference [27] in which support for various model configurations is contrasted using Akaike's Information Criterion (AIC). Age, sex, and body mass variables, which we termed *individual attributes,* were included or excluded as a group; i.e., models included either all of these variables or none of them. We used singular and additive combinations of area and individual attribute effects, resulting in a candidate model set including EROD = area, EROD = individual attributes, and EROD = area + individual attributes. We also included a null model, which consisted of estimates of a mean and variance across all of the data; strong support for the null model would indicate that variables considered in other candidate models did not explain important variation in the response.

The model with the lowest AIC value corrected for small sample size (AIC_c) was considered to have the strongest support from the data among the models considered. Another metric, AIC_c weight (w), was calculated for each model; these sum to 1.0 across the entire model set and provide a measure of relative support for candidate models. The variables included in the models with highest support are considered to explain important variation in the response. Parameter likelihoods, which are the sums of w for all models including a given parameter, indicate the relative support for that variable, taking into account model uncertainty. Parameter likelihoods close to 1 indicate strong support. Finally, weighted parameter estimates and associated unconditional standard errors were calculated, which are estimates of the size, direction, and associated variation of effects of variables after accounting for model uncertainty.

EROD activity winter, 2006/2007

A similar analysis was conducted for data collected during November, 2006, and March and April, 2007. All of these samples were run concurrently at the UCD laboratory, so there was no interlaboratory or interrun variation to consider. Using GLMs and information-theoretic methods of model selection and inference, as described above, we evaluated variation in EROD in relation to singular and additive combinations of individual attributes, area, and, in this case, season (November vs. March and April). Therefore, the candidate set included the following eight models: EROD = individual attributes, EROD = area, EROD = season, EROD = individual attributes + area, EROD = individual attributes + season, EROD = area + season, EROD = individual attributes + area + season, and EROD = null.

EROD activity March, 2009

Our analysis of data from samples collected during March, 2009, was the same as that conducted for March, 2005. Note that

data were generated only from the UCD laboratory for March, 2009, samples.

RESULTS

Laboratory comparison

Based on paired liver biopsies from harlequin ducks captured in March, 2005, a strong correlation ($r^2 = 0.70$; Fig. 1) was found between results reported from WHOI and those reported independently from UCD. Averages (±SE) differed somewhat by laboratory (birds captured on areas oiled by the *Exxon Valdez* spill = 194.9 ± 30.1 at WHOI and 161.3 ± 31.2 at UCD, and those captured from unoiled areas = 96.6 ± 14.4 at WHOI and 55.3 ± 13.7 at UCD), although area differences were readily apparent in both data sets (Fig. 2). Similarly, the slope of the relationship between the two laboratories (using results from WHOI as the response) was less than one (0.71 ± 0.08), indicating that the different laboratories reported data with somewhat different absolute values, as reported elsewhere [26]. However, the close correlation, as well as the similarity in GLM results using both data sets (below), indicated that data from both laboratories supported the same inference about differences in EROD activity levels in relation to individual attributes and areas.

EROD activity March 2005

Variation in EROD activity of harlequin ducks captured in March, 2005, was strongly associated with whether they were from oiled or unoiled areas. Based on UCD analyses, the model with area as the only explanatory variable received nearly 10 times the support of any other model, with a *w* of 0.87 (Table 2). The group of individual attribute variables did not explain meaningful variation in EROD, insofar as both models including individual attributes had small *w* and received less support than the null model (i.e., had larger AIC_c values; Table 2). Analyses from WHOI corroborated these conclusions; the order of candidate models and the relative support for each closely matched those based on UCD analyses (Table 2).

Parameter likelihood values also supported the importance of area for explaining variation in March, 2005, EROD activity. With the UCD analysis results, the area parameter was strongly supported, with a parameter likelihood of 0.91 (Table 3). Also, the weighted parameter estimate indicated that areas differed by an average of 96.0 pmol/min/mg protein, with EROD activity markedly higher in oiled areas (Fig. 2). Parameter likelihood values for individual attributes were small, and the weighted parameter estimates were smaller than the corresponding unconditional standard errors (Table 3), further indicating that they did not have strong explanatory value. As with the model ranking described above, the patterns in parameter likelihoods, weighted parameter estimates, and unconditional SE based on WHOI results mirrored those from UCD (Table 3 and Fig. 2), strengthening confidence in the inference drawn from these analyses.

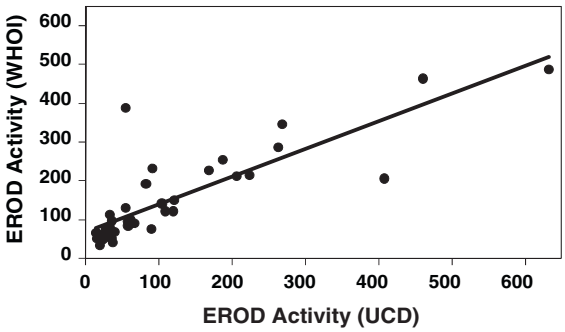

Fig. 1. Scatterplot of hepatic 7-ethoxyresorufin-*O*-deethylase (EROD) activity (pmol/min/mg protein) contrasting results from two laboratories (WHOI = Woods Hole Oceanographic Institution; UCD = University of California Davis) that independently analyzed subsamples of the same livers collected from harlequin ducks ($n = 40$) in Prince William Sound in March, 2005.

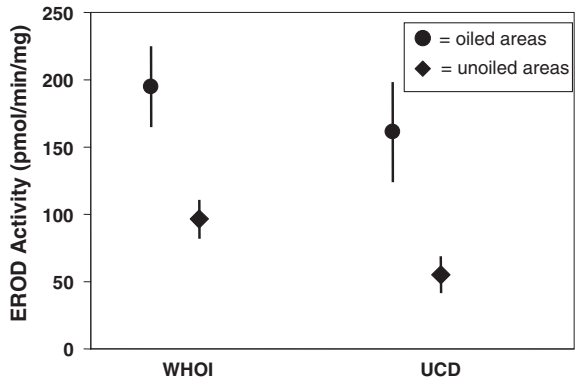

Fig. 2. Average (±standard error) hepatic 7-ethoxyresorufin-*O*-deethylase (EROD) activity of harlequin ducks ($n = 40$) captured in March, 2005, in areas of Prince William Sound, Alaska, USA, oiled during the *Exxon Valdez* spill and nearby unoiled areas. Results are presented for two laboratories (WHOI = Woods Hole Oceanographic Institution; UCD = University of California Davis) that independently analyzed subsamples of the same livers.

EROD activity winter, 2006/2007

For samples from winter, 2006 to 2007, analyzed only in the UCD laboratory, we found that the model with area as the only explanatory variable was best supported, with a *w* of 0.51 (Table 4). However, the model with area and season as explanatory variables also received considerable support, with an AIC_c value that was only 0.4 from the best-supported model and a *w* of 0.41. None of the other candidate models received substantial support, including all other models with season as an explanatory variable. Consistent with these findings, the parameter likelihood value for area was 1.0, indicating that only models including area received any meaningful support from the data (Table 3). The weighted parameter estimate for area indicated that EROD was significantly higher in oiled areas compared with unoiled (Table 3 and Fig. 3). Parameter likelihoods for individual attributes were low, and the size of unconditional SE exceeded the weighted parameter estimates in all cases (Table 3), which confirmed that these variables did not explain important variation in the response. The parameter likelihood for season indicated moderate support (Table 3), based on the inclusion of season in the second-best model; however, the weighted parameter estimate for season was small (−7.7 pmol/min/mg protein), with an unconditional SE larger than the estimate, further suggesting the lack of importance of season for explaining variance in EROD. Figure 3 graphically illustrates the modest seasonal difference in EROD as well as the consistent and large difference by area.

EROD activity March, 2009

Consistent with earlier sampling periods, the best-supported model for March, 2009, data indicated differences in average EROD activity between areas, with a *w* of 0.73 (Table 5). As in previous winters, average EROD activity was higher in oiled

Table 2. Results of information-theoretic analyses using general linear models to evaluate variation in hepatic 7-ethoxyresorufin-*O*-deethylase (EROD) activity of harlequin ducks ($n = 40$) captured in Prince William Sound, Alaska, USA, during March, 2005[a]

		UCD			WHOI		
Model	K[b]	AIC_c[c]	ΔAIC_c[d]	w[e]	AIC_c	ΔAIC_c	w
EROD = area[f]	3	391.1	0.0	0.87	377.3	0.0	0.91
EROD = null	2	395.7	4.6	0.09	383.2	5.9	0.05
EROD = area + individual[g]	6	397.4	6.3	0.04	383.6	6.5	0.04
EROD = individual	5	401.5	10.4	0.00	388.6	11.3	0.00

[a] Results are reported from two laboratories, University of California Davis (UCD) and Woods Hole Oceanographic Institution (WHOI), that independently analyzed paired samples from each individual.
[b] K = number of estimated parameters in the model.
[c] AIC_c = Akaike's information criterion, corrected for small sample size.
[d] ΔAIC_c = difference in AIC_c from the best supported model.
[e] $w = AIC_c$ weight.
[f] Area = categorical variable indicating areas either oiled during the *Exxon Valdez* spill or unoiled.
[g] Individual = a grouping of variables describing attributes of individuals (age, sex, and mass).

areas relative to unoiled areas (Fig. 3). The null model received a modest amount of support ($w = 0.21$), but less than one-third of the support received by the model including an area effect. The importance of the area term is reflected in the parameter likelihood of 0.76 (Table 3). Models including individual attributes received very little support, indicating that age, sex, and mass were not strong correlates with EROD, which was confirmed by the low parameter likelihood for these explanatory variables.

DISCUSSION

We found that hepatic CYP1A levels in harlequin ducks captured during 2005 to 2009, based on EROD activity, were unequivocally higher in areas oiled during the *Exxon Valdez* spill than in nearby unoiled areas. This conclusion was strongly supported over multiple sample periods, as well as by two independent laboratories for one of the sampling periods. Our results are consistent with the findings of Trust et al. [14] from 11 years prior that harlequin ducks were exposed to CYP1A inducers more frequently or in higher concentrations at oiled areas relative to unoiled areas. We interpret the current results as evidence that harlequin ducks continued to be exposed to residual oil from the *Exxon Valdez* spill through at least 2009, 20 years after the spill. This interval of time is much longer than conventional assumptions about the duration of bioavailability of spilled oil [3]. Evidence of continued exposure indicates that deleterious effects on individuals or populations possibly persisted over this time frame, although we recognize that exposure cannot necessarily be inferred to indicate damage [6].

Similar spatial patterns of CYP1A induction have been described for other vertebrates in Prince William Sound, including Barrow's goldeneyes (*Buchephala islandica* [14]), adult pigeon guillemots (*Cepphus columba* [28]), river otters (*Lontra canadensis* [29]), and two demersal fishes [8], masked greenlings (*Hexagrammos octogrammus*) and crescent gunnels (*Pholis laeta*), within a decade of the *Exxon Valdez* spill. This body of evidence overwhelmingly supports the conclusion that harlequin ducks, along with other near-shore vertebrates, were being exposed to CYP1A-inducing compounds in areas of Prince William Sound that had received oil during the *Exxon Valdez* spill.

Some authors have questioned the source of CYP1A-inducing compounds in Prince William Sound [30], recognizing that there may be multiple CYP1A-inducing compounds from multiple sources within a given area [6]. Several authors [30–33] have argued that non-*Exxon Valdez* sources of PAHs are more abundant and more likely to induce CYP1A responses than residual *Exxon Valdez* oil. However, the spatial correspondence between elevated CYP1A induction and history of contamination during the *Exxon Valdez* oil spill strongly suggests causation. Also, other studies have indicated that PAHs in the areas

Table 3. Parameter likelihoods (P.L.), weighted parameter estimates, and unconditional standard errors (SE) derived from information-theoretic analyses using general linear models to evaluate variation in hepatic 7-ethoxyresorufin-*O*-deethylase (EROD) activity (pmol/min/mg protein) of harlequin ducks captured in Prince William Sound, Alaska, USA, during March, 2005; winter, 2006 to 2007 (including samples from November, 2006, and March and April, 2007); and March, 2009[a]

	WHOI March, 2005		UCD March, 2005		UCD, 2006 and 2007		UCD March, 2009	
Parameter	P.L.	Estimate ± SE	P.L.	Estimate ± SE	P.L.	Estimate ± SE	P.L.	Estimate ± SE
Intercept	1.00	115.0 ± 51.7	1.00	70.4 ± 55.4	1.00	22.9 ± 24.1	1.00	50.37 ± 62.87
Area[b]	0.95	93.1 ± 32.0	0.91	96.0 ± 37.1	1.00	68.7 ± 13.4	0.76	50.23 ± 37.85
Sex[c]	0.04	−2.3 ± 3.1	0.04	−0.7 ± 3.0	0.09	−0.0 ± 2.1	0.06	−4.43 ± 9.46
Age[d]	0.04	−0.2 ± 2.4	0.04	−2.9 ± 4.2	0.09	2.2 ± 4.7	0.06	3.24 ± 7.87
Mass	0.04	−0.02 ± 0.04	0.04	−0.01 ± 0.04	0.09	0.00 ± 0.02	0.06	−0.04 ± 0.09
Season[e]	—	—	—	—	0.44	−7.7 ± 11.7	—	—

[a] Results from March, 2005, samples are presented for data reported from two laboratories, University of California Davis (UCD) and Woods Hole Oceanographic Institution (WHOI), that independently analyzed paired samples from each individual.
[b] Area = categorical variable indicating areas either oiled during the *Exxon Valdez* spill or unoiled, with unoiled as the reference value.
[c] Sex = categorical variable (male vs. female), with male as the reference value.
[d] Age = categorical variable (hatch-year vs. after-hatch-year), with hatch-year as the reference value.
[e] Season = categorical variable (November vs. March and April), with November as the reference value.

Table 4. Results of information-theoretic analyses using general linear models to evaluate variation in hepatic 7-ethoxyresorufin-*O*-deethylase (EROD) activity of harlequin ducks ($n = 90$) captured in Prince William Sound, Alaska, USA, during winter, 2006–2007

Model	K[a]	AIC_c[b]	ΔAIC_c[c]	w[d]
EROD = area[e]	3	750.4	0.0	0.51
EROD = area + season[f]	4	750.9	0.4	0.41
EROD = area + individual[g]	6	755.0	4.5	0.05
EROD = area + season + individual	7	755.9	5.4	0.03
EROD = null	2	771.7	21.3	0.00
EROD = season	3	772.5	22.1	0.00
EROD = individual	5	777.7	27.2	0.00
EROD = season + individual	6	778.5	28.1	0.00

[a] K = number of estimated parameters in the model.
[b] AIC_c = Akaike's information criterion, corrected for small sample size.
[c] ΔAIC_c = difference in AIC_c from the best supported model.
[d] $w = AIC_c$ weight.
[e] Area = categorical variable indicating areas either oiled during the *Exxon Valdez* spill or unoiled.
[f] Season = categorical variable indicating period of sample collection (November vs. March and April).
[g] Individual = a grouping of variables describing attributes of individuals (age, sex, and mass).

where elevated CYP1A was observed in vertebrates are predominantly from the *Exxon Valdez* spill [15], supporting the inference that *Exxon Valdez* oil was the inducing agent. Recent studies have indicated that sites with residual *Exxon Valdez* oil had bioavailable PAHs that elicited CYP1A induction when experimentally injected into fish [34]. Other potential CYP1A inducers, specifically PCBs, were very low and below concentrations that would induce CYP1A induction, which is consistent with broad-scale atmospheric deposition [35]. In addition, Trust et al. [14] considered the potential role of PCBs in observed CYP1A induction in sea ducks in Prince William Sound and found that plasma concentrations were very low and generally were not related to EROD activity. In addition, Short et al. [16] calculated that, given the distribution of residual *Exxon Valdez* oil through 2003, benthic foraging vertebrates were likely to encounter lingering oil, further suggesting that residual *Exxon Valdez* oil was the inducing compound.

Vertebrates that inhabit the intertidal and shallow subtidal environments, particularly those that consume benthic organisms, were most likely to have elevated CYP1A [2]. This presumably is due, in part, to the fact that intertidal areas of

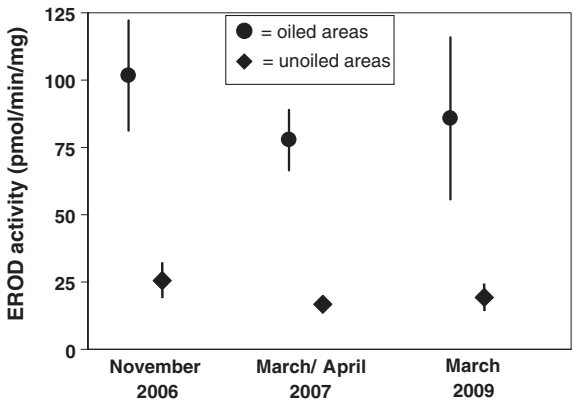

Fig. 3. Average (±standard error) hepatic 7-ethoxyresorufin-*O*-deethylase (EROD) activity of harlequin ducks captured during winter, 2006 to 2007 ($n = 90$), and March, 2009 ($n = 39$), in areas of Prince William Sound, Alaska, USA, oiled during the *Exxon Valdez* spill and nearby unoiled areas. Samples from November, 2006, and March/April, 2007, were analyzed concurrently, and results are presented separately for two capture seasons.

Table 5. Results of information-theoretic analyses using general linear models to evaluate variation in hepatic 7-ethoxyresorufin-*O*-deethylase (EROD) activity of harlequin ducks ($n = 39$) captured in Prince William Sound, Alaska, USA, during March, 2009

Model	K[a]	AIC_c[b]	ΔAIC_c[c]	w[d]
EROD = area[e]	3	358.3	0.0	0.73
EROD = null	2	360.9	2.5	0.21
EROD = individual	5	364.6	6.3	0.03
EROD = area + individual[f]	6	364.7	6.3	0.03

[a] K = number of estimated parameters in the model.
[b] AIC_c = Akaike's information criterion, corrected for small sample size.
[c] ΔAIC_c = difference in AIC_c from the best supported model.
[d] $w = AIC_c$ weight.
[e] Area = categorical variable indicating areas either oiled during the *Exxon Valdez* spill or unoiled.
[f] Individual = a grouping of variables describing attributes of individuals (age, sex, and mass).

Prince William Sound received a large portion of the spilled *Exxon Valdez* oil [18] and sequestered lingering oil a decade or more postspill [15]. Also, because certain molluscan invertebrates have a limited capacity to metabolize PAHs [36] and are known to bioaccumulate PAHs [37,38], predators such as harlequin ducks may be more likely to ingest PAHs with their prey. Also, some invertivores disturb sediment during foraging, which is a potential mechanism for release of hydrocarbons and ingestion.

Consistent with predictions of increased exposure to residual oil and vulnerability to subsequent effects, as well as empirical evidence of exposure [14], invertivorous, nearshore-dwelling vertebrates have been shown to have population demographic attributes outside of the normal range since the *Exxon Valdez* oil spill. For example, sea otter (*Enhydra lutris*) numbers in heavily oiled regions of Prince William Sound were well below estimates of prespill numbers [1]. Also, sea otter survival in oiled areas was depressed through at least 1998 [39]. Similar evidence of postspill demographic problems was described for harlequin ducks [2]. Densities of wintering harlequin ducks in 1996 and 1997 were lower than expected in oiled areas of Prince William Sound, after accounting for effects of differing habitat [20]. Also, survival of wintering female harlequin ducks was lower in oiled areas than in unoiled areas [21] during 1995 to 1998. More recent estimates have indicated that harlequin duck survival during winters, 2000 to 2003, did not differ between oiled and unoiled areas [40], suggesting that, despite the evidence of continued exposure reported herein, oil-induced effects on demographic rates may be diminishing.

In addition to potential relationships between oil exposure and demographic rates [2], more subtle effects at suborganismal and molecular levels are plausible. Rainbow trout (*Oncorhynchus mykiss*) showed increased mortality in response to viral challenge when they had been exposed to a CYP1A inducer [41]. In mammals, CYP1A1 is known to activate PAH to toxic and mutagenic derivatives [42]. In birds, Trust et al. [43] identified effects of PAHs on immune function and mixed-function oxygenase activity (e.g., EROD) in European starlings (*Sturnus vulgaris*). In controlled-dose experiments, crude oil and PAHs have been linked to impaired reproduction, depressed weight gain, increased organ weight, increased endocrine activity, or mixed-function oxygenase activity in several avian taxa [44–47]. Induction of CYP1A gene expression does not in itself represent an adverse effect and with some substrates or inducers could be principally an adaptive response. However, it can be a marker of exposure to PAHs demonstrated to have adverse effects on birds. Associations between aryl hydrocarbon recep-

tor agonist activation and subsequent effects, including possible involvement of the multiple CYP1 genes that are expressed in birds, have not been fully explored in relation to the effects of the *Exxon Valdez* oil spill, and research is warranted to assess appropriately those effects on harlequin ducks and other species at risk of exposure.

In summary, the EROD levels reported here provide strong evidence of CYP1A induction in harlequin ducks from oiled areas, which we conclude is due to continued exposure to residual *Exxon Valdez* oil and indicates that harlequin ducks remain at risk of potential deleterious consequences of that exposure. The present work extends the timeline of exposure to 20 years postspill and adds to the body of evidence describing the previously unanticipated duration of exposure and potential effects of the *Exxon Valdez* oil spill. We note that oil from other contamination events also has been reported to persist over long periods of time [48–52]. We agree with Peterson et al. [3] that it is important to recognize that the duration of presence of residual oil and potential for associated effects is not necessarily limited to a few years after spills; these may occur over decades for some vulnerable species. Continued monitoring of indicators of CYP1A induction in harlequin ducks in Prince William Sound will reveal when EROD in oiled areas has returned to background levels and will fully describe the timeline over which exposure occurs.

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REFERENCES

1. Bodkin JL, Ballachey BE, Dean TA, Fukuyama AK, Jewett SC, McDonald L, Monson DH, O'Clair CE, VanBlaricom GR. 2002. Sea otter population status and the process of recovery from the 1989 ''*Exxon Valdez*'' oil spill. *Mar Ecol Prog Ser* 241:237–253.
2. Esler D, Bowman TD, Trust K, Ballachey BE, Dean TA, Jewett SC, O'Clair CE. 2002. Harlequin duck population recovery following the *Exxon Valdez* oil spill: progress, process, and constraints. *Mar Ecol Prog Ser* 241:271–286.
3. 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.
4. Payne JF, Fancey LL, Rahimtula AA, Porter EL. 1987. Review and perspective on the use of mixed-function oxygenase enzymes in biological monitoring. *Comp Biochem Physiol C* 86:233–245.
5. Goksøyr A. 1995. Use of cytochrome P450 1A (CYP1A) in fish as a biomarker of aquatic pollution. *Arch Toxicol Suppl* 17:80–95.
6. Lee RF, Anderson JW. 2005. Significance of cytochrome P450 system responses and levels of bile fluorescent aromatic compounds in marine wildlife following oil spills. *Mar Pollut Bull* 50:705–723.
7. Carls MG, Heintz RA, Marty GD, Rice SD. 2005. Cytochrome P4501A induction in oil-exposed pink salmon *Oncorhynchus gorbuscha* embryos predicts reduced survival potential. *Mar Ecol Prog Ser* 301:253–265.
8. Jewett SC, Dean TA, Woodin BR, Hoberg MK, Stegeman JJ. 2002. Exposure to hydrocarbons ten years after the *Exxon Valdez*: evidence from cytochrome P4501A expression and biliary FACs in nearshore demersal fishes. *Mar Environ Res* 54:21–48.
9. Kammann U, Lang T, Vobach M, Wosniok W. 2005. Ethoxyresorufin-*O*-deethylase (EROD) activity in dab (*Limanda limanda*) as biomarker for marine monitoring. *Environ Sci Pollut Res* 12:140–145.
10. Peakall DB, Norstrom RJ, Jeffrey DA, Leighton FA. 1989. Induction of hepatic mixed function oxidases in the herring gull (*Larus argentatus*) by Prudhoe Bay crude oil and its fractions. *Comp Biochem Physiol C* 94: 461–463.
11. Ben-David M, Kondratyuk T, Woodin BR, Snyder PW, Stegeman JJ. 2001. Induction of cytochrome P4501A1 expression in captive river otters fed Prudhoe Bay crude oil: evaluation by immunohistochemistry and quantitative RT-PCR. *Biomarkers* 6:218–235.
12. Kubota A, Iwata H, Goldstone HM, Kim EY, Stegeman JJ, Tanabe S. 2006. Cytochrome P450 1A4 and 1A5 in common cormorant (*Phalacrocorax carbo*): Evolutionary relationships and functional implications associated with dioxin and related compounds. *Toxicol Sci* 92:394–408.
13. Miles AK, Flint PL, Trust KA, Ricca MA, Spring SE, Arietta DE, Hollmén T, Wilson BW. 2007. Polycyclic aromatic hydrocarbon exposure in Steller's eiders (*Polysticta stelleri*) and harlequin ducks (*Histrionicus histrionicus*) in the eastern Aleutian Islands, Alaska. *Environ Toxicol Chem* 26:2694–2703.
14. 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.
15. Short JW, Lindeberg MR, Harris PM, Maselko JM, Pella JJ, Rice SD. 2004. Estimate of oil persisting on the beaches of Prince William Sound 12 years after the *Exxon Valdez* oil spill. *Environ Sci Technol* 38:19–25.
16. Short JW, Maselko JM, Lindeberg MR, Harris PM, Rice SD. 2006. Vertical distribution and probability of encountering intertidal *Exxon Valdez* oil on shorelines of three embayments within Prince William Sound. *Environ Sci Technol* 40:3723–3729.
17. McKnight A, Sullivan KM, Irons DB, Stephensen SW, Howlin S. 2006. Marine bird and sea otter population abundance of Prince William Sound, Alaska: Trends following the *T/V Exxon Valdez* oil spill, 1989–2005. *Exxon Valdez* Oil Spill Restoration Project Final Report (Restoration Projects 040159/050751). U.S. Fish and Wildlife Service, Anchorage, AK.
18. Wolfe DA, Hameedi MJ, Galt JA, Watabayashi G, Short J, O'Claire C, Rice S, Michel J, Payne JR, Braddock J, Hanna S, Sale D. 1994. The fate of the oil spilled from the *Exxon Valdez*. *Environ Sci Technol* 28:561–568.
19. Rosenberg DH, Petrula MJ. 1998. Status of harlequin ducks in Prince William Sound, Alaska, after the *Exxon Valdez* oil spill, 1995–1997. *Exxon Valdez* Oil Spill Restoration Project Final Report 97427. Alaska Department of Fish and Game, Anchorage, AK, USA.
20. Esler D, Bowman TD, Dean TA, O'Clair CE, Jewett SC, McDonald LL. 2000. Correlates of harlequin duck densities during winter in Prince William Sound, Alaska. *Condor* 102:920–926.
21. 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.
22. Iverson SA, Esler D. 2006. Site fidelity and the demographic implications of winter movements by a migratory bird, the harlequin duck. *J Avian Biol* 37:219–228.
23. Mather DD, Esler D. 1999. Evaluation of bursal depth as an indicator of age class of harlequin ducks. *J Field Ornithol* 70:200–205.
24. Smith CM, Goudie RI, Cooke F. 1998. Winter age ratios and the assessment of recruitment of harlequin ducks. *Waterbirds* 24:39–44.
25. Head JA, Kennedy SW. 2007. Differential expression, induction, and stability of CYP1A4 and CYP1A5 mRNA in chicken and herring gull embryo hepatocytes. *Comp Biochem Physiol C Toxicol Pharmacol* 145:617–624.
26. Esler D. 2008. Quantifying temporal variation in Harlequin Duck cytochrome P4501A induction. *Exxon Valdez* Oil Spill Trustee Council Gulf Ecosystem Monitoring and Research Project Final Report (GEM Project 050777). Centre for Wildlife Ecology, Simon Fraser University, Delta, British Columbia, Canada.
27. Burnham KP, Anderson DR. 2002. *Model Selection and Multimodel Inference: A Practical Information Theoretic Approach*, 2nd ed. Springer-Verlag, New York, NY, USA.
28. Golet GH, Seiser PE, McGuire AD, Roby DD, Fischer JB, Kuletz KJ, 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.
29. Bowyer RT, Blundell GM, Ben-David M, Jewett SC, Dean TA, Duffy LK. 2003. Effects of the *Exxon Valdez* oil spill on river otters: Injury and recovery of a sentinel species. *Wildl Monogr* 153:1–53.
30. Harwell MA, Gentile JH. 2006. Ecological significance of residual exposures and effects from the *Exxon Valdez* oil spill. *Integr Environ Assess Manage* 2:204–246.

31. Page DS, Boehm PD, Douglas GS, Bence AE, Burns WA, Mankiewicz PJ. 1996. The natural petroleum hydrocarbon background in subtidal sediments of Prince William Sound, Alaska, USA. *Environ Toxicol Chem* 15:1266–1281.
32. Page DS, Boehm PD, Douglas GS, Bence AE, Burns WA, Mankiewicz PJ. 1997. An estimate of the annual input of natural petroleum hydrocarbons to seafloor sediments of Prince William Sound, Alaska. *Mar Pollut Bull* 34:744–749.
33. Boehm PD, Page DS, Burns WA, Bence AE, Mankiewicz PJ, Brown JS. 2001. Resolving the origin of the petrogenic hydrocarbon background in Prince William Sound, Alaska. *Environ Sci Technol* 35:471–479.
34. Springman KR, Short JW, Lindeberg MR, Maselko JM, Khan C, Hodson PV, Rice SD. 2008. Semipermeable membrane devices link site-specific contaminants to effects: Part I—Induction of CYP1A in rainbow trout from contaminants in Prince William Sound, Alaska. *Mar Environ Res* 66:477–486.
35. Short JW, Springman KR, Lindeberg MR, Holland LG, Larsen ML, Sloan CA, Khan C, Hodson PV, Rice SD. 2008. Semipermeable membrane devices link site-specific contaminants to effects: Part II—A comparison of lingering *Exxon Valdez* oil with other potential sources of CYP1A inducers in Prince William Sound, Alaska. *Mar Environ Res* 66:487–498.
36. Chaty S, Rodius F, Vasseur P. 2004. A comparative study of the expression of CYP1A and CYP4 genes in aquatic invertebrate (freshwater mussel, *Unio tumidus*) and vertebrate (rainbow trout, *Oncorhynchus mykiss*). *Aquat Toxicol* 69:81–93.
37. Fukuyama AK, Shigenaka G, Hoff RZ. 2000. Effects of residual *Exxon Valdez* oil on intertidal *Protothaca staminea*: mortality, growth, and bioaccumulation of hydrocarbons in transplanted clams. *Mar Pollut Bull* 40:1042–1050.
38. Rust AJ, Burgess RM, Brownawell BJ, McElroy AE. 2004. Relationship between metabolism and bioaccumulation of benzo[*a*]pyrene in benthic invertebrates. *Environ Toxicol Chem* 23:2587–2593.
39. Monson DH, Doak DF, Ballachey BE, Johnson A, Bodkin JL. 2000. Long-term impacts of the *Exxon Valdez* oil spill on sea otters, assessed through age-dependent mortality patterns. *Proc Natl Acad Sci U S A* 97: 6562–6567.
40. Esler D, Iverson SA. 2010. Female harlequin duck winter survival 11 to 14 years after the *Exxon Valdez* oil spill. *J Wildl Manage* 74: (in press).
41. Springman KR, Kurath G, Anderson JJ, Emlen JM. 2005. Contaminants as viral cofactors: assessing indirect population effects. *Aquat Toxicol* 71:13–23.
42. Nebert DW, Dalton TP, Okey AB, Gonzalez FJ. 2004. Role of aryl hydrocarbon receptor-mediated induction of the CYP1 enzymes in environmental toxicity and cancer. *J Biol Chem* 279:23847–23850.
43. Trust KA, Fairbrother A, Hooper MJ. 1994. Effects of 7,12-dimethylbenz[*a*]anthracene on immune function and mixed-function oxygenase activity in the European starling. *Environ Toxicol Chem* 13:821–830.
44. Hoffman DJ. 1979. Embryotoxic and teratogenic effects of petroleum hydrocarbons in mallards (*Anas platyrhynchos*). *J Toxicol Environ Health* 5:835–844.
45. Naf C, Broman D, Brunstrom B. 1992. Distribution and metabolism of polycyclic aromatic hydrocarbons (PAHs) injected into eggs of chicken (*Gallus domesticus*) and common eider duck (*Somateria mollissima*). *Environ Toxicol Chem* 11:1653–1660.
46. Peakall DB, Miller DS, Butler RG, Kinter WR, Hallett DJ. 1980. Effects of ingested crude oil on black guillemots: A combined field and laboratory study. *Ambio* 9:28–30.
47. Peakall DB, Tremblay J, Miller DS, Kinter WB. 1981. Endocrine dysfunction in seabirds caused by ingested oil. *Environ Res* 24:6–14.
48. Corredor JE, Morell JM, Castillo CE. 1990. Persistence of spilled crude oil in a tropical intertidal environment. *Mar Pollut Bull* 21:385–388.
49. Burns KA, Garrity SD, Jorissen D, MacPherson J, Stoelting M, Tierney J, Yelle-Simmons L. 1994. The Galeta oil spill. 2. Unexpected persistence of oil trapped in mangrove sediments. *Estuar Coast Shelf Sci* 38:349–364.
50. Vandermeulen JH, Singh JG. 1994. ARROW oil spill, 1970–1990: Persistence of 20-yr weathered Bunker C fuel oil. *Can J Fish Aquat Sci* 51:845–855.
51. Reddy CM, Eglinton TI, Hounshell A, White HK, Xu L, Gaines RB, Frysinger GS. 2002. The West Falmouth oil spill after thirty years: The persistence of petroleum hydrocarbons in marsh sediments. *Environ Sci Technol* 36:4754–4760.
52. Peacock EE, Nelson RK, Solow AR, Warren JD, Baker JL, Reddy CM. 2005. The West Falmouth oil spill: 100kg of oil persists in marsh sediments. *Environ Forensics* 6:273–281.