Exxon Valdez Oil Spill
Restoration Project Final Report

Long-term Monitoring: Lingering Oil
Evaluating Chronic Exposure of Harlequin Ducks and Sea Otters to Lingering *Exxon Valdez* Oil in Western Prince William Sound

Restoration Project 12120114-Q
Final Report

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PART ONE: HARLEQUIN DUCKS

Study History: Harlequin ducks have been studied extensively in Prince William Sound following the *Exxon Valdez* oil spill, leading to one of the most thorough considerations of wildlife population injury and recovery following a major oil spill ever undertaken. These efforts have included population monitoring by the U.S. Fish and Wildlife Service and the Alaska Department of Fish and Game, as well as a series of directed research projects designed to elucidate the process of, and constraints to, population recovery. These studies demonstrated that harlequin ducks were exposed to lingering oil over a much longer time frame (i.e., through at least 2011, 22 years following the spill) than expected at the time of the spill, based on elevated levels of cytochrome P4501A induction in birds from oiled areas. In addition, several lines of evidence suggested that direct population injury persisted through at least 1998. Specifically, female winter survival probabilities were found to differ between oiled and unoiled areas, and densities were shown to be lower in oiled than unoiled areas after accounting for habitat-related effects. More recent data have indicated that female winter survival did not differ between oiled and unoiled sites during 2000-03, suggesting that direct effects of oil exposure on demographic properties had abated. Using demographic data, a population model was constructed to estimate timeline until recovery of numbers to pre-spill levels, which was projected to be 24 years post-spill or 2013. However, persistence of oil in the environment and evidence of exposure of harlequin ducks to that oil through 2011 has led to continued monitoring to evaluate the timeline of exposure. The current work was designed as another data point in that time series for 2013.

Abstract: For the first time since the 1989 *Exxon Valdez* Oil Spill, we found that average cytochrome P4501A induction (as measured by EROD activity) during March 2013 was not elevated in wintering harlequin ducks captured in areas of Prince William Sound oiled by the spill, relative to those captured in unoiled areas. Another metric of oil exposure (the incidence of individuals with elevated cytochrome P4501A induction) was consistent with this result, as it also showed similar values between oiled and unoiled areas. We interpret these findings to indicate that exposure of harlequin ducks to residual *Exxon Valdez* oil abated within 24 years after the original spill. These findings follow results from 2011, which showed reductions since 2009 in these metrics on oiled areas relative to unoiled and hence progress towards abatement of exposure, despite continued differences between areas in 2011,. The data presented in this report add to a growing body of literature indicating that persistence of oil in the environment, and exposure of wildlife to that oil, can occur over much longer time frames than previously assumed.

These data may be used to define the duration of exposure (nearly two and a half decades) for one of the wildlife species most likely to suffer extended exposure.

Key Words: biomarker, cytochrome P4501A, *Exxon Valdez* oil spill, harlequin ducks, *Histrionicus histrionicus*, oil exposure, Prince William Sound, recovery.

Project Data: Data will be kept in digital format (MS Excel) at the Alaska Science Center, U.S. Geological Survey, Anchorage, Alaska.

Citation:

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PART ONE: HARLEQUIN DUCKS

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EXECUTIVE SUMMARY

Extensive research and monitoring supported by the *Exxon Valdez* Oil Spill Trustee Council has led to a thorough understanding of the response of harlequin duck populations to the 1989 *Exxon Valdez* spill, and the process of (and constraints to) recovery. The information presented in this report adds to that body of work.

Induction of cytochrome P4501A (CYP1A) in vertebrates occurs in response to exposure to a limited number of compounds, including polycyclic aromatic hydrocarbons such as those found in crude oil. Because CYP1A induction is both specific and sensitive, it has been used to evaluate exposure to inducing compounds in many cases of environmental contamination, including that of the *Exxon Va*ldez oil spill. Elevated CYP1A has been demonstrated in several species in areas of Prince William Sound oiled by the *Exxon Valdez* spill relative to unoiled areas, including harlequin ducks.

In this study, CYP1A induction was determined by measuring hepatic 7-ethoxyresorufin-O-deethylase (EROD) activity, which is a well-established method and is the same approach used in earlier *Exxon Valdez* studies and in similar studies of harlequin ducks and other sea ducks elsewhere. During March 2013, we captured 25 harlequin ducks in oiled areas of Prince William Sound and 25 in unoiled areas. Small liver biopsies were surgically removed from each individual, frozen immediately in liquid nitrogen, and subsequently shipped to the University of California Davis for EROD analysis.

We found that CYP1A induction was not related to area, with average (pmol/min/mg ± SE) EROD activity of 17.8 (± 3.0) in oiled areas and 27.7 (± 5.9) in unoiled areas. This represents the first occasion since sampling was initiated in 1998 that CYP1A induction was not statistically higher in oiled areas than unoiled areas. This critical result follows the observation during 2011 that, although CYP1A induction was higher on oiled areas, the magnitude of the difference was reduced relative to previous years (1998 to 2009). We also considered the incidence of elevated exposure (defined as the number of individuals with EROD activity ≥ 2 times the average on unoiled areas for that year); for 2013 samples, we found that 4% of individuals captured in oiled areas had elevated EROD, compared to 12% in unoiled areas. As in previous years, we found that attributes of individuals (age, sex, and mass) were not related to variation in EROD.

We interpret these results to indicate that harlequin ducks were no longer exposed to residual *Exxon Valdez* oil as of March 2013, 24 years after the spill. Additional sampling in 2014 to confirm this finding is recommended.

This work adds to the body of literature evaluating cytochrome P4501A induction in several nearshore vertebrates in Prince William Sound, and defines the timeline over which exposure to lingering oil was evident for a species particularly vulnerable to long-term exposure.

INTRODUCTION

Effects of the 1989 *Exxon Valdez* oil spill on wildlife populations and communities in Prince William Sound, Alaska have been intensively studied, to document the process and timeline of population and ecosystem recovery. As part of that research, 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 (Payne et al. 1987, Goksøyr 1995, Whitlock 1999). Because CYP1A is strongly induced by a limited number of compounds, it can be a particularly useful biomarker for evaluating exposure to those chemicals (Whyte et al. 2000). Although CYP1A induction does not necessarily indicate deleterious effects on individuals or populations (Lee and Anderson 2005), 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 (Carls et al. 2005). 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 mRNA, protein or activity have been used routinely to evaluate exposure to PAHs, PCBs, and dioxins in fish (Stegeman et al. 1986, Gooch et al. 1989, Goksøyr 1995, Spies et al. 1996, Marty et al. 1997, Woodin et al. 1997, Collier et al. 1996, Wiedmer et al. 1996, Jewett et al. 2002, Carls et al. 2005). 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 (Lee et al. 1985, Peakall et al. 1989, Rattner et al. 1994, Trust et al. 1994; Ben-David et al. 2001; Miles et al. 2007; Esler et al. 2010; Esler et al. 2011; Flint et al. 2012).

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 (e.g., Trust et al. 2000, Jewett et al. 2002; Esler et al. 2010; Esler et al. 2011). 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 nearshore vertebrates. 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 (Short et al. 2004), as well as calculations that intertidal-foraging vertebrates would be likely to encounter lingering oil repeatedly through the course of a year (Short et al. 2006, Bodkin et al. 2012).

Harlequin ducks (*Histrionicus histrionicus*) were one of the species showing indication of elevated CYP1A induction in oiled areas of Prince William Sound relative to unoiled areas (Trust et al. 2000; Esler et al. 2010). 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 (Robertson and Goudie 1999). They are common in Prince William Sound during the nonbreeding season (average of 14,500 individuals between 1990 and

2005; McKnight et al. 2006), and are at higher risk of exposure to residual *Exxon Valdez* oil than many other seabirds, given their exclusive occurrence in nearshore habitats where a disproportionate amount of oil was deposited (Galt et al. 1991, Wolfe et al. 1994) and where lingering oil has remained (Hayes and Michel 1999, Short et al. 2004).

In addition to higher likelihood of exposure, a number of natural history and life history characteristics make harlequin duck individuals and populations particularly sensitive to oil pollution (Esler et al. 2002). These include a diet consisting of invertebrates that live on or in nearshore 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 (Rosenberg and Petrula 1998), densities (Esler et al. 2000a), and female survival (Esler et al. 2000b) relative to unoiled areas. It was concluded that continued exposure to lingering oil was likely a constraint on population recovery (Esler et al. 2002). A population model built with available demographic information was used to estimate the timeline to numeric population recovery, which was estimated to be 24 years after the *Exxon Valdez* spill, or the year 2013 (Iverson and Esler 2010).

Because of the history of elevated indicators of CYP1A induction (Trust et al. 2000; Esler et al. 2010), continued occurrence of lingering oil in intertidal habitats where harlequin ducks occur (Short et al. 2004), and vulnerability of harlequin ducks to effects of oil exposure (Esler et al. 2002), the present study was conducted to follow up on research describing elevated biomarkers of CYP1A in this species. In previous studies, Trust et al. (2000) and Esler et al. 2010 found that average CYP1A expression levels, measured by hepatic 7-ethoxyresorufin-*o*-deethylase (EROD) activity, were significantly higher in wintering harlequin ducks captured in areas oiled by the *Exxon Valdez* spill than those captured in nearby unoiled areas through 2009. In 2011, average EROD activity was higher in harlequin ducks from oiled areas than those from unoiled, although the magnitude of the difference was smaller than during previous sample years (Esler 2011). The primary objective for the present study was to add to the monitoring timeline during 2013, 24 years after the *Exxon Valdez* oil spill, to evaluate whether differences in EROD activity persisted.

In addition to assessment of temporal variation, potential effects of individual attributes (age, sex, and body mass) on variation in CYP1A induction also were considered. Age, sex, and season have been shown to affect CYP1A induction in some fish (Sleiderink et al. 1995, Goksøyr and Larsen 1991, Lindstrom-Seppa and Stegeman 1995, Whyte et al. 2000, Kammann et al. 2005), and thus these factors should be accounted for when evaluating sources of variation in CYP1A induction (Lee and Anderson 2005).

METHODS

Capture and Sample Collection

To facilitate comparisons, the present study closely followed the design and procedures of previous work (Trust et al. 2000; Esler et al. 2010). We captured wintering harlequin ducks using a modified floating mist net (Kaiser et al. 1995) during March 2013. Birds were captured in a number of areas oiled during the *Exxon Valdez* spill, including Crafton Island (60.5º N, 147.9º W), Green Island (60.3º N, 147.4º W), Foul Pass (60.5º N, 147.6º W), and Herring Bay (60.5º N, 147.7º W). Also, birds were captured on nearby northwestern Montague Island (60.3º N, 147.3º 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 94% remaining all winter on the same island or coastline region where they were originally captured and only 2% moving between oiled and unoiled areas (Iverson and Esler 2006). 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. Fish and Wildlife 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 (Mather and Esler 1999, Smith et al. 1998). 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 age class, sex, and area (oiled versus unoiled).

Small (< 0.5 g) liver biopsies were surgically removed by a veterinarian from each harlequin duck while they 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 lab in liquid nitrogen.

Laboratory Analyses

CYP1A induction was determined by measuring hepatic 7-ethoxyresorufin-*o*-deethylase activity, which is a catalytic function principally of hydrocarbon-inducible CYP1A enzymes. In studies of captive harlequin ducks, EROD activity was confirmed to be significantly higher in birds chronically ingesting weathered Prudhoe Bay crude oil, compared to controls (Esler 2008). Similarly, oil-dosed Steller's eiders (*Polysticta stelleri*), another sea duck, had roughly 4-fold increased EROD activity compared to controls (Miles et al. 2007). EROD activity analysis procedures followed standard methods used in previous studies, described in detail by Miles et al. (2007). The measure of EROD activity is expressed in picomoles per minute per milligram of protein, i.e., pmol/min/mg protein.

Statistical Analyses

Variation in EROD activity was analyzed in relation to capture location and individual attributes for birds captured during March 2013. Our primary interest was to determine whether area (oiled versus 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 (Burnham and Anderson 2002) 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 either included 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: (1) EROD = area; (2) EROD = individual attributes; and (3) EROD = area + individual attributes. We also included a null model, which consisted of estimates of a mean and variance across all of the data; 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.

RESULTS

Variation in EROD activity of harlequin ducks captured in March 2013 was not strongly associated with any of the explanatory variables. The best supported model included only the parameter indicating whether harlequin ducks were captured from oiled or unoiled areas (*w* = 0.43; Table 2). However, support for that model was virtually indistinguishable from the null model (*w* = 0.43), which indicated that none of the explanatory variables was strongly supported. In addition, average EROD activity was lower on oiled areas than on unoiled (Table 3; Figure 1); therefore, the moderate support for an area effect was in the opposite direction than expected under a hypothesis of continued oil exposure. As in previous years (Esler et al. 2010, Esler 2011), the group of individual attribute variables did not explain meaningful variation in EROD, 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).

Parameter likelihood values also supported the inference that none of the variables had strong value for explaining variation in March 2013 EROD activity. The area parameter was moderately

supported, with a parameter likelihood of 0.49 (Table 3). However, the weighted parameter estimate indicated that EROD activity was slightly higher on unoiled areas than on oiled areas (Figure 1), by an average of 4.8 pmol/min/mg protein (Table 3). The corresponding unconditional standard error for the area variable (6.5; Table 3) was larger than the parameter estimate, further indicating the lack of strong support for an area effect. Parameter likelihood values for individual attributes were small, and the weighted parameter estimates were smaller than the corresponding unconditional standard errors (Table 3), indicating that they did not have strong explanatory value.

Several measures of CYP1A induction suggested that the degree and incidence of oil exposure on oiled areas was indistinguishable from, or lower than, that on unoiled areas in 2013, which is in stark contrast to previous years. First, average (pmol/min/mg ± SE) EROD activity on oiled areas was 17.8 (± 3.0) in 2013, compared to point estimates > 40 pmol/min/mg in the previous 4 sampling periods, in contrast to consistent estimates of EROD activity in unoiled areas over that same period (Figure 1). Similarly, when data were scaled relative to the reference values from birds captured on unoiled areas, findings from 2013 stand out as being the first time since sampling was initiated that EROD activity on oiled areas was similar to or lower than that on unoiled areas (Figure 2). Finally, the incidence of elevated EROD activity was 4% of individuals from oiled areas in 2013 (Figure 3), which was lower than estimates from oiled areas in previous years and similar to results from unoiled areas across all years.

DISCUSSION

We found that hepatic CYP1A levels in harlequin ducks captured in March 2013, based on EROD activity, were similar between areas oiled during the *Exxon Valdez* spill and in nearby unoiled areas. In fact, the point estimate of average EROD activity was slightly lower in oiled areas than in unoiled. This March 2013 sample constitutes the first time since initiation of harlequin duck CYP1A sampling in 1998 that EROD activity has not been higher in oiled areas than in unoiled areas of Prince William Sound. We interpret this to indicate that harlequin ducks are no longer exposed to residual oil from the 1989 *Exxon Valdez* spill. The timeline over which the observed return to baseline has occurred (24 years) is longer than anticipated at the time of the spill, given conventional assumptions at that time about duration of bioavailability of spilled oil (Peterson et al. 2003). Abatement of exposure to lingering oil implies that any potential direct, deleterious effects on individuals or populations also must have ceased. We recognize that evidence of exposure through 2011 could not necessarily be inferred to indicate ongoing damage (Lee and Anderson 2005), but absence of exposure in 2013 assumes that any remaining damage is due to demographic or toxicological effects of previous exposure.

The observation of similar average EROD activity between oiled and unoiled areas in 2013 follows observations in 2011 of reductions in both average and incidence of CYP1A induction of harlequin ducks in oiled areas, compared to previous years. This suggests that the degree of exposure was declining at that time. This pattern of declines in metrics of exposure also was observed in Barrow's goldeneyes (*Buchephala islandica*), another nearshore-dwelling sea duck, although evidence of lack of exposure by all metrics occurred earlier in this species, by 2009 (Esler et al. 2011).

Differential CYP1A induction between oiled and unoiled areas has been described for other vertebrates in Prince William Sound, including Barrow's goldeneyes (Trust et al. 2000; Esler et al. 2011), adult pigeon guillemots (*Cepphus columba*; Golet et al. 2002), river otters (*Lontra canadensis*; Bowyer et al. 2003), and two demersal fishes (Jewett et al. 2002), masked greenlings (*Hexagrammos octogrammus*) and crescent gunnels (*Pholis laeta*). This body of evidence strongly supports the conclusion that harlequin ducks, along with other nearshore vertebrates, were being exposed to CYP1A-inducing compounds in areas of Prince William Sound, Alaska that received oil during the *Exxon Valdez* spill. It also demonstrates that the timeline for cessation of exposure varies across species, with harlequin ducks being one of the last to show cessation of exposure, likely due to natural history features that enhance risk of exposure (Esler et al. 2002).

Some authors have questioned the source of CYP1A inducing compounds in Prince William Sound (Harwell and Gentile 2006), recognizing that there may be multiple CYP1A-inducing compounds from multiple sources within a given area (Lee and Anderson 2005). Several authors (Page et al. 1996, 1997, Boehm et al. 2001, Harwell and Gentile 2006) 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 where elevated CYP1A was observed in vertebrates are predominately from the *Exxon Valdez* spill (Short et al. 2004), 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 (Springman et al. 2008). Other potential CYP1A inducers, specifically PCBs, were very low and below concentrations that would induce CYP1A induction, consistent with broad-scale atmospheric deposition (Short et al. 2008). In addition, Trust et al. (2000) and Ricca et al. (2010) 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. (2006) 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. Finally, our results indicating declines in CYP1A induction in both harlequin ducks and Barrow's goldeneye over time, and subsequent return to baseline, were consistent with exposure to a source declining in availability over time, as would be expected with *Exxon Valdez* oil, rather than compounds predicted to be constant over time such as atmospheric PCBs or oil from natural seeps.

Vertebrates that inhabit intertidal and shallow subtidal environments, particularly those that consume benthic organisms, were most likely to have prolonged, elevated CYP1A (Esler et al. 2002). This is presumably due, in part, to that fact that intertidal areas of Prince William Sound received a large portion of the spilled *Exxon Valdez* oil (Galt et al. 1991, Wolfe et al. 1994) and sequestered lingering oil a decade or more post-spill (Hayes and Michel 1999, Short et al. 2004). Also, because certain molluscan invertebrates have a limited capacity to metabolize PAHs (e.g., Chaty et al. 2004) and are known to ingest and accumulate PAHs (Short and Harris 1996, Fukuyama et al. 2000, Rust et al. 2004), predators such as harlequin ducks may be more likely to

ingest PAHs with their prey. Also, invertivores disturb sediment during foraging, which is a potential mechanism for release of hydrocarbons and ingestion (Bodkin et al. 2012).

Consistent with predictions of increased exposure to residual oil and vulnerability to subsequent effects, as well as empirical evidence of exposure (Trust et al. 2000, Bodkin et al. 2002, Esler et al. 2010, Esler et al. 2011), invertivorous, nearshore-dwelling vertebrates have been shown to have population demographic attributes outside of the normal range during the period since the *Exxon Valdez* oil spill. For example, sea otter numbers in heavily oiled regions of Prince William Sound were well below estimates of pre-spill numbers (Bodkin et al. 2002). Also, sea otter survival in oiled areas was depressed through at least 1998 (Monson et al. 2000). Similar evidence of post-spill demographic problems was described for harlequin ducks (Esler et al. 2002). 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 (Esler et al. 2000a). Also, survival of wintering female harlequin ducks was lower in oiled areas than unoiled (Esler et al. 2000b) 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 (Esler and Iverson 2010), suggesting that despite the evidence of continued exposure reported by Esler et al. (2010), oil-induced effects on demographic rates were diminishing. Given observed demographic rates, Iverson and Esler (2010) projected numeric population recovery would occur by approximately 2013.

In addition to potential relationships between oil exposure and demographic rates (Esler et al. 2002), more subtle effects at the suborganismal and molecular level are plausible. Rainbow trout (*Oncorhynchus mykiss*) showed increased mortality in response to viral challenge when they had been exposed to a CYP1A inducer (Springman et al. 2005). In mammals, CYP1A1 is known to activate PAH to toxic and mutagenic derivatives (Nebert et al. 2004). In birds, Trust et al. (1994) 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 (Hoffman 1979, Naf et al. 1992, Peakall et al. 1980, Peakall et al. 1981). However, given the lack of CYP1A induction observed in 2013, both lethal and sublethal direct effects of oil exposure can be considered to have ceased.

In summary, the EROD levels reported here provide evidence that CYP1A induction is similar between harlequin ducks from oiled areas and those from unoiled areas, which we conclude is due to lack of continued exposure to residual *Exxon Valdez* oil. This suggests the period of exposure of this species to lingering oil was between 22 and 24 years. We note that oil from other contamination events also has been reported to persist over long periods of time (Corredor et al. 1990, Burns et al. 1994, Vandermeulen and Singh 1994, Reddy et al. 2002, Peacock et al. 2005). We agree with Peterson et al. (2003) that the conventional paradigm that the duration of presence of residual oil and associated effects is limited to a few years should be abandoned and replaced with the recognition that these may occur over decades in certain, vulnerable species. We recommend that monitoring of indicators of CYP1A induction in harlequin ducks in Prince William continue for at least one more year, to confirm that EROD in oiled areas has returned to background levels.

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TABLES AND FIGURES

Table 1. Sample sizes of harlequin ducks captured in Prince William Sound, Alaska for analyses of cytochrome P4501A induction in March 2013. Numbers are listed by sex and age class cohort, and capture area (oiled during *Exxon Valdez* oil spill versus unoiled).

Cohort[a]	Oiled	Unoiled
AHY M	18	15
HY M	0	2
AHY F	7	7
HY F	0	1
TOTAL	25	25

[a]Cohort consists of an age class designation (HY = hatch-year, i.e., within one year of hatching; AHY = after-hatch-year) and sex (M = male; F = female).

Table 2. Results of information-theoretic analyses using general linear models to evaluate variation in hepatic7-ethoxyresorufin-*O*-deethylase (EROD) activity of harlequin ducks (n = 50) captured in Prince William Sound, Alaska during March 2013.

Model	K[a]	AIC_c[b]	ΔAIC_c[c]	w[d]
EROD = Area[e]	3	319.9	0.0	0.43
EROD = null	2	319.9	0.0	0.43
EROD = Area + Individual[f]	6	321.9	3.4	0.08
EROD = Individual	5	324.1	4.2	0.05

[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).

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 hepatic7-ethoxyresorufin-*O*-deethylase (EROD) activity (pmol/min/mg protein) of harlequin ducks captured in Prince William Sound, Alaska during March 2011.

	P.L.	Estimate ± SE
Intercept	1.00	25.99 ± 13.25
Area[a]	0.49	-4.76 ± 6.50
Sex[b]	0.13	1.57 ± 3.42
Age[c]	0.13	-1.12 ± 3.31
Mass (g)	0.13	-0.00 ± 0.02

[a]Area = categorical variable indicating areas either oiled during the *Exxon Valdez* spill or unoiled, with unoiled as the reference value.
[b]Sex = categorical variable (male versus female), with male as the reference value.
[c]Age = categorical variable (hatch-year versus after-hatch-year), with hatch-year as the reference value.

Figure 1. Average (± SE) hepatic7-ethoxyresorufin-O-deethylase (EROD) activity (pmol/min/mg protein) of harlequin ducks (n = 50) captured in Prince William Sound, Alaska in March 2013, contrasted with results from previous years (Esler et al. 2010, Esler 2011).

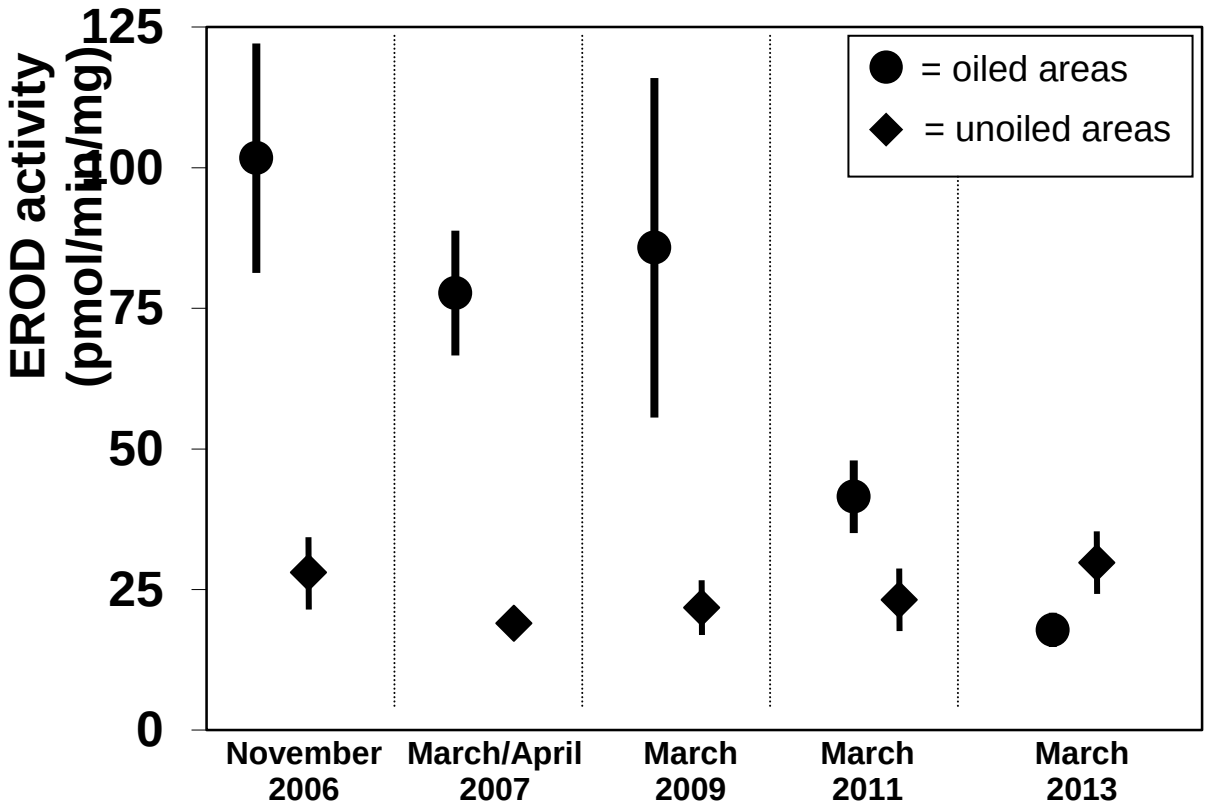


Figure 2. Average (± 95% CI) scaled hepatic7-ethoxyresorufin-O-deethylase (EROD) activity of harlequin ducks (n = 50) captured in March 2013 in areas of Prince William Sound, Alaska oiled during the Exxon Valdez spill relative to nearby unoiled areas, contrasted with results from previous years (Esler et al. 2010, Esler 2011). Results are scaled such that the average on unoiled areas for each year is set to 1; therefore, the data point for each year represents the multiplicative degree to which EROD is elevated on oiled areas (e.g., in 2011, EROD activity was approximately 2 times higher on oiled areas than on unoiled areas).

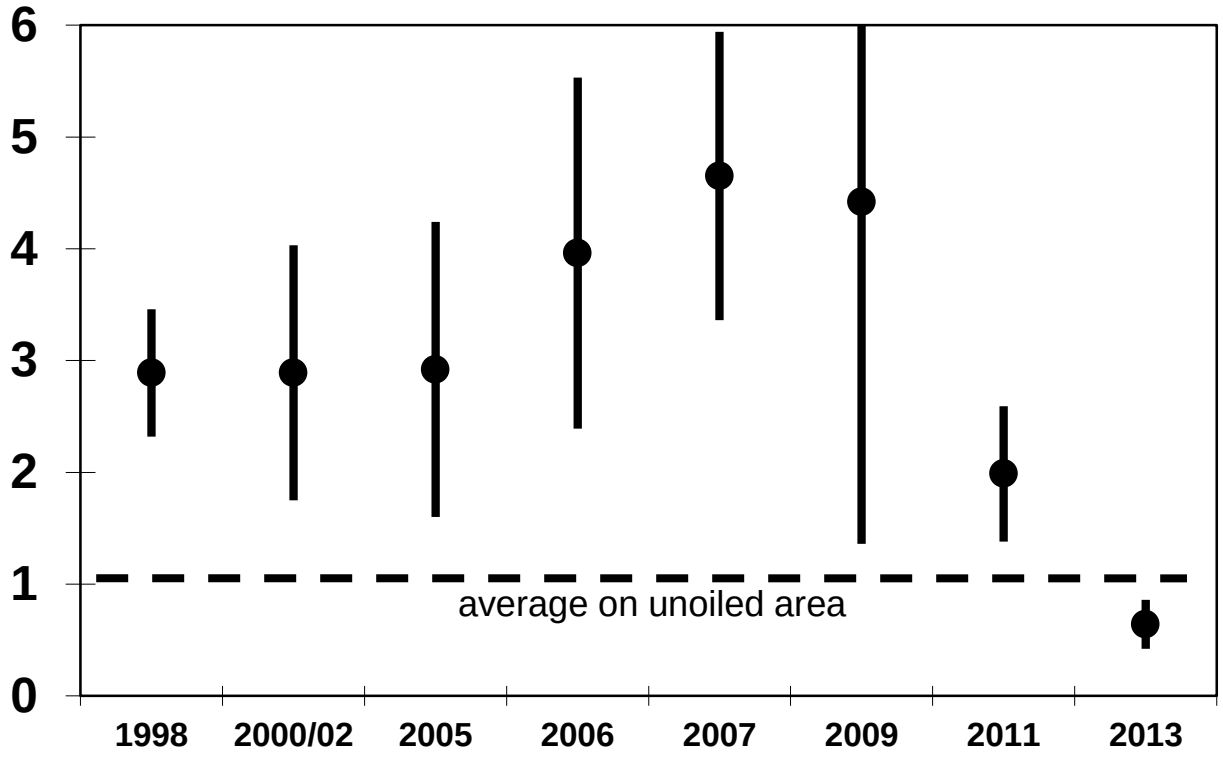
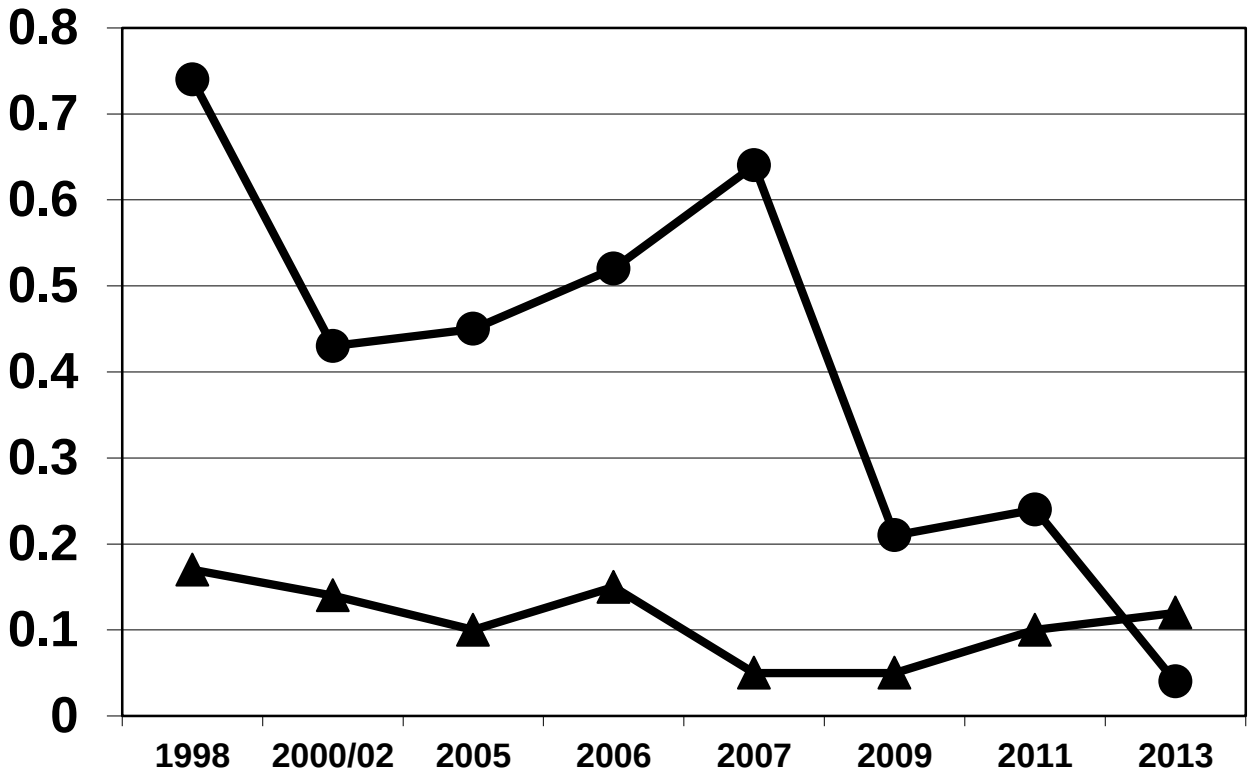


Figure 3. Proportion (y-axis) of captured harlequin ducks with elevated hepatic7-ethoxyresorufin-O-deethylase (EROD) activity, defined as 2 times the average among birds from unoiled areas. Data include results from this study (March 2013) contrasted against findings from previous studies (Esler et al. 2010, Esler 2011).



Long-Term Monitoring –
Evaluating Chronic Exposure of Harlequin Ducks and Sea Otters to
Lingering *Exxon Valdez* Oil in Western Prince William Sound

Project 12120114-Q
Final Report

PART TWO: SEA OTTERS

Study History: Harlequin ducks and sea otters have been studied extensively in Prince William Sound following the 1989 *Exxon Valdez* oil spill (EVOS), leading to one of the most thorough considerations of wildlife population injury and recovery ever undertaken following an oil spill. For sea otters, these efforts have included surveys of abundance and studies of demographics, foraging behavior, and habitat use. Findings indicated that sea otters were at risk of chronic exposure to lingering oil on shorelines, and that their recovery was constrained for about two decades after the initial spill. However, by about 2011, sea otters showed signs of population recovery based on abundance and survival metrics and, as of 2014, are considered to have recovered from the spill. The work presented in this report provides further information on the status of sea otters. Specifically, we provide a final report on the results of gene transcription analyses to evaluate health and exposure of sea otters sampled in western Prince William Sound (WPWS), Alaska, in 2012. This study was conducted as a necessary progression of findings from Restoration Project 090841, which examined gene transcription patterns in sea otters captured in WPWS in 2008.

Abstract: Gene transcription patterns in sea otters were used to evaluate recovery of sea otters from the 1989 EVOS. In 2008, we sampled sea otter blood from oiled and unoiled areas of WPWS and compared gene transcripts from these samples to those from sea otters in captivity and wild sea otters from the Alaska Peninsula. We concluded that sea otters from oiled areas had gene transcription patterns consistent with chronic, low-grade exposure to organic compounds (Miles et al. 2012). In 2012, we resampled sea otters from the same areas of WPWS to determine if gene transcription patterns observed in 2008 persisted. Herein we present gene transcription data on sea otters from WPWS in 2008 and 2012, and include results from sea otters sampled from the Alaska Peninsula, Katmai, Clam Lagoon (Adak Island), Kodiak Island, and captive aquaria populations, as well as results from sea otters captured in WPWS in 2006, 2007 and 2010 but not reported previously. The 2006, 2007, and 2008 WPWS samples are referred to as PWS1, and the 2010 and 2012 samples as PWS2. Cluster analysis of gene transcription patterns was used in two statistical multivariate approaches, non-metric multidimensional scaling (NMDS) and heatmap analysis, and revealed similar results. The majority of sea otters sampled separated into 3 distinctive clusters: Cluster 1 - Kodiak and PWS1; Cluster 2 - Clam Lagoon and PWS2; and Cluster 3 - Katmai, Alaska Peninsula, and captive sea otters. Heatmap analysis showed lower relative transcription in Cluster 2 sea otters, higher relative transcription in Cluster 1 otters, and mixed transcriptional responses in Cluster 3 sea otters. The PWS2 sea otters exhibited transcript patterns consistent with a nutritional deficit or alternate resource allocation regime, which may be associated with an inability to mount effective responses to pathogens, contaminants, or stress. Overall suppression of the transcription response precluded concluding that sea otters from WPWS in 2012 showed no molecular signs of exposure to lingering oil.

Key Words: *Enhydra lutris*, *Exxon Valdez* oil spill, gene transcription, oil exposure, Prince William Sound, recovery, sea otter.

Project Data: Data are kept in digital format (csv files with metadata) at the U.S. Geological Survey, Alaska Science Center, Anchorage, Alaska. Data custodian – Daniel Esler, Research Wildlife Biologist and Project Leader, Nearshore Marine Ecosystem Research Program, U.S. Geological Survey, Alaska Science Center, Anchorage, Alaska. Project data and associated metadata files also are stored on the Alaska Ocean Observing System Workspace as part of the Gulf Watch Alaska Data Management program (EVOS Restoration Project 12120114).

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EXECUTIVE SUMMARY

To assess recovery of sea otters from the 1989 EVOS, the EVOS Trustee Council established demographic and physiological (biomarker-based) criteria to evaluate individual and population health. To examine the physiological status of the sea otters from the oiled area of WPWS, we used molecular gene transcription studies. Gene transcription is the process by which information from the DNA template of a particular gene is transcribed into messenger RNA (mRNA) and eventually translated into a functional protein. The amount of mRNA transcribed from a particular gene is physiologically dictated by a number of intrinsic and extrinsic factors, including stimuli such as infectious agents, toxin exposure, trauma, or neoplasia. Altered levels of gene transcripts provide the earliest observable signs of health impairment, discernable prior to clinical manifestation.

In 2008, we sampled sea otters from oiled and unoiled areas of WPWS and compared them to captive and wild reference otters from the Alaska Peninsula. We concluded that sea otters from oiled areas had gene transcription patterns consistent with chronic, low-grade exposure to organic compounds (Miles et al. 2012). In 2012, we resampled sea otters from the same areas of WPWS to evaluate whether gene transcription patterns observed in 2008 persisted. For the second analysis, we included gene transcription data on sea otters from the Alaska Peninsula (2009), Katmai (2009), Clam Lagoon (Adak Island; 2012), Kodiak (2005), and captive aquaria populations (2008-2010), and additional captures in WPWS (2006, 2007, 2010) with the WPWS data from 2008 and 2012.

Preliminary analyses using nonparametric, multi-dimensional scaling analysis (NMDS) in conjunction with cluster analysis, SIMPROF, and ANOSIM (*R Core Team, 2012*), indicated gene expression profiles of otters sampled in 2006, 2007, and 2008 in WPWS differed from those collected in 2010 and 2012; thus they are split into two groups: PWS1 (2006-2008) and PWS2 (2010 and 2012). Cluster analysis of gene transcription patterns used in two statistical multivariate approaches, non-metric multidimensional scaling (NMDS) and heatmap analysis, revealed similar results. The majority of sea otters sampled separated into 3 distinctive clusters: Cluster 1, Kodiak and PWS1; Cluster 2, Clam Lagoon and PWS2; and Cluster 3, Katmai, Alaska Peninsula, and captive sea otters. Heatmap analysis showed lower relative transcription in Cluster 2 sea otters, higher relative transcription in Cluster 1 otters, and mixed transcriptional responses in Cluster 3 sea otters.

We suggest that the PWS2 sea otters exhibit transcript patterns consistent with a nutritional deficit or alternate resource allocation regime. Implications of this type of molecular profile can include an inability to mount effective responses to pathogens, contaminants, or stress. In effect, overall suppression of the transcription response precludes our evaluation of whether or not individual sea otters show continued signs of exposure to lingering oil. However, related studies on sea otter demographics indicate that by 2012, numbers and mortality patterns had returned to pre-spill conditions (Ballachey et al. 2014); this result is supported by harlequin duck findings in 2013 and 2014 that indicated cessation of oil exposure for that species (Esler and Ballachey 2014). Overall, the gene transcription studies indicate that in 2008 sea otters in WPWS were still subject to lingering oil exposure, while for the 2012 samples, interpretation of the gene expression data is complicated by general decreased transcription but associated sea otter studies indicate no continuing oil exposure.

INTRODUCTION

To assess recovery of sea otters from the 1989 EVOS, the EVOS Trustee Council established demographic and physiological (based on biomarkers indicating exposure to aromatic hydrocarbons) criteria to evaluate population health. To examine the physiological status of the sea otters in the oiled area of WPWS, we used molecular gene transcription studies. Exposure to petroleum hydrocarbons has the potential to cause not only catastrophic short-term effects but also important, and often underappreciated, long-term damage to individuals, populations, and ecosystems (Peterson et al. 2003). The question of extent and duration of long-term effects is difficult to answer, as the pathophysiological changes within an individual may be significant yet subtle, and consequently undetectable using classical diagnostic methods. Alterations in levels of gene transcription can provide the earliest observable signs of health impairment, discernable prior to clinical manifestation (Farr and Dunn 1999, McLoughlin et al. 2006, Poynton and Vulpe 2009). Gene transcription is the process by which information from the DNA template of a particular gene is transcribed into messenger RNA (mRNA) and eventually translated into a functional protein. The amount of mRNA transcribed from a particular gene is physiologically dictated by a number of intrinsic and extrinsic factors, including stimuli such as infectious agents, toxin exposure, trauma, or neoplasia. As a result of this keystone function, analysis of mRNA can provide information about dynamic changes in the physiological state of an organism. The utility of the methodology used in our study relies on the assumption that oil-induced pathology in sea otters is accompanied by predictable and specific changes in gene transcription.

In 2008, we sampled sea otters in oiled and unoiled areas of WPWS and compared these to samples from reference (i.e., deemed clinically normal) wild sea otters from the Alaska Peninsula and captive aquaria sea otters. We concluded that sea otters in oiled areas had gene transcription patterns consistent with chronic, low-grade exposure to organic compounds (Miles et al. 2012). In 2012, we resampled sea otters in the same areas of WPWS to evaluate whether gene transcription patterns observed in 2008 persisted. To provide a broader context for the analysis of 2012 data, we included comparable gene transcription data on sea otters from the Alaska Peninsula, Katmai, Clam Lagoon on Adak Island (Aleutians), Kodiak Island, and captive normal populations from aquaria.

Here, we provide results of gene transcription analyses on sea otters sampled in the summer of 2008 and 2012, analyzed with data from sea otter populations sampled across southwest Alaska and from aquaria.

METHODS

Sea Otter Samples

Free-Ranging Sea Otters

Free-ranging sea otters were sampled from five locations: (1) WPWS in 2006, 2007, and 2008 (n = 80), and in 2010 and 2012 (n = 88), (2) Alaska Peninsula (AP) in 2009 (n = 25), (3) Katmai

(KAT) in 2009 (n = 32), (4) Kodiak (KOD) in 2005 (n = 25), and (5) Clam Lagoon (CL) at Adak Island, Aleutians, in 2012 (n = 24). Preliminary analyses (Figure 1) indicated a significant difference between WPWS sea otters sampled during 2006-2008 compared to those from the same general area in 2010-2012, hence we have assigned these sea otters to two separate groups, PWS1 and PWS2, respectively, for further analyses. Sea otters were captured, anesthetized with fentanyl citrate and midazolam hydrochloride (Monson et al. 2001), and blood drawn by jugular venipuncture within 1-2 hours of the initial capture. Capture methods are presented in detail in Miles et al. (2012) and Bodkin et al. (2012).

Captive Reference Sea Otters

Blood samples from 17 captive reference sea otters were obtained from the Monterey Bay Aquarium, Monterey, CA (n = 9), Shedd Aquarium, Chicago, IL, (n = 4), Oregon Coast Aquarium, Newport, OR (n = 2), and the Vancouver Aquarium, Vancouver, BC (n = 2) in 2008, 2009, and 2010 (Bowen et al. 2011). These animals were identified as clinically normal by staff veterinarians at these aquaria at the time of blood collection.

Blood Collection and RNA Extraction

A 2.5 mL sample from each sea otter was drawn directly into a PAXgene™ blood RNA collection tube (PreAnalytiX©, Switzerland) from either the jugular or popliteal vein and then frozen at – 20^{o}C until extraction of RNA (Bowen et al. 2011). The PAXgene™ tube contains RNA stabilizing reagents that protect RNA molecules from degradation by RNases and prevents further induction of gene transcription. Without stabilization, copy numbers of individual mRNA species in whole blood can change many-fold during storage and transport. The RNA from blood in PAXgene™ tubes was isolated according to manufacturer's standard protocols (Bowen et al. 2007). All RNA was checked for quality by running on both an agarose gel and on a nanodrop 2000 and achieved A260/A280 ratios of approximately 2.0 and A260/A230 ratios of less than 1.0. A standard cDNA synthesis was performed on 2 µg of RNA template from each animal (Bowen et al. 2007). Quantitative real time polymerase chain reaction (qPCR) systems for the individual, sea otter-specific reference or housekeeping gene (S9) and genes of interest (Table 1) were run in separate wells (Bowen et al. 2007). Amplifications were conducted on a 7300 Real-time Thermal Cycler (Applied Biosystems™, Foster City, Calif.) with reaction conditions identical to those in Bowen et al. (2007, 2012) and Miles et al. (2012).

Targeted Genes

The 10 genes targeted in our study represent multiple physiological systems that play a role in immuno-modulation, inflammation, cell protection, tumor suppression, cellular stress-response, xenobiotic metabolizing enzymes, and antioxidant enzymes. These genes can be modified by biological, physical, or anthropogenic impacts and consequently provide information on the general type of stressors present in a given environment (Table 1).

Statistical Analyses

We used nonparametric statistical analyses because the cycle threshold (CT) measure of gene transcription provided by qPCR may have a lognormal distribution (McLoughlin et al. 2006). We used conventional nonparametric mean comparison tests (Kruskal-Wallis with Dunns' Multiple Comparison; NCSS© Statistical Software, 2007, Kaysville, Utah) to evaluate transcript values of each gene by classification groups (7 groups, based on location, including captives as a reference "location" group, and including 2 temporal groups from WPWS). We conducted multivariate, nonparametric, multi-dimensional scaling analysis (NMDS) in conjunction with cluster analysis for statistical and graphical representation of individual sea otters clustered by similarity in transcription and not by pre-defined groups such as location. Statistical comparisons of individuals grouped by clusters were made using SIMPROF, which is a similarity profile permutation test for significance among *a priori*, unstructured clusters of samples. We used ANOSIM (*R Core Team, 2012*), a nonparametric analogue to a 2-way ANOVA, to test for differences in gene transcription among years, between sexes and among three age groups based primarily on potential reproductive status, i.e., juvenile, adult, and aged adult (Monson et al. 2000). Statistical significance was based on p values ≤ 0.05 (*R Core Team, 2012)*.

RESULTS

Gene transcription (C_T) values differed among sea otters sampled in WPWS, in 2006, 2007, 2008, 2010, and 2012 (ANOSIM, $p < 0.001$, Global $R = 0.594$). When analyzed without *a priori* structure (i.e., year), sea otters separated into two well-defined groups as depicted by NMDS (3d R = 0.08; Figure 1) and confirmed by cluster analysis (SIMPROF, $p < 0.001$). These well-defined groups were designated PWS1 (2006, 2007, 2008) and PWS2 (2010, 2012). Transcript patterns were not influenced by sex ($p = 0.08$) or age ($p = 0.16$).

For the analysis of all groups, patterns depicted by the NMDS analyses were similar to those reported in Miles et al. (2012), with differences attributable to the inclusion of the additional groups (Figure 2). Groups generally separated into three distinctive clusters: (1) KOD and PWS1, (2) CL and PWS2, and (3) KAT, AP, and captive sea otters (2d R = 0.15; SIMPROF, p < 0.001; Figure 2).

Overall gene transcription (CT) values differed among groups analyzed (Figure 2). The transcript profiles from the AP, KAT and clinically normal captive groups were relatively similar, and differed from the other 3 groups. Profiles of the PWS2 and CL groups were similar to each other. In general, gene transcription patterns in the PWS1 group of sea otters (captured 2006-2008) were indicative of molecular reactions to organic exposure, tumor formation, inflammation, and viral infection that may be consistent with chronic, low-grade exposure to an organic substance (Bowen et al. 2012, Miles et al. 2012). The PWS2 group (captured 2010 and 2012), in contrast, had a general pattern of lower transcription, with 8 of the 10 genes showing significant down-regulation compared to PWS1, and grouped statistically with the CL sea otters.

Using Kruskal-Wallis, nine of the ten genes evaluated had significant differences between at least two classification groups; only CYT did not differ among groups (Table 2). Geometric mean transcript values were highest (i.e., lowest C_T values) at KOD for seven of the nine genes showing significant differences (HDC, COX2, AHR, THRβ, HSP70, IL10, MX1). Geometric mean transcript values for IL18 were highest in the PWS1, AP and CAP groups. Lowest

geometric mean transcript values among groups generally were found in CL and PWS2 sea otters for seven of the nine genes (HDC, COX2, AHR, IL10, MX1 at CL, and THRβ, HSP70 at PWS2). The lowest geometric mean transcript value for IL18 was in the KOD group. The largest ranges of geometric means among groups (most variable expression) were identified for HDC and IL10, while the small ranges occurred for DRB, IL18, and CYT (the latter gene showing no variation among any groups). Genes with larger ranges may be subject to greater environmental variation in a particular system than genes with smaller ranges.

DISCUSSION

The genes examined in our study can be grouped into functional categories that include immune-modulation, pathogen response, inflammation, cell signaling, xenobiotic metabolizing enzymes, and cellular stress response (Table 1). Although transcription studies generally focus on genes that are differentially transcribed among groups, genes which show no difference among groups are also of importance. Of particular note in this study was the lack of statistical difference in gene transcription between the AP and clinically normal captive sea otters (Table 2, Figure 3). The interpretation of the high similarity of wild-captured sea otters to documented clinically normal, healthy sea otters is that individuals in the AP subpopulation are healthy, and do not appear to be responding to contaminant exposure, disease, or nutritional deficits. Transcript patterns from the KAT subpopulation of sea otters also were similar to those of the AP and captive populations (Figure 3). These findings are supported by population status data, indicating that the KAT and AP populations are below carrying capacity and have ample prey resources available (Bodkin 2015, Tinker 2105).

Two other groups with remarkably similar transcript patterns were CL and PWS2, both exhibiting relatively low levels of transcription in most genes examined. Relatively low levels of select gene transcripts have been described in mice experiencing a nutritional deficit (Saucillo et al. 2014). Alternatively, low transcription may be the result of unbalanced physiological resource allocation. For example, immune defense exists to impede infections, but other ecological demands (i.e., stressors related to nutrition, weather, and predation) can supersede this, causing immune defenses to be compromised (Martin et al. 2010). This is consistent with findings on comparative rates of energy recovery of sea otter populations throughout their range, indicating food resources for sea otters at both CL and WPWS were limited, relative to other groups sampled in this study (Tinker 2015, USGS unpublished data).

Distinct transcript patterns also existed among groups, and while it can be difficult to disentangle the effects of environmental factors on the underlying pathways, many marked physiological responses were evident. For example, transcriptional differences among sea otters from KOD, PWS1, and the other groups were evident, with sea otters from KOD, in particular, having very high transcription levels in relation to those of all other sea otters. The PWS1 and KOD groups appeared to have immunological responses that indicated greater organic compound exposure relative to the other populations examined. However, their profile motifs appeared to be quite different, suggesting unique environmental inputs at each site. Genomic profiling has successfully linked specific signatures to unique combinations of chemical contaminants in other species (Menzel et al. 2009, Steinberg et al. 2008, Yang et al. 2007, Poynton et al. 2008). In fact,

the transcription profile of the KOD otters is consistent with that of a dioxin-induced profile, while the transcription profile of PWS1 otters (in particular, those from the area that received heaviest shoreline oiling in 1989) is consistent with a PAH-induced profile (Zeytun et al. 2002). Up-regulation of AHR is indicative of immediate exposure to classes of environmental toxicants including polycyclic aromatic hydrocarbons, polyhalogenated hydrocarbons, dibenzofurans, and dioxin (Oesch-Bartlomowicz and Oesch 2005). Chronic exposure to specific toxicants may not necessarily cause a sustained increase in AHR transcription (Bowen et al. 2007, Miles et al. 2012), but can be associated with potential downstream consequences [e.g., modulation of T-regulatory (T_{REG}) (immune-suppressive) or T-helper type 17 (T_H17) (pro-inflammatory) immunologic activity (Quintana et al. 2008; Veldhoen et al. 2008); however, these were not specifically analyzed in this study]. The lack of up-regulation of AHR in WPWS sea otters may reflect findings of Bodkin et al. (2012), which demonstrate a pathway of chronic exposure from lingering intertidal oil to foraging sea otters in WPWS during the same time period. However, sea otters from the spill area in WPWS in 2008 demonstrated elevated transcription of several genes including HDC and THRβ, and down-regulation of the DRB gene. Dong et al. (1997) reported down-regulation of DRB by a dioxin compound, and both polycyclic aromatic hydrocarbons (constituents of crude oil) and dioxin-like compounds have been implicated in similar physiologic detoxification responses.

In summary, we found that the 2010 and 2012 WPWS sea otters exhibited gene transcription patterns consistent with a nutritional deficit or alternate resource allocation regime. Implications of this type of molecular profile can include an inability to mount effective responses to pathogens, contaminants, or stress. In effect, the overall dampening of the molecular response precludes determination of whether or not sea otters showed a continued response to lingering oil in 2010-2012. However, related studies on sea otter demographics indicated that by 2012, numbers and mortality patterns had returned to pre-spill conditions (Ballachey et al. 2014, Ballachey and Bodkin 2015); this result is supported by comparable harlequin duck findings in 2013 and 2014 (Esler and Ballachey 2014). Overall, the gene transcription studies suggest that in 2008, sea otters in WPWS were still subject to lingering oil exposure, while for the 2012 samples, interpretation of the gene transcription data is complicated by a general decreased transcription.

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TABLES AND FIGURES

Table 1. Documented function of 10 genes identified in free-ranging sea otters sampled at Alaska Peninsula, Katmai, Kodiak, Clam Lagoon, Prince William Sound 2006 – 2008, Prince William Sound 1010 – 2012, and in clinically normal captive sea otters.

Gene	Gene function
HDC	The HDCMB21P gene codes for a translationally controlled tumor protein (TCTP) implicated in cell growth, cell cycle progression, malignant transformation, tumor progression, and in the protection of cells against various stress conditions and apoptosis (Bommer and Thiele, 2004, Tuynder et al. 2004, Ma et al. 2010). Environmental triggers may be responsible for population-based, up-regulation of HDC. HDC transcription is known to increase with exposure to carcinogenic compounds such as polycyclic aromatic hydrocarbons (Bowen et al. 2007, Raisuddin et al. 2007, Zheng et al 2008).
COX2	Cyclooxygenase-2 catalyzes the production of prostaglandins that are responsible for promoting inflammation (Goldsby et al. 2003). Cox2 is responsible for the conversion of arachidonic acid to prostaglandin H2, a lipoprotein critical to the promotion of inflammation (Harris et al. 2002). Up-regulation of Cox2 is indicative of cellular or tissue damage and an associated inflammatory response.
CYT	The complement cytolysis inhibitor protects against cell death (Jenne and Tschopp 1989). Up-regulation of CYT is indicative of cell or tissue death.
AHR	The arylhydrocarbon receptor responds to classes of environmental toxicants including polycyclic aromatic hydrocarbons, polyhalogenated hydrocarbons, dibenzofurans, and dioxin (Oesch-Bartlomowicz et al. 2005). Depending upon the ligand, AHR signaling can modulate T-regulatory (T_{REG}) (immune-suppressive) or T-helper type 17 (T_H17) (pro-inflammatory) immunologic activity (Quintana et al. 2008, Veldhoen et al. 2008).
THRβ	The thyroid hormone receptor beta can be used as a mechanistically based means of characterizing the thyroid-toxic potential of complex contaminant mixtures (Tabuchi et al. 2006). Thus, increases in THRβ transcription may indicate exposure to organic compounds including PCBs, and associated potential health effects such as developmental abnormalities and neurotoxicity (Tabuchi et al. 2006). Hormone-activated transcription factors bind DNA in the absence of hormone, usually leading to transcriptional repression (Tsai and O'Malley 1994).
HSP 70	The heat shock protein 70 is produced in response to thermal or other stress including hyperthermia, oxygen radicals, heavy metals, and ethanol (Iwama et al. 1999, Tsan and Gao 2004).
IL-18	Interleukin-18 is a pro-inflammatory cytokine (Goldsby et al. 2003). IL-18lays an important role in inflammation and host defense against microbes (Krumm et al. 2008).
IL-10	Interleukin-10 is an anti-inflammatory cytokine (Goldsby et al. 2003). Levels of IL-10 have been correlated with relative health of free-ranging harbor porpoises, *e.g*., increased amounts of IL-10 correlated with chronic disease whereas the cytokine was relatively reduced in apparently fit animals experiencing acute disease (Beineke et al. 2007). Association of IL-10 transcription with chronic disease has also been documented in humans (Rigopoulou et al. 2005).
DRB	A component of the major histocompatibility complex, the DRB class II gene, is responsible for the binding and presentation of processed antigen to T_H lymphocytes, thereby facilitating the initiation of an immune response (Goldsby et al. 2003, Bowen et al. 2006). Up-regulation of MHC genes has been positively correlated with parasite load (Wegner et al. 2006), whereas down-regulation of MHC has been associated with contaminant exposure (Dong et al. 1997).
Mx1	The Mx1 gene responds to viral infection (Tumpey et al. 2007). Vertebrates have an early strong innate immune response against viral infection, characterized by the induction and secretion of cytokines that mediate an antiviral state, leading to the up-regulation of the MX-1 gene (Kibenge et al. 2005).

Table 2. Geometric mean (normalized to the S9 housekeeping gene in each animal) cycle threshold (C_T) transcription values (and 95% confidence intervals) for targeted genes (see Table 1) in sea otters sampled at Alaska Peninsula (AP), Katmai (KAT), Kodiak (KOD), Clam Lagoon (CL), Prince William Sound 2006 – 2008 (PWS1), Prince William Sound 2010 – 2012 (PWS2), and clinically normal captive otters (CAP). Letters denote significant differences among populations (Kruskal-Wallis with Dunns' Multiple Comparison); lack of a letter denotes no significant difference from any other group. Note that the **smaller** the mean value, the **higher** the level of transcription.

Group	Gene									
	HDC	COX2	CYT	AHR	THRβ	HSP70	IL18	IL10	DRB	MX1
CAP	5.90^{ad} (5.02 - 6.94)	6.78^{abcd} (6.02 – 7.64)	2.41 (1.91 – 3.04)	11.01^{abe} (10.56 – 11.48)	13.30^{a} (12.49 – 14.56)	9.62^{ab} (8.74 – 10.59)	1.65^{acde} (1.05 – 2.60)	13.70^{a} (13.01 – 14.44)	-0.33 (-0.86 – 0.21)	10.99^{ab} (9.95 – 12.15)
AP	6.26^{a} (5.47 - 7.16)	6.60^{abcd} (5.92 – 7.36)	1.90 (1.45 – 2.52)	10.67^{ab} (9.94 – 11.46)	13.32^{a} (12.19 – 14.56)	8.58^{abd} (7.97 – 9.23)	1.68^{acde} (1.30 – 2.17)	13.21^{a} (12.21 – 14.30)	-0.91^{a} (-1.57 - -0.26)	12.61^{a} (11.42 – 13.93)
KAT	4.54^{ab} (4.06 - 5.08)	7.68^{bd} (7.10 – 8.30)	1.96 (1.54 – 2.50)	10.36^{ab} (9.79 – 10.96)	12.53^{a} (11.86 – 13.23)	8.26^{abd} (7.56 – 9.04)	2.78^{ce} (2.15 – 3.59)	13.45^{a} (12.62 – 14.33)	-0.56^{a} (-1.09 - -0.03)	12.56^{a} (11.99 – 13.16)
KOD	-1.84^{e} (-2.33 - -1.35)	5.44^{c} (4.79 – 6.16)	2.59 (2.04 – 3.28)	8.80^{d} (8.09 – 9.57)	9.50^{c} (8.62 – 10.46)	5.48^{d} (4.86 – 6.18)	5.19^{b} (4.61 – 5.85)	9.03^{c} (8.26 – 9.87)	1.29 (0.78 – 2.13)	8.26^{b} (7.64 – 8.92)
CL	10.30^{c} (10.06 - 10.54)	9.45^{e} (8.91 – 10.02)	1.53 (1.28 – 1.83)	12.78^{c} (12.38 – 13.18)	16.85^{b} (15.83 – 17.93)	14.07^{c} (13.17 – 15.02)	2.49^{acde} (2.06 – 3.01)	22.09^{b} (20.85 – 23.40)	0.43^{b} (0.22 – 0.84)	16.89^{c} (15.36 – 18.57)
PWS1	4.01^{bde} (2.89 – 5.57)	7.98^{ad} (7.60 – 8.38)	1.88 (1.60 – 2.21)	10.17^{abd} (9.75 – 10.62)	11.35^{ac} (10.74 – 11.99)	9.76^{ab} (9.17 – 10.39)	1.60^{d} (1.24 – 2.07)	13.34^{a} (12.77 – 13.94)	1.08^{b} (0.80 – 1.44)	10.41^{b} (10.05 – 10.78)
PWS2	8.94^{c} (8.46 – 9.46)	9.30^{e} (8.96 – 9.65)	1.62 (1.45 – 1.80)	12.07^{c} (11.79 – 12.36)	16.09^{b} (15.54 – 16.66)	13.62^{c} (13.10 – 14.01)	2.38^{e} (2.21 – 2.56)	20.28^{b} (19.44 – 21.16)	-0.071 (-0.25 – 0.10)	14.95^{c} (14.50 – 15.41)

Figure 1. Multivariate, nonparametric, multi-dimensional scaling (NMDS) of gene transcription profiles (see Table 2) of sea otters captured in five different years (2006, 2007, 2008, 2010, 2012) in western Prince William Sound. Due to the NMDS configuration, three-dimensional visualization was necessary to view separation in this case.

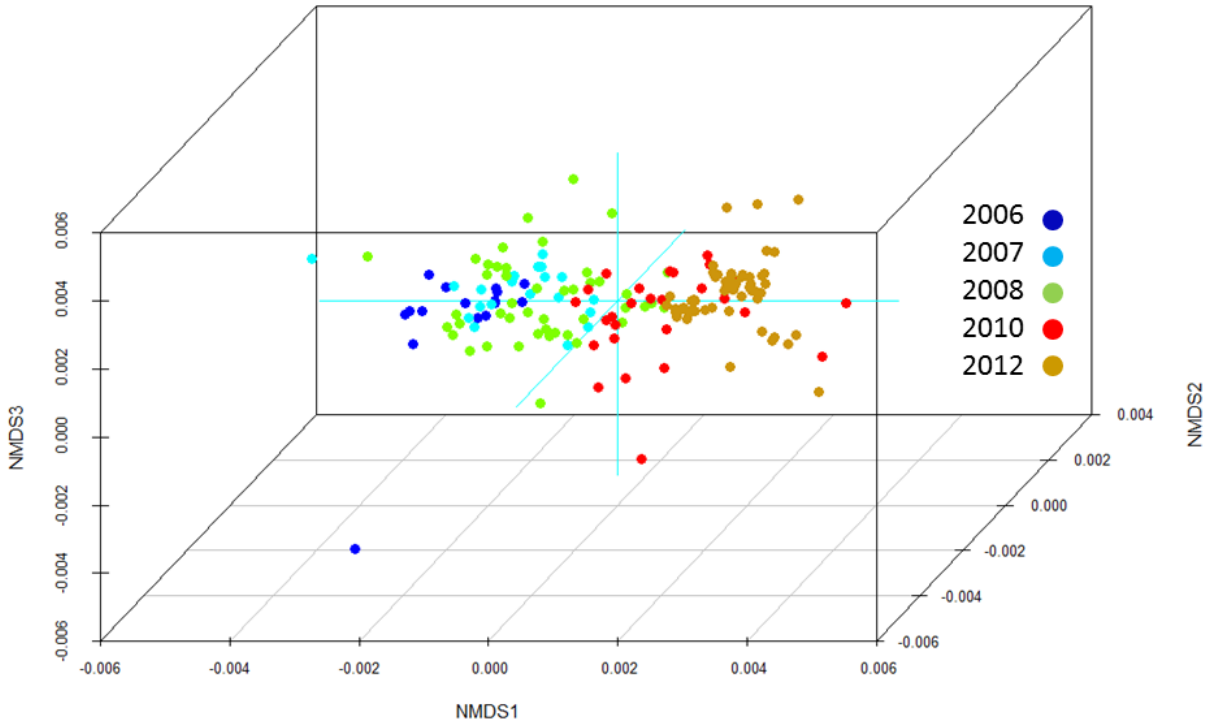

Figure 2. Multivariate, nonparametric, multi-dimensional scaling (NMDS) of gene transcription profiles (see Table 2) of sea otters sampled at Alaska Peninsula (AP), Katmai (KAT), Kodiak (KOD), Clam Lagoon (CL), western Prince William Sound 2006, 2007 & 2008 (PWS1), western Prince William Sound 2010 & 2012 (PWS2), and clinically normal captive otters (CAP).