

Variations of transcript profiles between sea otters *Enhydra lutris* from Prince William Sound, Alaska, and clinically normal reference otters

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ABSTRACT: Development of blood leukocyte gene transcript profiles has the potential to expand condition assessments beyond those currently available to evaluate wildlife health, including sea otters *Enhydra lutris*, both individually and as populations. 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. We compared gene transcript profiles of sea otters sampled in 2008 among areas within Prince William Sound impacted to varying degrees by the 1989 'Exxon Valdez' oil spill with those of captive and wild reference sea otters. Profiles of sea otters from Prince William Sound showed elevated transcription in genes associated with tumor formation, cell death, organic exposure, inflammation, and viral exposure when compared to the reference sea otter group, indicating possible recent and chronic exposure to organic contaminants. Sea otters from historically designated oiled areas within Prince William Sound 19 yr after the oil spill had higher transcription of genes associated with tumor formation, cell death, heat shock, and inflammation than those from areas designated as less impacted by the spill.

KEY WORDS: Gene transcription · Sea otter · *Enhydra lutris* · Prince William Sound · Exxon Valdez

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INTRODUCTION

Oil spills have the potential to cause catastrophic short-term and dramatic, but often unexpected, long-term damage to individuals, populations, and ecosystems (Eppley & Rubega 1989, Jackson et al. 1989,

Guzmán et al. 1991, Anderson et al. 1996, Bodkin et al. 2002, Romero & Wikelski 2002, National Research Council 2003, Rice et al. 2007). The question of extent and duration of long-term effects is difficult to answer. Several recent studies have shown that long-term effects were evident for a variety of species after

an oil spill (Monson et al. 2000, Peterson et al. 2003, Esler et al. 2002, 2010, 2011); such effects may result from sublethal pathology in individuals exposed to oil at the time of the spill or chronic physiological stresses from continued exposure to oil remaining in the environment. Polycyclic aromatic hydrocarbons, key constituents of crude oil, are a class of environmental pollutants that are known to be carcinogenic and immunotoxic, and can lead to increased disease susceptibility. Whatever the mechanism behind these long-term effects, the pathophysiological changes within an individual may be significant but subtle, and consequently undetectable with classical diagnostic methods. Hence, biological markers that identify oil-induced sublethal pathology in susceptible species are needed.

In recent decades, abundances in several populations of northern sea otters *Enhydra lutris kenyoni* have declined (Estes et al. 1998, Bodkin et al. 2002). Suggested causes for these declines are complex and have been attributed to a variety of ecological or anthropogenic pressures. For example, the Alaska Peninsula sea otter population has declined and remained far below equilibrium density, despite sufficient prey resources (Burn & Doroff 2005). Sea otters in historically heavily-oiled areas of Prince William Sound (PWS), Alaska, have failed to recover from the 1989 'Exxon Valdez' oil spill (EVOS; Bodkin et al. 2002), which may be a consequence of effects from lingering oil (Short et al. 2004). The acute effects of crude oil on sea otters have been evaluated by mortality, necropsy, and clinical estimations (Garrott et al. 1993, Lipscomb et al. 1993, Ballachey et al. 1994, Bodkin & Udevitz 1994, Garshelis 1997). Chronic effects are more difficult to identify, and studies often rely on demographic modeling, estimations of reproductive efficiency and age-specific survival rates, or limited health examination and morphometric measures of captured sea otters in the field (Monson et al. 2000, 2011, Bodkin et al. 2002, Ballachey et al. 2003). The goal of our study was to evaluate the utility of sensitive genetic markers responsive in various ways to the effects of environmental stressors on sea otters.

Crude petroleum oil has multiple aromatic and aliphatic hydrocarbon constituents, and the toxic effects of exposure and ingestion can be diverse and extensive (Rebar et al. 1995, Mazet et al. 2000, Mazet et al. 2001, Quintana et al. 2008, Veldhoen et al. 2008, Carls & Meador 2009). Under these circumstances, molecular investigation of subtle alterations of expressed genes indicative of multiple

physiological processes at the cellular level is particularly useful and can elucidate the mechanisms by which oil may have deleterious effects over a long period, including increased tumor formation or disease susceptibility. Ultimately, these methods may provide an understanding of the susceptibility of individuals and, subsequently, populations at risk from chronic oil exposure. Furthermore, sampling blood for molecular investigations is minimally invasive, which is important when federally protected, free-ranging species, such as sea otters, are the subject of study.

Gene expression is the process by which information from the DNA template of a particular gene is transcribed into mRNA and eventually translated into a functional protein. The amount of a particular gene that is expressed is physiologically dictated by a number of intrinsic and extrinsic factors, including stimuli such as infectious agents, toxin exposure, trauma, or neoplasia. The earliest observable signs of health impairment are altered levels of gene transcripts, evident prior to clinical manifestation (McLoughlin et al. 2006). As a result of this keystone function, analysis of mRNA can provide information not only about genetic potential but also about dynamic changes in the functional state of an organism. In fact, mRNA levels closely approximate functional protein levels, and specific changes in mRNA have been identified following heat shock, drug treatment, and metabolic and disease states (Wu et al. 2008, Miller et al. 2011). The utility of the methodology proposed in our study relies on the assumption that oil-induced sublethal pathology in sea otters is accompanied by predictable and specific changes in gene transcription.

Marine mammal toxicology has historically relied heavily on the identification or burden of foreign chemicals (xenobiotics) within specific tissues as an indicator of a toxic insult. These assays do not measure the influence of xenobiotics on the welfare of the individual and therefore are limited in interpretation. The advantage of using gene expression assays is the ability to measure the acute or chronic physiologic responses of an individual, as manifested by levels of gene transcripts, to stimuli. Impact-specific, gene-expression patterns can be identified either on free-ranging animals opportunistically (such as animals caught in a chemical spill) or under experimental conditions using model organisms (Mancia et al. 2007, 2008).

Peripheral blood leukocytes are a primary focus of gene transcription research. These leukocytes react to chemical compounds absorbed into the blood-

stream from the digestive tract, from dermal contact, from inhalation exposure, or are released from lipid-rich tissues. For example, cytochrome P450 enzymes (Stephen et al. 1997) and transferases are present in leukocytes that are important in Phase II reactions of xenobiotics (Landi et al. 1998). Leukocytes produce heat shock proteins (Haire et al. 1988), antioxidant enzymes (Pereira et al. 1999), and metallothioneins (Garte et al. 1995, Yurkowi & Makhijani 1998). Leukocytes are also capable of DNA repair (Hallberg et al. 1997) and have multidrug resistance transporters (Legrand et al. 1996); both processes are of high adaptive value when cells are stressed by xenobiotic chemicals.

A change in gene transcription in peripheral blood leukocytes was evident in American mink *Mustela vison* exposed to petroleum oil (Bowen et al. 2007). Sensitive and specific gene markers were developed using samples previously collected from mink with subtle petroleum oil-associated pathophysiological anomalies and altered lymphocyte function (Schwartz et al. 2004a,b). The effects of oil exposure, as with other toxins, could involve multiple organ systems and change with dose and route of exposure. Bowen et al. (2007) identified genes in the mink that were significantly altered in transcription by exposure to oil. These genes play a role in immunomodulation, inflammation, cytoprotection, tumor suppression, reproduction, cellular stress-response, metal metabolism, xenobiotic metabolizing enzymes, antioxidant enzymes, and cell-cell adhesion (Bowen et al. 2011).

The close phylogenetic relationship between mink and sea otters facilitated our development of a similar set of gene probes for the sea otter; gene sequences were sufficiently conserved across the 2 species so that the majority of the degenerate primers used to develop the mink panels were used to establish sea otter-specific primers (Bowen et al. 2006a). Using these degenerate primers on sea otter complementary DNA (cDNA), we sequenced 10 target genes and 1 reference gene identified in the mink panel (Bowen et al. 2006a, 2007, 2011). The objective of our study was to compare transcription of targeted genes in sea otters sampled in 2008 among areas within PWS that had been impacted to varying degrees by the EVOS with reference groups of sea otters diagnosed as 'clinically normal', consisting of captive aquarium animals sampled from 2008 to 2010, and free-ranging animals from the Alaska Peninsula sampled in 2009 from an area with no known, large-scale anthropogenic impacts (Bowen et al. 2011).

MATERIALS AND METHODS

Free-ranging target otters

A total of 45 sea otters *Enhydra lutris* from 3 different areas of western PWS were captured in summer 2008. Sea otters were captured at Knight Island (heavily oiled in the 1989 spill) (n = 16), Prince of Wales Passage (moderate level of oil contamination) (n = 15), and Montague Island (least oiled, reference area) (n = 14) (Galt & Payton 1990, Bodkin & Udevitz 1994). Lingering oil from the EVOS was more prevalent at Knight Island than Prince of Wales Passage, at least through 2002 (Short et al. 2004). Approximate age of otters was determined by analysis of cementum annuli in extracted premolar teeth (Bodkin et al. 1997). Sea otters were captured using a Wilson trap (Wendell et al. 1996) and brought immediately to a shipboard station for processing. All target, as well as captive and free-ranging reference sea otters, were anesthetized with fentanyl citrate and midazolam hydrochloride (Monson et al. 2001) prior to processing.

Captive and free-ranging reference otters

Seventeen blood samples from 17 captive sea otters were obtained from the Monterey Bay Aquarium (Monterey, California), Shedd Aquarium (Chicago, Illinois), Oregon Coast Aquarium (Newport, Oregon), and the Vancouver Aquarium (Vancouver, British Columbia) in 2008, 2009, and 2010, and included both northern and southern subspecies (Bowen et al. 2011). These animals were identified as clinically normal by staff veterinarians at these aquaria during the time interval of blood collection.

Wild reference sea otters were captured along the southwestern Alaska Peninsula (n = 28) in summer 2009, and 25 of these sea otters used in our study were deemed clinically normal by the attending veterinarian. The remaining 3 sea otters from this population were deemed not clinically normal due to bad teeth, multiple wounds, or an abnormal blood panel. Alaska Peninsula sea otters were chosen as reference animals because they were from an area not affected by the EVOS, were far removed from any known human perturbations, and were at or below equilibrium density. The Alaska Peninsula sea otters were captured and processed exactly as the PWS sea otters.

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) from either the jugular or popliteal veins and then frozen at -20°C until extraction of RNA (Bowen et al. 2011). Rapid RNA degradation and induced transcription of certain genes after blood draws has led to the development of methodologies for preserving the RNA transcription profile immediately after blood is drawn. The PAXgene tube contains a blend of RNA stabilizing reagents that protect RNA molecules from degradation by RNases and prevents further induction of gene transcription. Without this stabilization, copy numbers of individual mRNA species in whole blood can change more than 1000-fold during storage and transport. The RNA from blood in PAXgene tubes was isolated according to manufacturer's standard protocols, which included an on-column DNase treatment to remove contaminating genomic DNA (silica-based microspin technology) and the extracted RNA stored at -80°C until analysis. All RNA was checked for quality on a NanoDrop 2000™ and achieved A260/A280 ratios of approximately 2.0 and A260/A230 ratios of less than 1.0.

cDNA creation

A standard cDNA synthesis was performed on 2 μg of RNA template from each animal. Reaction conditions included 4 units reverse transcriptase (Omniscript, Qiagen), 1 μmol random hexamers, 0.5 mM each dNTP, and 10 units RNase inhibitor, in RT buffer (Qiagen). Reactions were incubated for 60 min at 37°C , followed by an enzyme inactivation step of 5 min at 93°C , and then stored at -20°C until further analysis.

Real-time PCR

Real-time PCR systems for the individual, sea otter-specific reference or housekeeping gene known as S9 and genes of interest (Table 1) were run in separate wells (Bowen et al. 2011). Briefly, 1 μl of cDNA was added to a mix containing 12.5 μl of QuantiTect SYBR Green Master Mix 5 mM Mg^{2+} (Qiagen), 0.5 μl each of forward and reverse sequence specific primers, 0.5 μl of Uracil-N-Glycosylase (Invitrogen), and 10.0 μl of RNase-free water; total reaction mixture was 25 μl . The reaction mixture cDNA samples for each gene of interest and the S9 gene were

loaded into 96 well plates in duplicate and sealed with optical sealing tape (Applied Biosystems). Reaction mixtures containing water, but no cDNA, were used as negative controls; thus approximately 3 to 4 individual sea otter samples were run per plate.

Amplifications were conducted on a 7300 Real-time Thermal Cycler (Applied Biosystems). Reaction conditions were as follows: 50°C for 2 min, 95°C for 15 min, 40 cycles of 94°C for 30 s, 58°C for 30 s, 72°C for 31 s, an extended elongation phase at 72°C for 10 min. Reaction specificity was monitored by melting curve analysis using a final data acquisition phase of 60 cycles of 65°C for 30 s and verified by direct sequencing of randomly selected amplicons (Bowen et al. 2007). Cycle threshold crossing values (C_T) for the genes of interest were normalized to the S9 housekeeping gene.

Statistical analysis

Analysis of quantitative PCR (qPCR) data was conducted using normalized values, i.e. housekeeping gene threshold crossing (in qPCR, the point at which amplification is exponential) subtracted from the gene of interest threshold crossing for each animal (McLoughlin et al. 2006).

We used nonparametric statistical analyses because the cycle threshold (C_T) measure of gene transcription provided by qPCR may have a lognormal distribution (McLoughlin et al. 2006). We used ANOSIM (Primer v6 software) analysis of variance to test for differences: in gene transcription among locations, i.e. the PWS subpopulations, Alaska Peninsula, and captive sea otters; between sexes and among 3 age groups based primarily on potential reproductive status, i.e. juvenile, adult, and aged adult (Monson et al. 2000) (Table 2). Examination of sex or age group differences was exploratory, as a disproportionate number of females were sampled and age groups were not evenly distributed across locations. We conducted multivariate, multi-dimensional scaling (MDS) analysis 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 by clusters were made using SIMPROF (Primer v6), which is a similarity profile permutation test for significance among *a priori*, unstructured clusters of samples. Statistical significance was based on p-values ≤ 0.05 , and in the case of the ANOSIM tests, relative to the R statistic value.

Table 1. *Enhydra lutris*. Documented function of 10 genes identified in free-ranging sea otters sampled at Prince William Sound in 2008, in clinically normal free-ranging reference otters at Alaska Peninsula in 2009, and in clinically normal captive reference otters sampled in 2008, 2009, or 2010

Gene	Gene function
HDC	The HDCMB21P gene (HDC) 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 & Thiele 2004, Tuynder et al. 2004, Ma et al. 2010). Up-regulation of HDC is indicative of the development or existence of cancer. 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 (COX2) 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 H ₂ , 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 (CYT) protects against cell death (Jenne & Tschopp 1989). Up-regulation of CYT is indicative of cell or tissue death.
AHR	The arylhydrocarbon (AHR) receptor responds to classes of environmental toxicants including polycyclic aromatic hydrocarbons, polyhalogenated hydrocarbons, dibenzofurans, and dioxin (Oesch-Bartlomowicz & Oesch 2005). Depending upon the ligand, AHR signaling can modulate T-regulatory (T _{REG}) (immune-suppressive) or T-helper type 17 (T _H 17) (pro-inflammatory) immunologic activity (Quintana et al. 2008, Veldhoen et al. 2008).
THR	The thyroid hormone (THR) 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 THR 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).
HSP70	The heat shock protein 70 (HSP70) is produced in response to thermal or other stress (Iwama et al. 1999, Tsan & Gao 2004). In addition to being expressed in response to a wide array of stressors (including hyperthermia, oxygen radicals, heavy metals, and ethanol) heat shock proteins act as molecular chaperones (De Maio 1999). For example, heat shock proteins aid the transport of the AHR/toxin complex in the initiation of detoxification (Tanabe et al. 1994).
IL-18	Interleukin-18 (IL-18) is a pro-inflammatory cytokine (Goldsby et al. 2003). Plays an important role in inflammation and host defense against microbes (Krumm et al. 2008).
IL-10	Interleukin-10 (IL-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. 2006b). Up-regulation of major histocompatibility complex (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).
MX-1	The MX-1 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).

RESULTS

Veterinary diagnoses

Health evaluations of the sea otters captured in PWS were performed by the same veterinarian using the same clinical paradigm used to assess sea otters from the Alaska Peninsula and from the Monterey Bay Aquarium (Bowen et al. 2011). More females than males were captured in PWS, ranging from one

to 12 yr old, and most sea otters were in the adult age group (Table 2). Of the 45 PWS animals sampled, 15 (33.3%) were found to have clinically significant anomalies, with 5 (11.1%) having more than one anomaly. Eight animals (17.8%) had clinically significant dental disease which may be associated with gingival or periodontal disease. Three animals (6.7%) had either clinical or laboratory changes suggestive of bacterial infection (abscessation, cellulitis); 2 (4.4%) had overt evidence of nasal acariasis. One

Table 2. *Enhydra lutris*. Location, number sampled (n), age (in yr; <4 = juvenile, 4–8 = adult, ≥9 = aged adult), and sex (f = female, m = male) of sea otters in Prince William Sound (PWS) in May 2008, Alaska Peninsula in July 2009, and aquaria in 2008, 2009, or 2010. (Alaska Peninsula and captive otters together comprise the reference group)

Sampling location	n	Sex		Age (yr)		
		f	m	<4	4–8	9+
Knight Island, PWS	16	12	4	4	7	5
Montague Island, PWS	14	11	3	9	2	3
Prince of Wales Passage, PWS	15	14	1	1	12	2
Alaska Peninsula	25	12	13	8	15	2
Captive	17	10	7	3	6	8
PWS proportions		0.82	0.18	0.31	0.47	0.22
Reference proportions		0.52	0.48	0.26	0.50	0.24

otter (2.2%) had a uterine mass suggestive of a leiomyoma, and 1 had oral papillomatosis (possible viral etiology).

Four PWS otters (8.9%) had serum chemistry parameters of clinical significance outside of the reference range (Rebar et al. 1995): 2 (4.4%) with slightly elevated alanine aminotransferase, 1 (2.2%) with a slightly elevated blood urea nitrogen, and 1 (2.2%) with a slightly elevated sodium value. While these values were outside of published reference ranges, there was no evidence to corroborate the presence of liver, kidney, or adrenal gland disease, respectively. Evaluation of serial samples over time may have offered more insight.

Two PWS otters (4.4%) had anomalies in the complete blood count (CBC). Both had increased numbers of band neutrophils present, suggesting infectious disease. Interestingly, both had dramatically worn and damaged teeth suggestive of active, advanced dental disease, likely associated with tooth root infection.

Gene transcription profiling

Overall gene transcription C_T values differed among sea otters sampled in PWS, Alaska Peninsula, and captives (ANOSIM, $p < 0.001$, Global $R = 0.267$, with 0 permuted statistics \geq Global R ; Table 3). As a group, the 3 PWS subpopulations differed significantly ($p < 0.001$) from the Alaska Peninsula and captive sea otters, with indication of less chance of overlap in transcription

profiles with Knight Island ($R = 0.41$ to 0.56) than with Montague Island ($R = 0.25$ to 0.41) or Prince of Wales Passage ($R = 0.27$ to 0.41) sea otters. Transcription profiles also differed slightly within the 3 subpopulations and between Alaska Peninsula and captive sea otters ($p < 0.03$), but the R statistics (-0.002 to 0.04) indicated high probability of overlap in transcription profiles within the 3 PWS subpopulations and between the Alaska Peninsula and captive sea otters. Most notable among the PWS subpopulations was high transcription (characterized by low C_T values) of HDC (associated with tumor formation) in Knight Island sea otters relative to those from other locations (Table 3). Gene transcription profiles did not differ between sexes ($p = 0.80$) or age group ($p = 0.16$).

When analyzed without *a priori* structure (e.g. location), sea otters separated into well-defined groups as depicted by MDS (Fig. 1) and confirmed by cluster analysis (SIMPROF, $p < 0.001$ to 0.03 ; Table 4). Clusters 1 to 3 were dominated by Knight Island ($n = 13$), Prince of Wales Passage ($n = 8$), and Montague Island ($n = 7$) sea otters, with no captive sea otters and only 1 Alaska Peninsula sea otter in Cluster 3 (Fig. 1). Sea otters in Cluster 1 had high gene transcription of HDC, CYT (protects against cell death), and HSP70

Table 3. *Enhydra lutris*. Geometric mean ($\pm 95\%$ CI) normalized (to the S9 housekeeping gene in each animal) cycle threshold (C_T) transcription values for targeted genes (see Table 1) in sea otters sampled at Alaska locations, in Prince William Sound (PWS) in 2008 and in clinically normal Alaska Peninsula (sampled 2009) and captive reference animals sampled in 2008, 2009, or 2010 (Bowen et al. 2011). PWS sea otters were captured at either Knight Island (heavily oiled in the 1989 spill), Prince of Wales Passage (moderate level of oil contamination), or Montague Island (least oiled). Note that the smaller the mean value, the higher the level of transcription

Gene	Knight Island (n = 16)	Prince of Wales Passage (n = 15)	Montague Island (n = 14)	Reference (n = 42) Geometric mean	Range
HDC	2.80 (2.12)	7.28 (3.36)	5.61 (3.00)	6.11	5.85–6.92
COX2	8.34 (1.10)	8.25 (0.72)	8.58 (0.91)	6.67	6.36–7.38
CYT	2.20 (1.76)	1.69 (0.88)	2.23 (1.78)	2.09	2.03–2.86
AHR	10.6 (1.36)	9.87 (0.63)	10.7 (1.06)	10.81	10.45–11.37
THR	11.7 (1.48)	12.3 (1.91)	11.8 (0.98)	13.12	12.65–13.91
HSP70	9.54 (1.42)	9.68 (1.30)	9.70 (1.50)	8.99	8.61–9.68
IL-18	1.46 (0.74)	0.89 (0.53)	1.73 (0.99)	1.67	1.34–2.20
IL-10	14.5 (0.58)	13.4 (1.09)	14.1 (1.31)	13.22	12.77–13.91
DRB	1.77 (0.99)	1.09 (0.57)	1.34 (0.85)	-0.64	-1.11–-0.24
MX-1	10.8 (0.63)	10.7 (0.91)	10.4 (0.65)	11.52	11.08–12.28

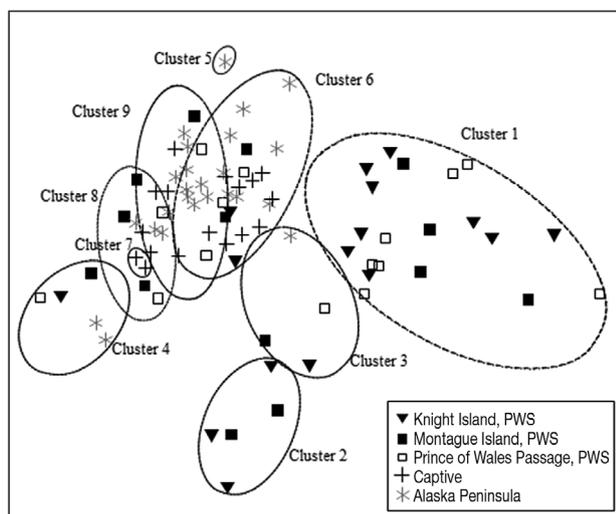


Fig. 1. *Enhydra lutris*. Multivariate, nonparametric, multi-dimensional scaling of gene transcription profiles (see Table 1) of sea otters sampled at 3 locations in Prince William Sound (PWS), at the Alaska Peninsula, and in captivity. Interpretive cluster analysis and SIMPROF (similarity profile permutation test, Primer v6) indicated significant ($p < 0.001$ to 0.034) separation between all clusters depicted by circles. The figure is 2-dimensional (Stress = 0.11); however, 3-dimensional (Stress = 0.07) representation depicted distinct separation among Clusters 6 to 9

(heat shock protein) compared to sea otters in all remaining clusters. High transcription of IL-18 (pro-inflammatory) and DRB (bacterial infection) were evident but not exclusive to this cluster (Table 4). Sea otters in Clusters 2 and 3 had high transcription of just the HDC gene alone, while those in Cluster 4 had notably low transcription of most targeted genes. The sole Alaska Peninsula sea otter in Cluster 5 had

the highest transcription of DRB and IL-18 genes. Refinement of the ordination from 2- to 3-dimensional space improved the adequacy of the MDS analysis (i.e. stress improved from 0.11 to 0.07, whereas 0.05 is defined as excellent representation), and the remaining clusters were visually separated in 3-dimensional space. Clusters 6 and 9 were among the 3 largest clusters and were dominated by 9 and 6 captive and 10 and 11 Alaska Peninsula sea otters, respectively. Cluster 6 had notably higher transcription values, except for HSP70, than Cluster 9, and included 3 sea otters from Montague Island and 4 from Prince of Wales Passage; both clusters included 1 Knight Island sea otter each (Table 4). Clusters 6, 9, and also 8 (which had 3 Montague Island and 4 Prince of Wales Passage sea otters) were characterized by comparable or high transcription of IL-18 and DRB relative to the other clusters. Cluster 7 had only 2 captive sea otters that, in general, had low transcription values, except for possibly DRB.

Gene transcription profiles were consistent with veterinarian diagnoses, and 87% of the sea otters with clinical anomalies fell within Clusters 1 to 4, with 60% in Cluster 1. Of the 15 sea otters with clinical anomalies, 13 had at least 50% or more genes with abnormal transcription levels, and in over half of these otters, almost every gene was abnormally transcribed (MX-1 was 33%). We defined abnormal levels of transcription as those falling outside the clinically normal reference range (Bowen et al. 2011). Of the 6 otters with blood panel anomalies, 5 had at least 50% or more genes with abnormal transcription levels, and every gene was abnormally transcribed in 50% or more of these otters.

Table 4. *Enhydra lutris*. Geometric mean ($\pm 95\%$ CI), normalized (to the S9 housekeeping gene in each animal) cycle threshold (C_T) transcription values for indicated genes (see Table 1) in sea otters sampled 2008, 2009, or 2010 at Prince of Wales Sound (Knight Island, Montague Island, and Prince of Wales Passage), the Alaska Peninsula, and aquaria. Multivariate, non-parametric, multi-dimensional scaling and cluster analyses (Primer v6) were used to separate individual sea otters into clusters of similar profiles. All clusters differed significantly from each other (SIMPROF, $p < 0.001$ to 0.034) (see Fig. 1). Note that the smaller the mean value, the higher the level of transcription; n = the number of sea otter samples in each cluster

Gene	Cluster 1 (n = 20)	Cluster 2 (n = 5)	Cluster 3 (n = 4)	Cluster 4 (n = 5)	Cluster 5 (n = 1)	Cluster 6 (n = 27)	Cluster 7 (n = 2)	Cluster 8 (n = 5)	Cluster 9 (n = 18)
HDC	-2.78 (1.0)	-0.58 (0.8)	-1.27 (1.6)	9.86 (1.3)	8.01	5.52 (0.5)	7.85 (0.2)	9.15 (1.0)	6.92 (0.6)
COX2	7.54 (0.5)	11.81 (1.2)	9.20 (0.9)	9.97 (0.6)	5.50	6.38 (0.6)	8.91 (0.0)	8.46 (1.7)	7.17 (0.5)
CYT	-1.24 (1.3)	4.94 (0.3)	3.31 (0.6)	4.06 (1.1)	1.05	1.85 (0.3)	5.75 (1.2)	2.59 (1.2)	2.01 (0.4)
AHR	9.39 (0.4)	15.13 (1.2)	12.13 (0.9)	12.43 (1.2)	8.22	9.69 (0.4)	11.12 (0.0)	10.91 (0.6)	11.60 (0.4)
THR	10.94 (1.1)	14.21 (0.9)	10.50 (0.5)	15.46 (0.9)	8.32	12.36 (0.7)	14.12 (0.7)	12.30 (0.8)	14.14 (0.6)
HSP70	7.91 (1.2)	13.11 (1.9)	11.05 (1.5)	12.67 (0.2)	7.21	9.23 (0.7)	9.56 (0.3)	10.95 (0.7)	8.82 (0.6)
IL-18	0.89 (0.5)	3.99 (0.5)	3.25 (1.4)	3.62 (0.4)	0.56	0.63 (0.4)	4.77 (1.2)	1.22 (0.8)	1.75 (0.4)
IL-10	13.87 (0.3)	17.34 (0.9)	13.82 (1.2)	16.97 (1.0)	11.47	11.91 (0.6)	12.75 (1.7)	15.17 (0.3)	14.53 (0.7)
DRB	-0.77 (0.3)	3.13 (0.6)	1.35 (0.5)	2.30 (0.4)	-4.62	0.66 (0.4)	1.75 (0.2)	0.84 (1.2)	0.91 (0.5)
MX-1	10.92 (0.6)	10.39 (1.1)	11.11 (1.0)	10.94 (2.8)	12.70	10.28 (0.6)	10.29 (1.2)	10.82 (0.9)	12.81 (0.5)

DISCUSSION

The genes selected in our study were based on a suite of genes transcribed in American mink (*Neovision vison*) experimentally exposed to crude oil (Bowen et al. 2007). Although targeted for oil exposure, these genes represent immune function responses to potential biological, physical, or anthropogenic inputs, and can be sensitive indicators of environmental stressors, particularly those that cause subtle or chronic effects on an organism.

Geographic location

There was relatively little difference in gene transcription, as a first-stage indicator of physiologic response within sea otters sampled at Knight Island, Montague Island, and Prince of Wales Passage in PWS, with the exception of the HDC gene, which was highest at Knight Island. Similarly, we found little difference between captive and Alaska Peninsula animals in gene transcription. However, we found significant differences in transcript profiles between reference (Alaska Peninsula and captive) and PWS sea otters.

Some of the similarity in the gene transcription profiles among sea otters from different areas in PWS could reflect movements of individuals among subpopulations; however, available data to support this were rare (Bodkin et al. 2002). In previous work, Montague Island was characterized as un-oiled (Bodkin et al. 2002, Ballachey et al. 2003). However, Short & Babcock (1994) indicated that portions of Montague Island experienced light EVOS contamination in 1989, and Short et al. (1994) identified detectable levels of non-EVOS oil at some locations in northern Montague Island. These studies support our findings, which associated transcript profiles of Montague sea otters with those of sea otters in PWS rather than with reference animals. Clinical diagnosis of sea otters was consistent with gene transcript analysis; while only 12% of sea otters captured at Alaska Peninsula showed clinical anomalies, 33% of PWS sea otters showed significant clinical anomalies. Interestingly, neither wild population is recovering. The PWS otters, especially those at Knight Island, had an abnormal transcription profile, while sea otters at the Alaska Peninsula appear, in general, to be clinically normal, based on veterinary diagnostic evaluations as well as gene transcription profiles. This suggests an alternative hypothesis for the lack of

recovery in the Alaska Peninsula population (see for example Williams et al. 2004).

Twelve sea otters (5 Knight, 3 Montague, 2 Prince of Wales, and 2 Alaska Peninsula) had profiles with at least 7 gene transcripts that were notably quiescent, that is, falling below baseline transcription levels (Bowen et al. 2011). Although one would doubt a complete lack of stimulation as the cause of abnormally low transcription in free-ranging sea otters in particular, there may be various reasons for this occurrence. Potential causes of exceedingly low transcription include nutritional deficiencies (Kilberg et al. 2005) related to food availability or inherent illness or exposure to xenobiotics (Dong et al. 1997, Laupeze et al. 2002). While defining the exact cause of this quiescence is beyond the scope of this study, there does appear to be a geographical association which would indicate a potential environmental influence. Without experimentation, the potential effects of abnormally quiescent transcription are unknown but may indicate acute immune system dysfunction (J. L. Stott unpubl. data). Further study of captive animals will be necessary to understand the implications of transcription values below suggested baselines, and provide clarification of interpretation of these values relative to the health of sea otters.

Statistical clusters

Nonparametric multivariate analyses provided more ecologically relevant insight into transcription differences among free-ranging sea otters that probably have inconsistent exposure to anthropogenic contaminants (Bodkin et al. 2012, Monson et al. 2011). Most striking in these analyses was the association of clinically normal free-ranging Alaska Peninsula sea otters with captive sea otters. These analyses further defined a majority of sea otters from the 3 different locations in PWS that differed from clinically normal reference animals based on higher transcription of certain genes. The 3 largest separations based on nonparametric analysis were Cluster 1 ($n = 20$), Cluster 6 ($n = 27$), and Cluster 9 ($n = 18$). More sea otters from the area that had been most severely impacted by the 'Exxon Valdez' oil spill (Knight Island) were aligned with the higher expressing (particularly HDC and CYT) Cluster 1, whereas more sea otters from Montague Island and Prince of Wales Passage were aligned with other clusters including the apparently healthier Cluster 6. Furthermore, only 1 Knight Island sea otter each aligned with Cluster 6 and also Cluster 9, which had transcription levels

closer to baseline across the profile with the exception of HSP70 (Bowen et al. 2011).

Clusters 1 to 4 were primarily PWS otters and clusters 6 to 9 were primarily reference otters. Consistent with the patchiness of residual oil found in PWS (Bodkin & Udevitz 1994, Short et al. 2004, Monson et al. 2011), otters from the different locations within PWS demonstrated considerable overlap in their clustering. For example, Cluster 1 was comprised of only PWS sea otters (45% Knight, 35% Prince of Wales, 20% Montague), reflective of previously described classifications of heavily oiled, moderately oiled, and un-oiled area reference. Consistent with this grouping, Cluster 1 transcript profiles were potentially indicative of chronic organic compound exposure and subsequent malignant transformation, inflammation, and immunologic impairment. Transcription of most genes in Cluster 1, with the exception of COX2, IL-10, and DRB, was high compared to baseline levels proposed by Bowen et al. (2011). Sea otters in Clusters 2 and 3 had high transcription of HDC but along with Cluster 4 displayed low expression by most genes relative to proposed baseline levels. The lone Alaska Peninsula sea otter in Cluster 5 had notably low expression of HDC and possibly MX-1 but high transcription of the remaining genes. Cluster 6 was comprised of 70% reference, 4% Knight, 15% Prince of Wales, and 11% Montague sea otters. Consistent with this grouping, Cluster 6 transcript profiles were likely indicative of relatively little extrinsic stimuli from environmental stressors. Clusters 7 (2 reference animals) and 8 (3 Montague Island and 4 Prince of Wales Passage animals) had most genes within baseline, indicating little to no environmental or physiologic perturbation. Cluster 9 ($n = 18$), comprised of 94% reference sea otters, was closest to a 'clinically normal' transcript profile (Bowen et al. 2011).

In general, gene transcription patterns in the PWS sea otters 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. This is consistent with findings from Bodkin et al. (2012), which documents a pathway of exposure from lingering intertidal oil to foraging sea otters in PWS. In particular, sea otters from the oil spill area in western PWS demonstrated elevated transcription of several of the genes measured, including HDC and THR, and down-regulation of DRB. 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 biochemical detoxification responses.

Chronic transcription of genes responsible for immunologic function, including detoxification, can be physiologically costly (Graham et al. 2010). Perhaps the largest cost is the reallocation of nutrients and energy from one portion of an individual's resource budget to other functions. For example, sea otters at Knight Island required less time foraging to meet energetic requirements; despite having adequate (or even excess) energy resources (greater at Knight Island than at Montague Island). Bodkin et al. (2002) observed increased mortality and reduced population growth/ recovery at Knight Island in the early 2000s. Potential repercussions of lingering oil continue to persist in sea ducks in PWS (Esler et al. 2002, 2010, 2011).

Interestingly, the gene AHR was not strongly differentially expressed in this nor the mink study (Bowen et al. 2007). 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 & Oesch 2005). Chronic exposure to specific toxicants may not necessarily cause a sustained increase in the expression of AHR (Bowen et al. 2007), but can be associated with potentially severe downstream consequences, e.g. modulation of T-regulatory (T_{REG}) (immunosuppressive) or T-helper type 17 (T_{H17}) (pro-inflammatory) immunologic activity (Quintana et al. 2008, Veldhoen et al. 2008). Consistent with our findings of relatively normal transcription of AHR, Bodkin et al. (2012) indicated that average annual oil encounter rates ranged from 2 to 24 times yr^{-1} for females, and 2 to 4 times yr^{-1} for males.

Mitigation of detrimental effects imposes demands on animals above those normally required to sustain life and may result in reduction of fitness evidenced by decreased reproductive capability, increased susceptibility to disease, or disadvantageous behavioral changes (Graham et al. 2010, Martin et al. 2010). Such effects may ultimately be manifested in reduced survival rates (Ballachey et al. 2003, Monson et al. 2011) that contributed to the delayed recovery of sea otters observed in areas of PWS initially oiled in 1989 (Bodkin et al. 2002).

Our results describe a sensitive tool for differentiating between sea otters diagnosed as clinically normal and those with sub-lethal chronic signatures that could be associated with environmental stressors. Although the PWS otter population is a wild population and thus subject to a variety of stressors, the

strong organic-induced gene transcript profile of the PWS otters was consistent with the profiles of oiled mink in the Bowen et al. (2007) model study and bivalves in the Downs et al. (2002) PWS study. Additionally, the strong organic-induced gene transcript profile was not ubiquitous throughout free-ranging otters but was focused on PWS. Further efforts will focus on examination of individual otters including histopathology, serum and blood chemistries, and information on specific habitat use. Overall the gene transcription technique has the exciting potential to be utilized for elucidation of oil-related perturbations that exist globally, and with multiple species, populations, and ecosystems.

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