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Effects of wildfire on sea otter (*Enhydra lutris*) gene transcript profiles

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Abstract

Wildfires have been shown to impact terrestrial species over a range of temporal scales. Little is known, however, about the more subtle toxicological effects of wildfires, particularly in downstream marine or downwind locations from the wildfire perimeter. These down-current effects may be just as substantial as those effects within the perimeter. We used gene transcription technology, a sensitive indicator of immunological perturbation, to study the effects of the 2008 Basin Complex Fire on the California coast on a sentinel marine species, the sea otter (*Enhydra lutris*). We captured sea otters in 2008 (3 mo after the Basin Complex Fire was controlled) and 2009 (15 mo after the Basin Complex Fire was controlled) in the adjacent nearshore environment near Big Sur, California. Gene responses were distinctly different between Big Sur temporal groups, signifying detoxification of PAHs, possible associated response to potential malignant transformation, and suppression of immune function as the primary responses of sea otters to fire in 2008 compared to those captured in 2009. In general, gene transcription patterns in the 2008 sea otters were indicative of molecular reactions to organic exposure, malignant transformation, and decreased ability to respond to pathogens that seemed to consistent with short-term hydrocarbon exposure.

Key words: eco-immunology, sea otter, *Enhydra lutris*, wildfire, hydrocarbon.

Wildfires are one of the most frequent ecological disturbances in North America, and have been shown to impact terrestrial organisms both positively and negatively over a range of temporal scales (Smith 2000). Documented wildfire effects most frequently include habitat alteration (Kotliar *et al*. 2002, Garvey *et al*. 2010, Doumas and Koprowski 2012), changes in dietary composition and food source availability (Kotliar *et al*. 2002), and changes in survival and reproduction rates

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(Bond *et al*. 2002, Burton 2005). Studies quantifying these affects have primarily focused on terrestrial systems and until recently, less on freshwater aquatic systems within or adjacent to the wildfire perimeter (Smith 2000). Only one study to our knowledge has considered potential impacts in coastal waters adjacent to wildfire events (Venn-Watson *et al*. 2013).

Little is known about subtle toxicological effects of wildfires on organisms, particularly downcurrent from the wildfire perimeter. These effects may be just as substantial as within the perimeter, and particularly detrimental to listed populations; far more species of concern and consequently wildfires occur in California than any other state. Ash deposition and toxic slurry can be immediately fatal or cause chronic stress to fish in freshwater systems (Spencer and Hauer 1991, Hauer and Spencer 1998). Influxes of fine sediment from increased runoff can fill the interstices of substrate and reduce macro-invertebrate density (Bjornn *et al*. 1977, Everest *et al*. 1987, Rinne and Medina 1988). Increases in stream pH and phosphorous concentrations from ash deposition, as well as increased ammonium and nitrate have been documented from wildfires (Cushing and Olson 1963, Spencer and Hauer 1991, Hauer and Spencer 1998). The manifestation of these effects in the marine environment and how they affect higher trophic level organisms has rarely been documented (Venn-Watson *et al*. 2013). As wildfires are widely projected to increase in size and intensity in North America through the 21st century (Spracklen *et al*. 2009), it is critical to understand how this trend may ultimately impact species of concern.

Polycyclic aromatic hydrocarbons (PAHs) are a major byproduct of wildfires. PAHs are a class of environmental pollutants known to be carcinogenic and immunotoxic that can lead to increased disease susceptibility. Although a number of studies have reported PAH concentrations in marine benthic invertebrates and higher trophic-level organisms such as marine mammals and birds, few studies have documented potential physiologic effects of PAHs on marine mammals. These include immunosuppression or tumorigenesis (Beland *et al*. 1993, De Guise *et al*. 1994, Martineau *et al*. 1994, Miles *et al*. 2012). Variations in the integrity of the immune response are very sensitive indicators of toxic insult (Luster and Rosenthal 1993) due to the complex nature of the immune system. However, the pathophysiological changes within an individual may be significant yet subtle, and consequently undetectable using classical diagnostic methods.

Under these circumstances, molecular investigation of subtle alterations of expressed genes indicative of multiple physiological processes at the cellular level is particularly useful, and may elucidate the mechanisms by which wildfires cause deleterious effects. Gene expression 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. How much a particular gene is expressed is physiologically dictated by a number of intrinsic and extrinsic factors, including stimuli such as infectious agents, toxin exposure, trauma, or neoplasia (Table 2). The earliest observable signs of health impairment are altered levels of gene transcripts that are evident prior to clinical manifestation (McLoughlin *et al*. 2006). As a result of this keystone function, analysis of mRNA can provide information not only of genetic potential but also of dynamic changes in the functional state of an organism. 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). In fact, PAH-induced sublethal pathology in sea otters (*Enhydra lutris*) has been shown to be

accompanied by predictable and specific changes in gene transcription (Bowen *et al*. 2007, Miles *et al*. 2012).

Marine mammal toxicology has historically relied heavily on the identification or concentration of xenobiotics within specific tissues as an indicator of toxic insult. These assays do not measure the influence of xenobiotics on the health status of free-ranging organisms and interpretation is often limited to effects extrapolated from laboratory surrogates. The advantage of using gene transcription 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-transcription patterns can be identified either on free-ranging animals opportunistically (such as those exposed to a toxic spill) or under experimental conditions using model organisms (Bowen *et al*. 2007; Mancia *et al*. 2007, 2008; Miles *et al*. 2012).

Big Sur Wildfires

The Basin and Chalk wildfires in the Big Sur region of central California in 2008 occurred just prior to a scheduled study of sea otters (*Enhydra lutris*) in the adjacent nearshore marine environment. This timing provided an opportunity to assess the potential effects of wildfire on the immunological response of sea otters just downslope from where the fire burned. The Basin fire burned approximately 66,500 ha between 21 June and late July, while the Chalk Fire consumed an additional 6,240 ha between ignition on 27 September and containment by the end of October. For the two wildfires combined, 45 percent of the burned area lay within the drainage basin that discharges into the range of the Big Sur sea otter population (Fig. 1). The fires burned with mixed severity, with partial to complete consumption of heavy vegetation within the perimeter (Fig. 2).

There are two potential indirect impacts of these fires to the sea otter population that may manifest in a genetic analysis of toxicological effects. First, during and immediately after the wildfires, prevailing night-time wind patterns consistently carried ash and debris from the smoke column off-shore and onto the surface waters (WRCC 2012). This ash was observed intermixed in the surface water column by us during the November 2008 sampling bout. Second, there are seven minor drainages that carry run-off into the adjacent nearshore environment from the burned area. From 1 to 3 November, the Big Sur Remote Automated Weather Station (RAWS) measured 6.6 cm of rain (WRCC 2012), the first appreciable rainfall in over 6 mo. This storm event subsequently produced a run-off spike in the Big Sur River that exceeded 5.7 m^3/s, compared to the 0.3–0.4 m^3/s discharge of the river in the summer and early autumn months (Fig. 3). Following this event, the Big Sur area experienced a slightly drier than normal winter, with 89 cm of (91% of normal) precipitation for the water year ending in October 2009 (WRCC 2012).

The objective of our study was to determine if wildfire in an adjacent watershed caused immune function suppression in sea otters. We compared transcription of targeted genes in sea otters sampled in Big Sur November 2008 and November 2009 with reference groups of sea otters diagnosed as clinically normal that consisted of captive aquaria animals sampled in 2008–2010 and free-ranging animals from the Alaska Peninsula sampled in 2009 in an area with no known, large-scale anthropogenic impacts, including forest fire or significant sources of PAHs (Bowen *et al*. 2012, Miles *et al*. 2012).

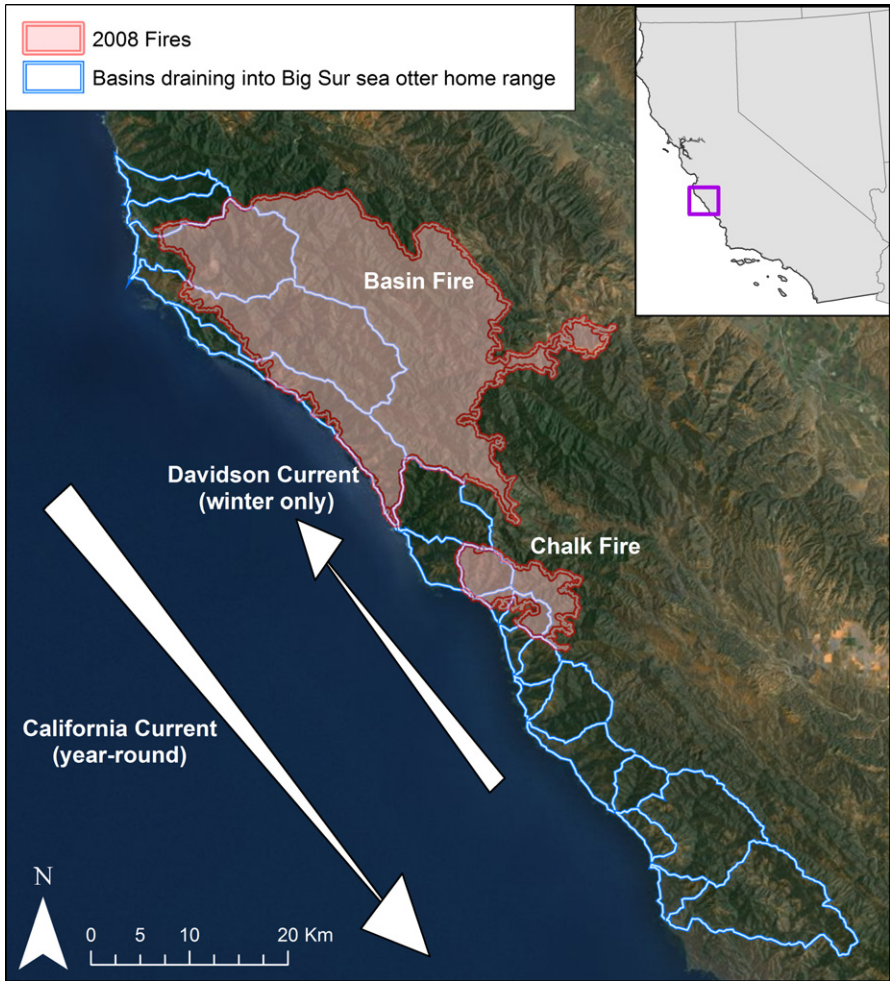

Figure 1. Basin and Chalk fire locations overlapping the primary watersheds draining into the Big Sur sea otter population range.

Materials and Methods

Big Sur Sea Otters

A total of 39 sea otters (*Enhydra lutris*) from Big Sur, California, were captured in November 2008 and 2009. Sea otters were captured in 2008, 1 mo after the Basin Complex Fire was declared under control ($n = 27$), and 2009, 15 mo after the fire was controlled ($n = 12$) (Table 1). Approximate age was determined by analysis of cementum annuli in extracted premolar teeth (Bodkin *et al*. 1997). Sea otters were captured with a Wilson trap (Wendell *et al*. 1996) and brought immediately to a shipboard station for processing. These sea otters (as well as reference sea otters) were anesthetized with fentanyl citrate and midazolam hydrochloride (Monson *et al*. 2001) prior to processing.

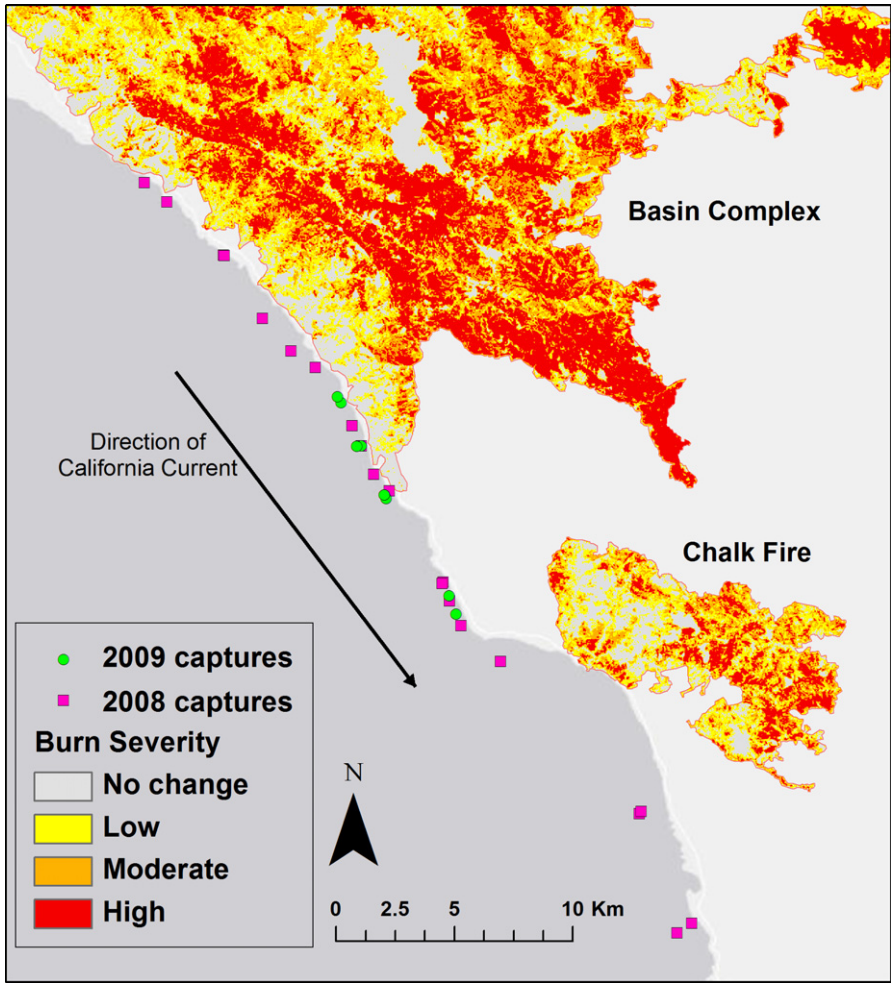

Figure 2. Wildfire burn severity for Basin and Chalk fires.

Captive and Free-ranging Reference Otters

Seventeen blood samples from captive sea otters were obtained from the Monterey Bay Aquarium (Monterey, CA), Shedd Aquarium (Chicago, IL), Oregon Coast Aquarium (Newport, OR), and the Vancouver Aquarium (Vancouver, BC) in 2008, 2009, and 2010, and included both northern and southern subspecies (Bowen *et al*. 2012). 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 ($n = 25$) in summer 2009 and deemed clinically normal by the attending veterinarian and the results of health blood tests. The Alaska Peninsula sea otters were captured and processed exactly as the Big Sur sea otters. There were no statistically significant

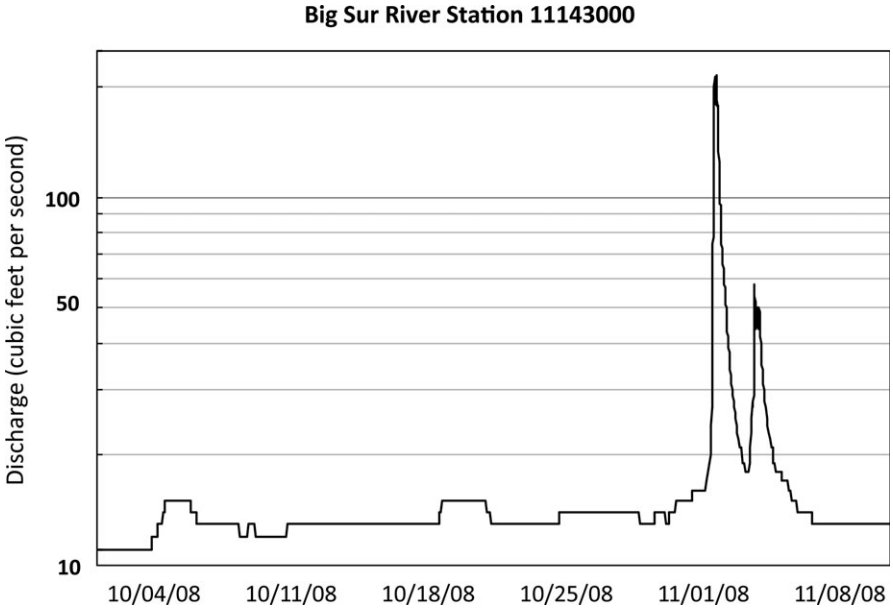

Figure 3. Discharge time series for October/November 2008 from USGS Big Sur River gauge showing peaks associated with 1–3 November 2008 precipitation event.

differences between transcript profiles from captive and free-ranging reference otters (Bowen *et al*. 2012).

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, Zurich, Switzerland) from either the jugular or popliteal veins and then frozen at –20°C until extraction of RNA (Bowen *et al*. 2012). 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. 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 gDNA (silica-based microspin technology), and the extracted RNA stored at –80°C until analysis. All RNA was checked for quality on a NanoDrop 2000 (Thermo Scientific, Wilmington, DE) and achieved A260/A280 ratios of approximately 2.0 and A260/A230 ratios of less than 1.0.

cDNA Synthesis

A standard cDNA synthesis was performed on 2 μg of RNA template from each animal. Reaction conditions included 4 units reverse transcriptase (Omniscript, Qiagen, Valencia, CA), 1 μM random hexamers, 0.5 mM each dNTP, and 10 units

Table 1. ID number, category, capture location, capture date, sex, and age estimate of captive reference sea otters, free-ranging reference sea otters, and Big Sur target sea otters.

Sample ID	Category	Capture location	Sampling date	Sex	Age estimate
1066-08	Free-ranging target	Big Sur	5 November 2008	M	9
1067-08-08	Free-ranging target	Big Sur	5 November 2008	F	5
1068-08	Free-ranging target	Big Sur	5 November 2008	F	5
1069-08	Free-ranging target	Big Sur	5 November 2008	F	8
1070-08	Free-ranging target	Big Sur	5 November 2008	F	A
1073-08	Free-ranging target	Big Sur	6 November 2008	n/a	4
1074-08-08	Free-ranging target	Big Sur	6 November 2008	F	10
1075-08	Free-ranging target	Big Sur	6 November 2008	F	3
1079-08	Free-ranging target	Big Sur	7 November 2008	F	2
1081-08	Free-ranging target	Big Sur	7 November 2008	F	10
1083-08	Free-ranging target	Big Sur	7 November 2008	F	8
1084-08	Free-ranging target	Big Sur	7 November 2008	F	6
1086-08	Free-ranging target	Big Sur	7 November 2008	F	2
1088-08	Free-ranging target	Big Sur	10 November 2008	F	4
1089-08	Free-ranging target	Big Sur	6 November 2008	F	6
1090-08	Free-ranging target	Big Sur	10 November 2008	F	12
1091-08	Free-ranging target	Big Sur	6 November 2008	M	9
1093-08	Free-ranging target	Big Sur	6 November 2008	M	8
1094-08	Free-ranging target	Big Sur	7 November 2008	M	8
1094-08-09	Free-ranging target	Big Sur	3 November 2009	M	9
1095-08	Free-ranging target	Big Sur	5 November 2008	M	6
1096-08-08	Free-ranging target	Big Sur	7 November 2008	M	9
1096-08-09	Free-ranging target	Big Sur	4 November 2009	M	10
1097-08	Free-ranging target	Big Sur	11 November 2008	F	5
1098-08	Free-ranging target	Big Sur	11 November 2008	F	2
1099-08	Free-ranging target	Big Sur	11 November 2008	M	6
1103-08	Free-ranging target	Big Sur	11 November 2008	F	9
1105-08	Free-ranging target	Big Sur	11 November 2008	F	4
1106-08	Free-ranging target	Big Sur	11 November 2008	F	9
1135-09	Free-ranging target	Big Sur	2 November 2009	F	4
1136-09	Free-ranging target	Big Sur	2 November 2009	F	4
1137-09	Free-ranging target	Big Sur	3 November 2009	M	8
1139-09	Free-ranging target	Big Sur	3 November 2009	F	4
1141-09	Free-ranging target	Big Sur	3 November 2009	F	5
1142-09	Free-ranging target	Big Sur	4 November 2009	F	3
1143-09	Free-ranging target	Big Sur	4 November 2009	M	7
1146-09	Free-ranging target	Big Sur	4 November 2009	F	1
1147-09	Free-ranging target	Big Sur	4 November 2009	M	2
1148-09	Free-ranging target	Big Sur	5 November 2009	F	5
1	Captive reference	Monterey Bay Aquarium	13 January 2010	F	7
2	Captive reference	Monterey Bay Aquarium	20 November 2010	F	1
3	Captive reference	Monterey Bay Aquarium	1 March 2010	M	1
4	Captive reference	Monterey Bay Aquarium	25 March 2010	F	1

(Continued)

Table 1. (Continued)

Sample ID	Category	Capture location	Sampling date	Sex	Age estimate
5	Captive reference	Monterey Bay Aquarium	29 December 2009	F	12
6	Captive reference	Monterey Bay Aquarium	27 October 2009	F	8
7	Captive reference	Monterey Bay Aquarium	19 November 2009	F	9
8	Captive reference	Monterey Bay Aquarium	8 March 2010	M	7
9	Captive reference	Monterey Bay Aquarium	20 October 2009	F	11
10	Captive reference	Vancouver Aquarium	10 April 2008	M	7
11	Captive reference	Vancouver Aquarium	16 January 2009	M	9
12	Captive reference	Oregon Coast Aquarium	25 March 2010	M	10
13	Captive reference	Oregon Coast Aquarium	25 March 2010	M	12
14	Captive reference	Shedd Aquarium	25 March 2009	F	20
15	Captive reference	Shedd Aquarium	25 March 2009	F	7
16	Captive reference	Shedd Aquarium	24 March 2009	F	5
17	Captive reference	Shedd Aquarium	24 March 2009	M	10
RefSO-09-31	Free-ranging reference	Alaska Peninsula	25 July 2009	M	1
RefSO-09-32	Free-ranging reference	Alaska Peninsula	25 July 2009	M	3
RefSO-09-33	Free-ranging reference	Alaska Peninsula	26 July 2009	M	1
RefSO-09-34	Free-ranging reference	Alaska Peninsula	26 July 2009	M	7
RefSO-09-35	Free-ranging reference	Alaska Peninsula	26 July 2009	F	1
RefSO-09-37	Free-ranging reference	Alaska Peninsula	26 July 2009	M	2
RefSO-09-38	Free-ranging reference	Alaska Peninsula	26 July 2009	F	6
RefSO-09-39	Free-ranging reference	Alaska Peninsula	27 July 2009	F	9
RefSO-09-40	Free-ranging reference	Alaska Peninsula	27 July 2009	M	1
RefSO-09-43	Free-ranging reference	Alaska Peninsula	28 July 2009	F	11
RefSO-09-45	Free-ranging reference	Alaska Peninsula	28 July 2009	F	8

(Continued)

Table 1. (Continued)

Sample ID	Category	Capture location	Sampling date	Sex	Age estimate
RefSO-09-46	Free-ranging reference	Alaska Peninsula	29 July 2009	F	12
RefSO-09-47	Free-ranging reference	Alaska Peninsula	29 July 2009	F	5
RefSO-09-48	Free-ranging reference	Alaska Peninsula	29 July 2009	F	8
RefSO-09-49	Free-ranging reference	Alaska Peninsula	29 July 2009	M	5
RefSO-09-51	Free-ranging reference	Alaska Peninsula	29 July 2009	M	1
RefSO-09-52	Free-ranging reference	Alaska Peninsula	29 July 2009	M	3
RefSO-09-53	Free-ranging reference	Alaska Peninsula	29 July 2009	F	6
RefSO-09-54	Free-ranging reference	Alaska Peninsula	29 July 2009	F	11
RefSO-09-55	Free-ranging reference	Alaska Peninsula	29 July 2009	F	3
RefSO-09-57	Free-ranging reference	Alaska Peninsula	30 July 2009	M	3
RefSO-09-58	Free-ranging reference	Alaska Peninsula	30 July 2009	F	5
RefSO-09-59	Free-ranging reference	Alaska Peninsula	30 July 2009	M	1
RefSO-09-60	Free-ranging reference	Alaska Peninsula	30 July 2009	M	3
RefSO-09-61	Free-ranging reference	Alaska Peninsula	30 July 2009	M	3

RNase inhibitor, in RT buffer (Qiagen, Valencia, CA). 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 (S9) and genes of interest were run in separate wells (primer sequences for these genes can be found in Bowen *et al*. 2012 and were used in Miles *et al*. 2012; Table 2). Briefly, 1 μL of cDNA was added to a mix containing 12.5 μL of Quanti-Tect SYBR Green Master Mix [5mM Mg^{2+}] (Qiagen, Valencia, CA), 0.5 μL each of forward and reverse sequence specific primers, 0.5 μL of Uracil-N-Glycosylase (Invitrogen, Carlsbad, CA), 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, Foster City, CA). Reaction mixtures containing water, but no cDNA, were used as negative controls; thus approximately 3–4 individual sea otter samples were run per plate.

Table 2. Documented function of 13 genes identified in free-ranging sea otters sampled at Big Sur in 2008 and 2009, and Alaska Peninsula in 2009, and in clinically normal captive reference animals sampled in 2008, 2009, or 2010.

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).
THR	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 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).
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-18 plays 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 antigenn 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).

(Continued)

Table 2. (Continued)

Gene	Gene function
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).
CCR3	The chemokine receptor 3 binds at least seven different chemokines and is expressed on eosinophils, mast cells (MC), and a subset of Th cells (Th2) that generate cytokines implicated in mucosal immune responses (Gurish *et al*. 2002, Kringel *et al*. 2006). Up-regulation of CCR3 occurs in the presence of parasites (Gurish *et al*. 2002, Kringel *et al*. 2006).
5HTT	The serotonin transport gene codes for an integral membrane protein that transports the neurotransmitter serotonin from synaptic spaces into presynaptic neurons. This transport of serotonin by the SERT protein terminates the action of serotonin and recycles it in a sodium-dependent manner (Jennings *et al*. 2006, Squire *et al*. 2008). Increased transcription of 5HTT confers a low anxiety phenotype (Jennings *et al*. 2006).
CaM	Calmodulin (CaM) is a small acidic Ca^{2+}-binding protein, with a structure and function that is highly conserved in all eukaryotes. CaM activates various Ca^{2+}-dependent enzyme reactions, thereby modulating a wide range of cellular events, including metabolism control, muscle contraction, exocytosis of hormones and neurotransmitters, and cell division and differentiation (Chen *et al*. 2012).

Amplifications were conducted on a 7300 Real-time Thermal Cycler (Applied Biosystems, Foster City, CA). 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 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). First, we used two-way analysis of similarities (ANOSIM, Primer E, Plymouth, U.K.) to examine potential interaction of sex, age, and year. Then we used conventional mean responses per classification group (Big Sur 2008, Big Sur 2009) with data assessed for statistical significance between classification ranks using Kruskal-Wallis with Dunns' Multiple Comparison Tests relative to the mean of the reference sea otters (NCSS 2007, Kaysville, UT).

Next, we used gene profiling based on per gene and per otter response correlation, using normalized qPCR data obtained from each individual otter, which were subjected to hierarchical clustering using Genesis software (Genesis, Graz, Switzerland). Average dot product metric, with complete linkage clustering, was used to generate a heatmap profile of gene expression (Connon *et al*. 2012).

Results

Veterinary Evaluations

Health evaluations of the sea otters captured in Big Sur in 2008/2009 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 Big Sur in 2008 and 2009, ranging from 1 to 12 yr old, and most sea otters were in the adult age group (Table 1). Of the 27 Big Sur sea otters sampled in 2008, 21 (72%) were found to have clinically significant anomalies. Nine of these animals had clinically significant dental disease which included marked wear and loss of the occlusal aspect of teeth, tooth fractures, loss of teeth, and gingival or periodontal disease. Three others had either clinical or laboratory changes suggestive of bacterial infection (abscessation, cellulitis), while four others had overt evidence of trauma. The remaining 5 of 21 otters with clinical anomalies showed evidence of anemia (1), chronic illness (1), emaciation (2), or liver abnormalities (1).

Although not indicative of a clinical anomaly, eight Big Sur 2008 otters had slightly elevated alanine aminotransferase and three others had slightly elevated blood urea nitrogen. While these values were outside published reference ranges, there was no evidence to corroborate the presence of liver or kidney disease. Evaluation of serial samples over time may have offered more insight. One Big Sur 2008 otter had anomalies in the complete blood count (CBC). Increased numbers of band (immature) neutrophils were present, suggesting infectious disease.

Of the 12 Big Sur sea otters sampled in 2009, 10 (83.3%) were found to have clinically significant anomalies. Four animals had clinically significant dental disease as described above. None of the animals had clinical or laboratory changes suggestive of bacterial infection. The remaining 6 of the 10 animals with clinically significant anomalies presented with trauma (1), pregnancy (1), anemia (1), emaciation (2), or liver abnormalities (1).

Five Big Sur 2009 otters had slightly elevated alanine aminotransferase but no supportive evidence to corroborate the presence of liver disease. Evaluation of serial samples over time may have offered more insight. One Big Sur 2009 otter had decreased hemoglobin and decreased hematocrit, indicative of anemia, however, the cause could not be determined.

Gene Transcription Profiling

Gene transcription profiles differed between the two sampling years (2008, 2009) and between Big Sur and reference sea otters (Table 3, Fig. 4). Transcription responses were distinctly different between Big Sur temporal groups, identifying detoxification of PAHs (up-regulation of AHR) and associated malignant transformation (up-regulation of HDC) as the primary responses to fire in the Big Sur 2008 sea otters compared to 2009 sea otters (Table 3). Downstream effects of exposure to PAHs were evidenced in the 2008 sea otters by immune suppression (down-regulation of IL-10, IL-18, and DRB), and an increased calmodulin response in 2008 otters (Table 3). Differences possibly unrelated to PAH exposure included an elevated response to viral infection (*i.e.*, up-regulation of MX1) in 2008 otters. Additionally, transcript values were significantly

Table 3. Geometric mean normalized (to the S9 housekeeping gene in each animal) cycle threshold (C_T) transcription values for targeted genes (see Table 2) in sea otters sampled at Big Sur in 2008, and 2009, in clinically normal Alaska Peninsula and captive reference animals sampled in 2008, 2009, or 2010 (Bowen *et al*. 2012). Note that the *smaller* the mean value, the *higher* the level of transcription.

	Big Sur 2008 ($n = 27$)	Big Sur 2009 ($n = 12$)	Reference ($n = 42$) Geometric mean	Reference range ($n = 42$)
HDC* ($P = 0.02$)	5.93	6.55	6.11	5.85–6.92
COX2	8.54[b] ($P = 0.00$)	7.83	6.67	6.36–7.38
CYT	3.19[b] (0.00)	3.03[b] ($P = 0.02$)	2.09	2.03–2.86
AHR[a] ($P = 0.00$)	10.01[b] ($P = 0.00$)	11.57	10.81	10.45–11.37
THR	12.93	12.73	13.12	12.65–13.91
HSP70	10.87[b] ($P = 0.00$)	10.60[b] ($P = 0.00$)	8.99	8.61–9.68
IL-18[a] ($P = 0.00$)	2.54[b] ($P = 0.00$)	1.17	1.67	1.34–2.20
IL-10[a] ($P = 0.00$)	14.16	12.10	13.22	12.77–13.91
DRB[a] ($P = 0.00$)	0.66[b] ($P = 0.00$)	−0.32	−0.64	−1.11–0.24
MX-1[a] ($P = 0.00$)	11.13[b] ($P = 0.02$)	12.72	11.52	11.08–12.28
CCR3	5.43	4.93	4.80	4.22–5.46
5HTT	12.11[b] ($P = 0.00$)	11.87[b] ($P = 0.00$)	10.20	9.76–10.67
CaM[a]($P = 0.02$)	−0.14	0.23[b] ($P = 0.00$)	−0.52	−0.82–0.22

[a] Indicates significantly different between 2008 and 2009.
[b] Indicates significantly different from reference.

different between 2008 Big Sur sea otters and clinically normal reference otters for COX2, CYT, AHR, HSP70, IL18, DRB, MX1, and 5HTT. Transcript values differed significantly between 2009 Big Sur sea otters and clinically normal reference otters for CYT, HSP70, 5HTT, and CaM (Table 3). Multivariate analysis of transcription patterns of sex and age indicated no difference. Although sexes were skewed toward more females captured, no marked or notable differences in transcription were apparent among the suite of genes.

Hierarchical cluster analysis and subsequent heat map generation was conducted using individual sea otter transcription data (Fig. 4). Heat map analysis was successful in identifying transcriptional differences between capture years and reference sea otters according to transcript profile (Fig. 4). Cluster 1 was comprised of 50% reference otters and 50% Big Sur 2008 otters. Clusters 2 and 6 were dominated by 78% and 88% reference otters, respectively. Clusters 3 and 4 were comprised of 67% and 71% Big Sur 2008 otters, respectively. Cluster 5 was dominated by 50% Big Sur 2009 otters. Reference otter RefSO-09-48 was an outlier and not included in any cluster.

Discussion

PAH levels are challenging to quantify from wildfires, as this estimation requires inputs of area burned, biomass density, burning efficiency, and emissions factors (Yuan *et al*. 2008). These measures were not quantified for the Basin or Chalk fires. However, PAH concentrations were estimated for two wildfires that burned in 2006

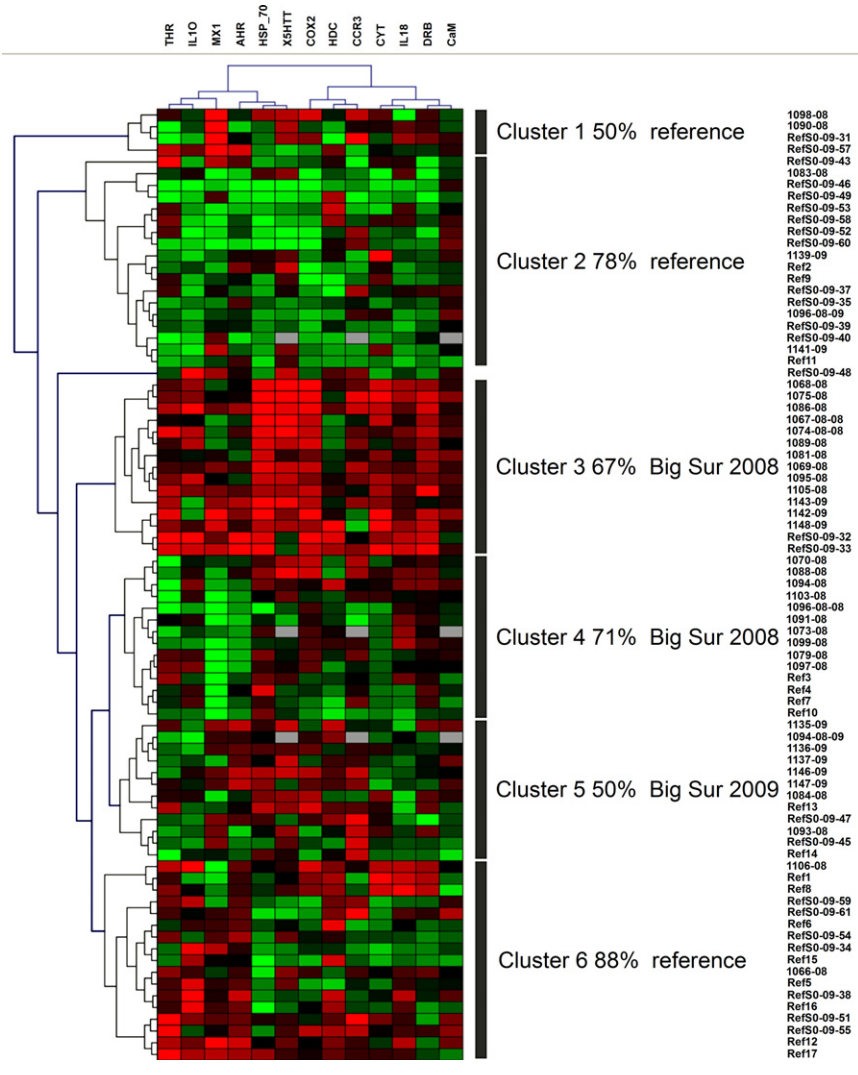

Figure 4. Gene profiling: Transcription matrix of 13 target genes in sea otters captured in 2008 (three months after the Basin Complex Fire was declared under control) (n = 27) and 2009 (15 mo after the Basin Complex Fire was declared under control) (n = 13) (Hierarchical clustering with complete linkage disequilibrium; Genesis, Graz, Switzerland). Green indicates higher relative transcription levels and red indicates lower relative transcription levels. Gray indicates missing data. Sea otter ID explanations: REFSO- indicates free-ranging Alaska Peninsula reference otters, REF# indicates captive reference otters, all other sea otter IDs are from Big Sur; each of these IDs ends with either 08 or 09 indicating capture year. See Table 1 for corresponding sea otter information (*i.e.*, age, sex, category, capture date). Primary group composition is indicated for each cluster. Animal RefSO-09-48 is an outlier, not included in any cluster.

and 2009 in the coastal mountains of southern California, and serve as analogs to the Big Sur to estimate potential PAH ranges.

The 2006 Day and the 2009 Station fires were remarkably similar in size and fire characteristics to the 2008 Basin Complex fire, with comparable geology, terrain, and vegetation. The Basin fire (665 km^2) burned primarily in chaparral and oak woodland at low-to-mid elevation and in ponderosa pine-dominated conifer stands at the highest elevations. The Day (657 km^2) and Station (668 km^2) fires burned across the same elevation and ecosystem gradients as the Basin fire, starting in chaparral shrublands and oak woodland savannahs, and burned into high-elevation stands of pine-dominated conifer (National Land Cover Database 2001, 2006). The Day and Station fires also burned with the same characteristics as the Basin and Chalk fires, *i.e.*, fuel-driven fires that burned with a normal distribution of fire severity over a month-long period. Stein *et al*. (2012) sampled multiple burned, unburned, natural, and urban watersheds downstream following the 2006 Day and the 2009 Station fires. They analyzed for concentrations and flux of known toxic constituents including PAHs. Flux was not measured in unburned natural watersheds, but was estimated to be four times greater than loads from urbanized watersheds. Strikingly, the authors compared the results of a well-tested runoff model of the entire Los Angeles River watershed (2,140 km^2) to their estimated loads from a single postfire storm event in a naturally vegetated watersheds, and found that runoff from burned watersheds transport 5%–40% of the total annual load of metals and 7%–35% of the total annual load of PAHs of the entire Los Angeles River.

Additionally, Hunsinger *et al*. (2008) found evidence of elevated PAH levels in shallow (<40 m) marine sediment cores collected in the nearshore following two 2003 wildfires in chapparal shrublands in southern California. These authors concluded that total PAH levels persist at high levels in nearshore areas such as the Big Sur coastline, primarily because of the contribution of carbon compounds derived from wildfires.

The genes selected in our study were based on a suite of genes transcribed in American mink (*Neovison vison*) experimentally exposed to crude oil (Bowen *et al*. 2007), and are thus sensitive indicators of subtle or chronic effects on an organism. We found subtle but significant differences in transcription between the Big Sur 2008 and Big Sur 2009 sea otters in 7 of the 13 target genes that were probably consistent with hydrocarbon exposure; 5 of these genes also had transcripts that differed significantly from clinically normal reference otters. In general, the Big Sur 2008 sea otters exhibited transcript profiles representative of recent hydrocarbon exposure (Bowen *et al*. 2007, Miles *et al*. 2012), which is consistent with the timing of sampling sea otters only a few weeks after the termination of the fires. The first substantial run-off of the season and winds likely carried high debris loads of ash and char coal into nearshore waters. By November 2009, these loads would have likely dissipated. Most notably, AHR transcription in 2008 otters was significantly higher than in 2009 otters. Transcription of CaM, a potential indicator of diet type, was higher in 2008, which could be a result of dietary shifts postfire. DRB transcription was significantly lower in 2008 otters; DRB can be down-regulated in the presence of hydrocarbons, conveying decreased ability to defend against pathogens. Also consistent with a hydrocarbon exposure, IL-18 and IL-10 transcriptions were significantly decreased in 2008 otters; both pro- and anti-inflammatory cytokines transcripts have been shown to be decreased subsequent to toxic exposures (Connon *et al*. 2012).

In general, gene transcription patterns in the 2008 Big Sur sea otters were indicative of molecular reactions to organic exposure, and decreased ability to respond to

pathogens, which may be consistent with short-term exposure to an organic substance. The AHR gene was strongly differentially transcribed in this study. 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). Previous, or even historic exposure to specific toxicants may not necessarily cause a sustained increase in the expression of AHR (Bowen *et al*. 2007, Miles *et al*. 2012), but can be associated with potentially severe 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).

Lower AHR transcription in 2009 Big Sur sea otters is consistent with the ephemeral nature of AHR signaling; sea otters living in close proximity to a historic oil spill had relatively normal levels of AHR while still exhibiting negative physiologic effects of exposure (Miles *et al*. 2012). Wildfire debris at Big Sur, including hydrocarbon-inducing ash and charcoal, likely continued to wash into the nearshore system throughout the winter of 2008–2009 with each subsequent storm event. Continued 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. 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).

Our results describe a physiologic perturbation to a sea otter population relative to a wildfire adjacent to their nearshore habitat. Sea otter samples taken prior to the Basin Complex Fire would have been beneficial to interpretation; however, the samples taken in 2009 demonstrated that sea otter transcript profiles generally returned to baseline levels more consistent with the reference sea otters than the transcripts analyzed in 2008 sea otters and provided a comparison highly suggestive of hydrocarbon exposure. Although the long-term population level effects of exposure to fire-generated hydrocarbon products remains to be seen, evidence exists for continued altered immune states in affected sea otters.

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