

## Original Contribution

# Differential Gene Expression Induced by Exposure of Captive Mink to Fuel Oil: A Model for the Sea Otter

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**Abstract:** Free-ranging sea otters are subject to hydrocarbon exposure from a variety of sources, both natural and anthropogenic. Effects of direct exposure to unrefined crude oil, such as that associated with the Exxon Valdez oil spill, are readily apparent. However, the impact of subtle but pathophysiologically relevant concentrations of crude oil on sea otters is difficult to assess. The present study was directed at developing a model for assessing the impact of low concentrations of fuel oil on sea otters. Quantitative PCR was used to identify differential gene expression in American mink that were exposed to low concentrations of bunker C fuel oil. A total of 23 genes, representing 10 different physiological systems, were analyzed for perturbation. Six genes with immunological relevance were differentially expressed in oil-fed mink. Interleukin-18 (IL-18), IL-10, inducible nitric oxide synthase (iNOS), cyclooxygenase 2 (COX-2), and complement cytolysis inhibitor (CLI) were down-regulated while IL-2 was up-regulated. Expression of two additional genes was affected; heat shock protein 70 (HSP70) was up-regulated and thyroid hormone receptor (THR) was down-regulated. While the significance of each perturbation is not immediately evident, we identified differential expression of genes that would be consistent with the presence of immune system-modifying and endocrine-disrupting compounds in fuel oil. Application of this approach to identify effects of petroleum contamination on sea otters should be possible following expansion of this mink model to identify a greater number of affected genes in peripheral blood leukocytes.

**Keywords:** differential gene expression, petroleum, sea otter, mink

## INTRODUCTION

The Southern sea otter (*Enhydra lutris nereis*) population has experienced recent dramatic declines (Bodkin et al.,

2002; USFWS, 2003). Because their ranges tend to be limited and concentrated near the coast, sea otters are vulnerable to runoff or shipping-related contamination with petroleum oil products (VanBlaricom and Jameson, 1982). The acute effects of petroleum oil exposure include disturbances in thermoregulation, respiration, and metabolism (Geraci and Williams, 1990; Rebar et al., 1995; Wil-

liams et al., 1995). These pathologies can be detected clinically, by hematological and serum chemical analyses, or at necropsy. Since the immediate effects of direct petroleum oil exposure are dramatic, the short-term impacts on individual or populations of sea otters in the spill area are relatively straightforward to record, monitor, or study. A number of studies have documented the long-term impacts of a catastrophic oil spill (Monson et al., 2000; Bodkin et al., 2002). These impacts may be a result of 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. Whatever the mechanism behind these long-term effects, the pathophysiological changes within an individual may be significant but subtle, and consequently undetectable using classical diagnostic methods. In fact, many of the studies investigating low-grade, long-term impacts of oil spills use statistical techniques to identify either changes in population demographics, patterns of mortality, reproductive efficiency, or survivability. While the conclusions from these studies are compelling, the supporting data are incomplete and complicated by confounding factors that also impact population demographics and survival.

Marine mammal toxicology has relied heavily on the identification of chemical contaminants within individual tissues as an indicator of toxic insult. Unfortunately, these assays are information-limited because the way xenobiotics affect the health of an individual is not assessed. Therefore, methods for measuring sensitive indicators of lingering, low-grade pathophysiological changes in oil-exposed individuals are urgently needed. Contemporary gene expression analysis used to identify an organism's genomic stress response to environmental contamination by individual chemicals or complex mixtures has the potential to transform marine toxicology research (Burczynski et al., 2000; Bartosiewicz et al., 2001). The advantage of using gene expression assays in marine mammal toxicology lies in the capability to measure the physiologic responses (acute or chronic) of an individual to toxic insults.

Microarray analysis and gene-specific quantitative real-time polymerase chain reaction (qRT-PCR) yields important information on the physiological mechanisms that orchestrate an integrated response to a variety of stressors (Marrack et al., 2000). The value of these novel technologies is that the up- or down-regulation of many genes, which provide the transcriptional messages important in mediating toxicological and immunological reactions, can be assayed from a single sample. This is ideal for wildlife

researchers where the amount of sample collected can be limiting. Gene expression analysis can be used to detect transcriptionally active genes that are up- or down-regulated by particular toxicants and this may give insights into changes in an animal's response to toxic insult.

The long-term goal of this study is to develop sensitive and specific markers that can be used to measure long-lasting pathophysiological changes associated with either acute or chronic low-grade exposure to petroleum oil. Since petroleum oil has multiple components, the toxic effects of exposure and ingestion are likely to be diverse and widespread within the body. For this reason, the utility of a single marker of sub-lethal oil-induced pathology would be limited. The development of molecular technique(s) capable of detecting toxin-specific patterns in gene expression would permit examination of animals for subtle alterations in multiple physiological processes. Such an approach would facilitate monitoring long-term effects of oil exposure in individual, free-ranging organisms.

Surrogate species are invaluable for defining immunologic changes associated with exposure to environmentally relevant chemical contaminants. Captive American mink (*Mustela vison*) have been successfully used as a model for sea otters to study the toxic effects of fuel oil (Mazet et al., 2000, 2001; Schwartz et al., 2004ab). Petroleum oil-induced perturbations were observed in both immune and endocrine systems. This article describes the further development of mink as a sensitive model for detecting petroleum oil-induced changes in gene expression. Captive ranch mink exposed to fuel oil were analyzed for alterations in gene expression by using a human microarray in combination with qRT-PCR. Such an approach would be useful for monitoring the long-term effects of an oil spill on the health of individual as well as populations of animals.

## METHODS

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### Animals and Oil Exposure Protocol

Ranch mink used in this study were part of a large fuel oil exposure experiment examining the chronic toxicological effects of bunker C fuel oil on the immune system. Full details of the exposure have been published elsewhere (Schwartz et al., 2004ab) and only information pertinent to the present study is described below. Animals (8-month-old males) were divided into two groups: one group ( $N = 9$ ) was maintained on a ranch feed (150–200 g/day) containing

500 ppm of Bunker C fuel oil for 113–118 days. The control group ( $N = 5$ ) was maintained on the same feed ration with mineral oil added instead of fuel oil. The concentration of fuel oil fed to the mink corresponded to the petroleum hydrocarbon concentrations measured in invertebrates sampled in the oiled Prince William Sound region 1 year after the *Exxon Valdez* spill (Mazet, 2001).

At the end of the exposure, venous blood from every animal was collected into cell-separation vacutainer tubes (8 ml) (CPT w/sodium citrate; Becton Dickinson, Franklin Lakes, NJ). Tubes were centrifuged at 1800g for 20 minutes. Isolated mononuclear cells were suspended in sterile PBS (phosphate buffered saline) containing 0.5 M EDTA (pH 7.4), centrifuged (250g, 8 minutes), resuspended in cryopreservation media (10% Dulbecco's modified Eagle's medium, 10% DMSO, and 80% fetal bovine serum), rate frozen at  $-80^{\circ}\text{C}$ , and then transferred to liquid nitrogen.

Animals were euthanized by  $\text{CO}_2$  asphyxiation following blood collection. Cells from the spleen obtained from each animal were flushed out with cell culture media, the mononuclear cells collected by density gradient centrifugation, and then cryopreserved in the same manner as PBMLs (peripheral blood mononuclear leukocytes).

### RNA Extraction and cDNA Synthesis

Total RNA was isolated from splenic mononuclear leukocytes and PBMLs using silica-based gel membranes combined with microspin technology (Qiagen, Valencia, CA) and stored at  $-70^{\circ}\text{C}$ . A standard cDNA synthesis was performed on 2 ( $\mu\text{g}$ ) of RNA template from each animal. Reaction conditions included 4 units reverse transcriptase (Omniscript®; Qiagen, Valencia, CA), 1 (M) random hexamers, 0.5 mM each dNTP, and 10 units RNase inhibitor, in RT buffer (Qiagen, Valencia, CA). Reactions were incubated for 60 minutes at  $37^{\circ}\text{C}$ , followed by an enzyme inactivation step of 5 minutes at  $93^{\circ}\text{C}$ , and stored at  $-20^{\circ}\text{C}$  until further analysis.

### Development of Mink-specific Quantitative PCR Primers

Microarray analysis was performed by Genome Explorations Inc. (711 Jefferson Ave., Suite 415, Memphis, TN). Mink-specific quantitative PCR systems were designed for: i) select genes identified by microarray as being differentially expressed between oiled and control mink; ii) genes representing six broad categories of biologically relevant

physiological systems; and iii) an endogenous control gene, ribosomal subunit S9. Degenerate primers were designed based upon multi-species alignments (GenBank) (Table 1). The six systems were selected based on petroleum oil's—or components of petroleum oil's—known effect on immune defense (Schwartz et al., 2004ab), cellular injury (Ghanem et al., 2006), signal transduction (Burchiel et al., 2004), xenobiotic and metal metabolism (Schwartz et al., 2004ab), tumorigenesis (Ramesh et al., 2004), and reproduction (Mazet et al., 2001). Briefly, degenerate primer pairs were utilized on cDNA generated from three mink spleen samples. PCR amplifications using these primers were performed on 20 ng of each cDNA sample in 50  $\mu\text{l}$  volumes containing 20–60 pmol of each primer, 40 mM Tris-KOH (pH 8.3), 15 mM KOAc, 3.5 mM  $\text{Mg}(\text{OAc})_2$ , 3.75  $\mu\text{g}/\text{ml}$  bovine serum albumin (BSA), 0.005% Tween-20, 0.005% Nonidet-P40, 200  $\mu\text{M}$  each dNTP, and 5U of Advantage® 2 Taq polymerase (Clontech, Palo Alto, CA). The PCR was performed on an MJ Research PTC-200 thermal cycler (MJ Research, Watertown, MA) and consist of 1 cycle at  $94^{\circ}\text{C}$  for 3 minutes, 40 cycles at  $94^{\circ}\text{C}$  for 30 seconds,  $60^{\circ}\text{C}$  for 30 seconds, and  $72^{\circ}\text{C}$  for 2 minutes, with a final extension step of  $72^{\circ}\text{C}$  for 10 minutes. The products of these reactions were electrophoresed on 1.5% agarose gels and visualized by ethidium bromide staining. Bands representing PCR products of the predicted size were excised from the gel, and extracted and purified using a commercially available nucleic acid-binding resin (Qiaex II Gel extraction kit, Qiagen).

Isolated fragments were ligated into a T/A type cloning vector (pGEM®-T Easy vector systems; Promega, Madison, WI). Following transformation, growth, and blue-white selection in competent cells (SE DH5 $\alpha$  competent cells, Life Technologies Inc, Rockville, MD), the DNA from positive clones was isolated. Nucleotide sequences of both strands were determined by dideoxy nucleotide methodology using an automated sequencer (Model 373; Applied Biosystems, Foster City, CA). Nucleotide sequences of the PCR products were analyzed using Align™ and Contig™ sequence alignment software programs (Vector NTI™; Informax Inc, North Bethesda, MD) and compared to known sequences using the NCBI BLAST program (Altschul et al., 1990), and the IMGT/HLA database (Robinson et al., 2001).

### Quantitative PCR

Real-time PCR systems for mink S9 and the genes of interest were run in separate wells. cDNA was examined using an intercalating fluorescent dye PCR (Bowen et al.,

**Table 1.** Degenerate Primer Sequences for Amplification of Mink Genes

Gene of interest	Forward primer	Sequence 5'-3'	Reverse primer	Sequence 5'-3'
Aryl hydrocarbon receptor	AHRF1	GGACAGAMAAGAAGGAAAGATG	AHRR1	GGTCTCTGAGTTRCAAATGATATAATC
Estrogen receptor beta	ERBF1	GGATATCACTATGGAGTCTGGTGG	ERBR1	CATCATCATGGAGGGCGTCGGTG
Glutathione-S-transferase	GLUF1	CCTGAATGCCAAGGAATCCGG	GLUR1	GCCAGATGAGGTAATCAATCATAG
UDP-glucuronyltransferase	UDPF2	GAGGACTCCACTGCAAACTGCG	UDPR2	GGGATCCCATGGTAGATTGCCCTC
Heat shock protein 90	HSP90F1	GCCTGAGGAAAGTGCACCAATGGA	HSP90R1	GATCACAAACCACCTTCTCTGCCAC
Heat shock protein 70	HSP70F1	ACCTGGGCACCACTACTCCTG	HSP70R1	GCTTGTCTGGCTGATGTCCTTCT
Interleukin-2	IL-2F1	CAAGTGCAGTCATTGCTGCAGGAT	IL-2R1	GTAAATCCATTTGTTTCAGAANTTCTACAG
Interleukin-12	IL-12F1	CMTCRTGGCCATRTGGAACTGGAG	IL-12R1	CACTGAATTTTCARATCAGTACTGATTGC
Interleukin-18	IL-18F1	GATGAARACCTGGAATCRGATYACT	IL-18R1	CATGTCWGGRACACTTCTYTGAA
Interleukin-10	IL-10F2	GACTTTAAGGGTTACCTGGGTTGC	IL-10R2	TCCACCGCCTTGCTCTTGTTTC
iNOS	INOSF1	CAGGAACTACCAGCTGACGG	INOSR1	GTGATGGCCGACCTGATGTTGC
TGFβ	TGFBF1	CAGTACAGCAAAGTCTCTGGCCC	TGFBR1	CTGCTCCACCCTGGGTTGCG
COX-2	COX-2F1	GAGCTCTTCTCTCTGTGCTGA	COX-2R1	CTTTGRCTGSGMGGATACAYCT
S9	S9F1	GTGGCCCGGARTGGGTTTG	S9R1	GGGYCTCTCYAARAAATCC
Metallothionein	metF1	AGCCTTCCACGTGCGCCTTATAG	metR1	GCACAGCAGCTGCACCTTSTCYG
Complement cytolytic inhibitor	CYTF1	ACGAGCTGCTAAAGTCTACCAGTG	CYTR1	ACTGAGGTGGTCTGTAAGCTCTTTG
HDCMB21P	HDCF1	ATGTTCTCCGACATCTACAAGATCC	HDCR1	CATGAAACCATCACCTGCAGGAAAC
DQA	DQAUD	CCAGTACACCCATGAATTTGATGG	DQALD	GAAACACAGTCACCTCAGGAACC
DRA	DRAU102	ATAAGTGGAGTCCCTGTGCTA	DRAL	CCCAGTGTCCACCCTTGCACTCATA
DRB	DRBU71	CGGGACSGAGGGGTTK	ZcDRB4L	CCACTTGGCAGGTGTAGACCTCTCC
Interleukin-6	IL-6F1	CYCTGGGRCTGCTYCTGGTG	IL-6R1	CTGACCAGARRRGGGAATGCC
Thyroid hormone receptor	THRBF1	GGACAAACCGAAAGCACTGTCCAG	THRBR1	GGAATATYAGCTAAAGTCCAAAGTGG
Cold inducible RNA-binding protein	CIRBPF1	TTCTGAGTGTAGTGTGGTAGGACCC	CIRBPR1	AGTGGCTGAGGAAATCTTGTACGCA
FoxP3	FOXP3F1	CAGGCACCTCCTCCAGGACAG	FOXP3R1	CTCCCTGGACACCCCATTTCCAG

2006). Each reaction contained 500 ng DNA in 25  $\mu$ l volumes with 20 pmol SSP, Tris-Cl, KCl,  $(\text{NH}_4)_2\text{SO}_4$ , 2.5mM  $\text{MgCl}_2$  (pH 8.7), dNTPs, HotStar Taq DNA Polymerase (Quantitect SYBR Green PCR Master Mix; Qiagen, Valencia, CA), and 0.5 units uracil-N-glycosylase (Roche, Indianapolis, IN). Amplifications were performed in an ABI 7300 (Applied Biosystems) under the following conditions: 2 minutes at 50°C, followed by 15 minutes at 95°C, and 35 cycles of 94°C for 30 seconds, 58°C for 30 seconds, and 72°C for 30 seconds, with a final extension step of 72°C for 10 minutes. Reaction specificity was monitored by melting curve analysis using a final data acquisition phase of 60 cycles of 65°C for 30 seconds and verified by direct sequencing of randomly selected amplicons (Bowen et al., 2006).

Gene expression was analyzed by relative quantitation, using the comparative  $C_T$  (cycle threshold) method; values are expressed relative to a calibrator (weakest signal of the normalized values) (Bowen et al., 2006). Amplification efficiencies of S9 and the other genes of interest (GOI) were determined using six dilutions of cDNA preparations (run in triplicate).

Statistical analysis was performed in NCSS (Number Cruncher Statistical System). Differences between GOI transcription were analyzed with standard *t*-tests. Differences were considered significant if  $P \leq 0.05$ .

## RESULTS

### Quantitative PCR

Twenty-three genes, representing approximately 10 physiological pathways and one endogenous control gene, were amplified and sequenced (Table 2). Genes were divided into broad functional categories based upon biological relevance, i.e., immunomodulation, inflammation, cellular stress-response, cytoprotection, tumor suppression, reproduction, xenobiotic and metal metabolism, antioxidant metabolism, and cell–cell adhesion.

Quantitative real-time PCR systems were developed and optimized based upon these previously unpublished sequences (Table 3). Gene expression differed between fuel oil-fed versus control mink; these differences were not always evident in cells derived both from blood and spleen (Table 4). Decreased expression of 11 genes was identified in oil-fed mink; results were significant for 6 of these genes (interleukin-18,  $P = 0.002$ ; interleukin-10,  $P = 0.04$ ;

inducible nitric oxide synthase (iNOS),  $P = 0.01$ ; cyclooxygenase-2 (COX-2),  $P = 0.047$ ; complement cytolysis inhibitor (CLI),  $P = 0.05$ ; thyroid hormone receptor,  $P = 0.01$ ). Increased expression of eight genes was identified in oil-fed mink; results were significant for two of these genes (heat shock protein 70 (HSP70),  $P = 0.02$ ; interleukin-2 (IL-2),  $P = 0.04$ ). Gene expression was virtually identical between fuel oil-fed and control mink in four of the genes (heat shock protein 90 [HSP90]; transforming growth factor-beta [TGF $\beta$ ]; and major histocompatibility complex class II DQA and DRB).

## DISCUSSION

The pathophysiologic effects of oil exposure undoubtedly impact multiple organ systems. Ingestion of low concentrations of petroleum hydrocarbons has been associated with reproductive failure, genotoxicity, hematological changes, or impaired immune function (Bickham et al., 1998; Mazet et al., 2000, 2001; Burchiel and Luster, 2001; Schwartz et al., 2004a,b). Exposure to xenobiotics also has been implicated in compromised immunological health as well as increased incidence of disease (Harvell et al., 1999).

Quantitative real-time PCR was employed as a sensitive and specific assay for detecting fuel oil-induced changes in gene expression in mink. Our study suggests that animals exposed to petroleum oil have alterations in multiple physiological pathways including immunomodulation, inflammation, cytoprotection, calcium regulation, cellular stress-response, metal metabolism, xenobiotic metabolizing enzymes, tumor suppression, reproduction, antioxidant enzymes, and cell–cell adhesion. A variety of immunologically relevant genes were differentially expressed in fuel oil exposed versus control mink. The T cell cytokine, IL-2, was up-regulated in mink exposed to fuel oil. This would suggest some level of increased T cell activation as IL-2 is classically considered a T cell growth factor (Kindt et al., 2007). Expression of the regulatory cytokines, IL-10 and IL-18, were decreased in exposed mink. IL-10 is largely of T cell origin and is typically considered an antiinflammatory mediator due to suppression of macrophage activation, whereas IL-18 is produced by macrophages and dendritic cells and is considered a proinflammatory cytokine by inducing production of interferon gamma by T cells (Kindt et al., 2007). While it is not possible to suggest a specific impact of these perturbations, due to the pleiotropic and often redundant

**Table 2.** American Mink (*Mustela vison*) Gene Sequences Amplified with Degenerate Primers

Gene of interest	<i>Mustela vison</i> gene sequence 5'-3'
Aryl hydrocarbon receptor	CGATAAGTGTCTGTAGTATTCAGGCTTCTTACCAAAGATAATCGATGGCCCTGGGTTTCAGTCTAATGCACGCTTAGTGA
Estrogen receptor beta	AATCAGTGTACAATAGATAAGAAATCGGGCAAGAGCTGCCAGGCTGCCAGGCTCCGGGCTCCGGAAGTGTCTATGAAGTGG GGATGGTGAAGT
Glutathione-S-transferase	TGATCTACGAATCTGGCATCACCTGTGAGTACCTGGATGATGTATATCCAGGAAAGAAGCTATT
UDP-glucuronyltransferase	GCCAGATGACCCCTATGAGAAAGCTCGTCAGAAGATGGTGTGAGTTT AGAGTTGTCCAGAGCTCTGGAGAAAATGGTATTGTGGTGTTCACACTAGGGTCTATGATCACAACCT GCCAGAGGAAAAAGCCAATGTGATTCAGCCCTTGCACAGATTCACAAAAGGTTCTATGGAGATTGTCTGGC AAGAAAACAGACAACTTAGGACCAAATCTCGGCTGTACTGGATCCCCCAGAAATGACCTTCTTGGTCAATCCAAAAACCAA GCCTTTGTAACTCATGGTGGAAACCAATGGCATCTATGAGGCAATCTACCATGGG
Heat shock protein 90	CCCAGGAACGCACTTGACTCTAGTGGACACAGGCATTTGGCATGACCAAGCCGATCTCATAAATAATTTGGGAACAATTTGCCAA GTCCGGAAACTAAAGCAATTCAT
Heat shock protein 70	TCGAGCGTAGCGGTGTCCGCCGAGTTCGGGACCCCGTGGTGCAGTCGGACATGAAGCACTGGCCCTTCCAGG TGATCAGCGATGGCGATAAGCCCAAGGTGCAGGTGAGCTACAAGGA
Interleukin-2	CCATAGTACTCAACTTTGGAGGAGTGTATATTTAGCTCAAGCAAAAACCTTTCACCTTGACAGACATCAAGGAACATAAT GAGCAATATCAATGTAACTCTGAAACTAAAGGATCTGAAACAAGA
Interleukin-12	TGGTGGTCTCACCTGCCAAAACCCCTGAAGAAATGACATCACCTGGACCTCGGACCAGAGCAGTGACGTCTTAGGC TCCGTTAAAACCTCTGACCAITCAAGTCAAAGAAATTTGGAGATGCTGGCCGGTATACCTGTCATAAAGGAGGCAAG GTTCTGAGCCATTCGCTCTGTGATTCACAAAAGGAAAGATGGAATTTGGTCCACTGATATCTTA AAGGAACAAGAAAGAAATCCAAAATAAGATCTTTCTAAAATGTGAGGCAAGAAATTAITCTGGACGTTTTCACCTGCG TGGTGGCTGACAGGAATCAGTACTGATC
Interleukin-18	ATCATAACGAAATTTGACGACCAAGTTCTCTTCGTTAACGAGGGCAATCAACCAAGTGTTCGAGGATATGCCT GATTTGACTGTACAGAAAACCGCATCCCATACCATATTTATCATAAAATATGTATAAAGATAAGCTCACCCAGAGGTCTGG CGGTAAACCATCTCTGTGATGTAAACAGAAATGTCTACTCTGCTGTAAAGAACAAAACATAATTCCTTTAAGGAAATGAGACCTC CAGACAGTATCAGCGACGAAGGAATGACATCATATTTCTTCAAAGAAAGTGT
Interleukin-10	CAGTTTTACTTTGGAGGAGTGTATGCCCAAGCCGAGAAACCAAGCCCTGAAAGTCAAGGAGCTCGTGAACCT CGCTGGGGGAAAAGCTGAAAGCCCTGCGGCTGAGGCTGCGGGCTGTCATCGATTTCTGCCCTGTGAGAACAA GAGCAAGGCCGTGGAA
iNOS	CCCCGCTGCATCGGGAGGATCCAGTGGTCCAACTGCAGGTCTTCGACGCCCGGAGCTGTTCACITGCCAAAGGAA ATGTTCCAGACACATCTGCAGACACCTGGGTTATGCCACCAACAAATGGCAACATCAGGTTCGGCCATCAC
TGF- $\beta$	AGACATCAGCTGTCTGCTTTCCGGGCAAGTTGGCCCTGCTGAGGCTCAAAGTTAAAAGCGGAGCAGCACCTGGAGCTGTAC CAGAAATA
COX-2	TTGCATTTTGCCAGCACCTTCACCCATCAATTTTTCAAGACAGATCATAAGCGGAGGCCAGGTTTCACCAAAG

Table 2. Continued

Gene of interest	<i>Mustela vison</i> gene sequence 5'-3'
S9	GACTCCATCCTTCTCGTCCGCTGGATTCCAGAAAGCACATTGACTTCTCCTTGCGGTCTCCGGCTATGGGGGTGGCCGGCCC AGGCCGGTGAAGAGGAAGAAATGCCAAAGAAAGGGCCAGGGA
Metallothionein	AGAGCTGCTGCTCCTGCTGCCAGTGGGCTGTGCCAAGTGTGCCAGGGGTGTGTTTGCAAAAGGGA
Complement cytotoxicity inhibitor	CGTCTCCTGCTGAGCAGCTGGACGAGCAATTTAGTGGGTGCCAGCTGGCGAATCTCACTCA GACTGAGGACCCGTTCTATCTCCAGGTACGACGCGTGTGAGTTCCAGACTTCTGACTCCAGTGTCTCCCTCIGGGGCTC ACTGAGGTGGTGTGAAGCTCTTTGA
HDCMB21P	CTGTGCTGGAGGTGGAGGGGAGATGGTCAAGTGGACAGAGGGTAACATTGATGACTC GCTCATTTGGTGGAAATGCCTCCGCTGAAGGCCCGGAGGGAAGGTACCGAAAGCACAGTCATCACTGGTGT
DQA	GATATTGTCATGAACCATCACCTGCAGGAAACA CAGCTTGATCACAGGTTCACTGAGAACTTGGCAATAGCGAAACAATACTTGAACAATCCTGACTAAACGGGCTCCAA CTATACCGCTGCTA
DRA	ATCAGGCCATGTGGACTGGGAGAGCCCCAACCCCTCATCTGCTTCAITTGATAAGTTCTCCCCAACCAGTGATCAATGTCACGTGG CTTGGAAATGGAACCCCTGTCAACCACA
DRB	GGATGACAGCTCTGACATTTGATTTGATGGTGTGACTCCCTCTTGGCTTGGGCCAGGGACACCCACCAC ATTTCTGKTYCTGRYGMGTGGAAATGCTACTTACCAAACGGCAACGGGGTGGGTTCTTGGAKAGGTATTTCTATAAC GGCGAGGAGTTC
Thyroid hormone receptor	STGCGCTTCGACACGACGTCGTGGGGAGTACCCKGSCGGTGACCCGAGCTGGGGGGCCGGACGCTCCGTACTGGAAACAGCC AGAAAGGACTTYGTGGAGCRGAMGGGGCCGAGGTGGACACGTACTGCAGACACAACTACGGGGTGGGTGAGAGCTTCAAG GTGCAGGGGGAGTGGAGCCTATAGTGACWGTGTATCCTKYGAAGACCCAGCCCTGAAGCACCAACCTCCTGGTCTG CTCTGTGAATGGTTTCTATCCAGGCCACATTGAAAGTCAAGTTCGGGAAATGGCCAGGAAGAGGAGTCTGGGGTCTGTGTC CACAGGTCTGATCCATAATGGAGACTGGACCTTCCAGACCCCTGGTGAATG CTGGAGACAGTTCCCTCAGAGTGGAGAGGTCTACACCT
Cold inducible RNA-binding protein	ACTGGTAGCTAGTAGGAATGTCTGAAGCCTGCCCTCCATAGGAAGAGCCATGGGGAGAGGGCAGGGCAITTGAAAAATGA ACAGTGTCAACACATCTCATCCAGGCCACTTGGACTTAGCTCAATATTCC AGGGCTCCGGTGGCTGGCCCTGGCGTGAACGGCCCTTCTATGGTATGATGACCCGAGTAGATC GCAGAGTCCCTCGAGAGGTCTTGAAAATGTTTATATATTGTCCTTTTTACTGGAAAGACGTATGCATATCTTATTGATGTTG TATTTGAAAGTGGCTGAGGAATTTCTGTACGCAAA
FoxP3	CCGCTGGCCATCCTGGAAAGCTCCCGAGAAGCAGCGGACACTCAAGAGACTTACCAGTGTTCACGGGCAITGT TGGCTTCTTCAGAAACCAACCTGCCACCTGGAAAAACGGCCATCCGCCACAACTGA

**Table 3.** Mink-specific Primer Pairs

Gene of interest	Derivation	Forward primer	Sequence 5'-3'	Reverse primer	Sequence 5'-3'
Aryl hydrocarbon receptor	Alignment	MvAHRF1	GTGCTGAGTACCATAATACGGATGA	MvAHRR1	GTTCAGTCTAATGCACGCCTTAGTG
Estrogen receptor beta	Alignment	MvERBF1	GCAITCAAGGACATAAACGATTACAT	MvERBR1	GTGGCTCCCGGAGGAAAGGT
Glutathione-S-transferase	Alignment	MvGLUF1	CTGGTCCCAGITTCGGAAAACAG	MvGLUR1	CTAAGGTCCCATCTTTGGTAAAC
UDP-glucuronyltransferase	Microarray	MvUDPF2	GAGCTCTGGAGAAAATGGTATTGT GGTGTTTACA	MvUDPR2	CCGAGTATTTGGTCTCTAAAGTTGTCGTG GTTCTCTG
Heat shock protein 90	Alignment	MvHSP90F1	CCAAAGTTGGACAGCGGTAAGAG	MvHSP90R1	GGAGGCTCTTCAGGCTGGTGC
Heat shock protein 70	Alignment	MvHSP70F1	CCAGGTGGGCTGAACCCCGC	MvHSP70R1	CAAGGTGCAGGTGAGCTACAAGG
Interleukin-2	Alignment	MvIL-2F1	GGCCACAGAAATTGACTCATCTTCA	MvIL-2R1	CTGAAACTAAAGGGATCTGAAACAAG
Interleukin-12	Alignment	MvIL-12F1	GCCATTCGCTCCTGCTGATTAC	MvIL-12R1	GTTTCACCTGCTGGTGGCTGAC
Interleukin-18	Alignment	MvIL-18F1	GTACAGAAAACGCATCCCATACC	MvIL-18R1	CCTTTAAGGAAATGAGACCTCCAG
Interleukin-10	Alignment	MvIL-10F2	GACTTTAAGRGTACCTGGGTTGC	MvIL-10R2	TCCACSGCCTTGCTCTTRTTYTC
iNOS	Alignment	MvINOSF1	CCGCTGCATCGGAGGATCC	MvINOSR1	GCAACATCAGGTGGCCCATCAC
TGFβ	Alignment	MvTGFBF1	GCTCTTCAACACATCGGAGCTC	MvTGFBR1	CAGCAACGATTCTGGCGCTAC
COX-2	Alignment	MvCOX-2F1	CAITTCCTGATCCCGAGGGAC	MvCOX-2R1	GACTGGGCCATGGGGTGGAC
S9	Alignment	MvS9F1	CCAGCGCCACATCAGGGTCCG	MvS9R1	GAATGCCAAGAAGGGCCAGGG
Metallothionein	Microarray	MvmetF1	GAGCTGCTGCTCCTGCTGCCCC	MvmetR1	CCAGGGCTGTGTTTGCAAAGGG
Complement cytolysis inhibitor	Microarray	MvCYTF1	GCTGGACGAGCAGITTAGCTGG	MvCYTR1	CCAGTGTCCCTCTGGCGTC
HDCMB21P	Microarray	MvHDCF1	CTGTGCTGGAGGTGGAGGGG	MvHDCR1	CAGTCATCACTGGTGTGATATTG
DQA	Alignment	MvDQAF1	CTGTCTGGCAGCTGCCTGTGTT	MvDQAR1	CCAATGAGGTCTCTGAGGTGAC
DRA	Alignment	MvDRAF1	CACCAATGTACCTCCGGAGGTG	MvDRAR1	GGAGTCTGGAGACAGTCTTCC
DRB	Alignment	MvDRBF1	CGGCGAGTGGAGCCTATAGTG	MvDRBR1	CGGAATGGCCAGGAAGAGGAG
Interleukin-6	Alignment	MvIL-6F1	CCTGCAGTTCAGCCTGAGGGC	MvIL-6R1	CATAAGTTATGTGCCCAATGGACAG
Thyroid hormone receptor	Alignment	MvTHRBF1	GGACAAAACCGAAGCAGTGTCCAG	MvTHRBR1	GGAATATYGAGCTAAGTCCAAGTGG
Cold inducible RNA-binding protein	Microarray	MvCIRBPF1	TTCTGAGTGTAGTGTGGTAGGACCC	MvCIRBPR1	TGCGTACAAGAATTCCTCAGCCACT
FoxP3	Alignment	MvFOXP3F1	CCGCTGGGCCATCCTGG	MvFOXP3R1	CAGGTGTGGGGGATGGC



**Table 4.** Differential Gene Expression in Oil-fed Mink<sup>a</sup>

Gene of interest	Spleen		CPT		Gene function	
	Average normalized C <sub>T</sub> values		Average normalized C <sub>T</sub> values			
	Oiled mink	Control mink	Oiled mink	Control mink		P-value
Aryl hydrocarbon receptor	15.78	14.88	16.13	15.1	0.19	Responds to classes of environmental toxicants including polycyclic aromatic hydrocarbons, and polyhalogenated hydrocarbons (Oesch-Bartlomowicz and Oesch, 2005)
Estrogen receptor beta	14.83	14.86	14.29	14.28	0.47	Endocrine-alterations in hormone synthesis, transport, receptor interaction, metabolism, excretion, or feedback regulation (Dahlman-Wright et al., 2006)
Glutathione-S-transferase	7.18	6.52	7.83	7.88	0.41	Important role in the detoxication and metabolism of many xenobiotic and endobiotic compounds (Armstrong et al., 1993)
UDP-glucuronyltransferase	22.25	21.50	NA	NA		Catalyzes the glucoronidation of xenobiotic compounds (Daidoji et al., 2005)
Heat shock protein 90	2.81	3.12	4.03	4.02	0.44	Produced in response to thermal or other cellular stress (Tsan and Gao, 2004)
Heat shock protein 70	2.57	3.8	8.13	7.6	0.10	Produced in response to thermal or other cellular stress (Tsan and Gao, 2004)
Interleukin-2	14.66	16.24	17.67	18.4	0.16	T cell growth factor (Kindt et al., 2007)
Interleukin-12	12.7	13.3	17.93	18.37	0.43	Proinflammatory cytokine (Kindt et al., 2007)
Interleukin-18	5.13	4.08	6.91	6.54	0.29	Proinflammatory cytokine (Kindt et al., 2007)
Interleukin-10	16.22	15.26	21.08	20.52	0.34	Antiinflammatory cytokine (Kindt et al., 2007)
iNOS	16.91	15.08	18.60	20.00	0.22	Induced upon macrophage activation (Kindt et al., 2007)
TGFβ	5.46	5.22	5.66	5.8	0.5	Antiinflammatory cytokine (Kindt et al., 2007)
COX-2	7.08	7.18	9.03	7.42	0.047*	Induced upon macrophage and neutrophil activation (Kindt et al., 2007)
S9	NA	NA	NA	NA		18S ribosomal subunit/housekeeping gene
Metallothionein	7.32	8.04	6.84	7.34	0.21	Modulates the bioavailability of physiological cations and the toxicity of heavy metals, and modulates immune functions (Andrews, 2000)
Complement cytotoxicity inhibitor	8.82	7.86	8.68	8.44	0.25	Interferes with cytolytic function of the membrane attack complex in the complement cascade (Jenne and Tschoopp, 1989)

Table 4. Continued

Gene of interest	Spleen		CPT		Gene function	
	Average normalized $C_T$ values		Average normalized $C_T$ values			
	Oiled mink	Control mink	Oiled mink	Control mink		$P$ -value
HDCMB21P	-0.49	-1.06	0.07	0.13	0.13	Translationally controlled tumor protein is implicated in cell growth, cell cycle progression, malignant transformation, and in the protection of cells against various stress conditions and apoptosis (Bommer and Thiele, 2004)
DQA	3.2	3.38	0.47	4.56	0.28	Binding of pathogens/initiation of immune response (Kindt et al., 2007)
DRA	4.28	3.88	0.16	5.46	0.31	Binding of pathogens/initiation of immune response (Kindt et al., 2007)
DRB	0.79	1.16	0.45	1.82	0.27	Binding of pathogens/initiation of immune response (Kindt et al., 2007)
Interleukin-6	9.36	9.26	0.42	13.16	0.5	Proinflammatory cytokine (Kindt et al., 2007)
Thyroid hormone receptor	15.39	15.02	0.16	17.01	0.01*	Hormone-activated transcription factors bind DNA in the absence of hormone, usually leading to transcriptional repression (Tsai and O'Malley, 1994)
CIRBP	9.46	9.78	0.33	9.74	0.44	Cold-shock protein (Nishiyama et al., 1997)
Fox FP3	11.12	11.28	0.32	10.68	0.32	Selectively expressed in a subpopulation of T cells with regulatory activity (Ziegler, 2007).

\*Standard  $t$ -tests were performed and statistical significance ( $P < 0.05$ ) indicated by an \*.  $C_T$  values are inversely proportional to the amount of mRNA present.

activity of cytokine activity, it is ample evidence of an immunologic insult. Two additional genes of immunologic interest, iNOS and COX-2, were down-regulated in fuel oil-fed mink. Both of these genes are induced upon activation of phagocytic cells, iNOS being associated with production of a bacteriocidal environment and COX-2 being integral in production of proinflammatory prostaglandins (Kindt et al., 2007). Thus, taken together, a reduction of the expression of these two genes would support compromised activity of macrophages and neutrophils. The differential expression of complement cytotoxicity inhibitor was initially observed using the human microarray and verified to be down-regulated by quantitative PCR. The product of this gene has been described as interfering with the cytolytic function of the membrane attack complex in the complement cascade (Jenne and Tschoopp, 1989). Again, while the significance of this perturbation is unknown, it provides additional evidence that ingestion of fuel will impact the immune system.

Two additional genes were identified with altered expression. HSP70 was up-regulated in fuel oil-fed mink. HSP70 is an intracellular molecular chaperone and is typically up-regulated by cells in response to an insult (Tsan and Gao, 2004). Up-regulation of this gene is good evidence that the ingestion of fuel oil resulted in cellular stress. Thyroid hormone receptor, a nuclear membrane receptor tightly associated with chromatin, was down-regulated. This receptor is a DNA-binding protein that regulates gene expression (Tsai and O'Malley, 1994). While the potential impact of this specific perturbation is unknown, its altered expression would be consistent with the presence of endocrine-disrupting compounds in fuel oil.

Results from quantitative PCR differed between samples taken from spleen and CPT tubes. While differential gene expression was most evident in spleen, the identification of two affected genes in the peripheral blood is encouraging; peripheral blood provides a much more accessible sample, as compared to internal tissues, when sampling free-ranging animals. The data generated in this project provides the justification for expansion of this mink model to identify additional genes affected in peripheral blood leukocytes from animals exposed to petroleum or other contaminants. Such future efforts will be complemented by the recent development of specialized blood collection tubes permitting "immediate" fixation of leukocyte mRNA upon blood collection that will facilitate identification of gene transcripts with short half-lives, the latter being readily degraded during routine transport of

necropsy, biopsy, and/or blood samples to the laboratory for processing.

The need to develop molecular tools for evaluating physiological, biochemical, and histopathological effects of chronic exposure to petroleum oil and other xenobiotics in the marine environment is obvious (Peterson et al., 2003). The quantitative real-time PCR assay developed in this study for detecting petroleum oil-induced changes in gene expression in mink provides a framework for monitoring the effects of sublethal levels of contaminants and for facilitating the assessment of ecosystem health.

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## REFERENCES

- Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ (1990) Basic local alignment search tool. *J Mol Biol* 215:403–410
- Andrews GK (2000) Regulation of metallothionein gene expression by oxidative stress and metal ions. *Biochem Pharmacol* 59:95–104
- Armstrong RN, Gilliland GL, Ji X, Johnson WW, Liu S (1993) Crystallographic and mechanistic studies of class mu glutathione-S-Transferases In: Tew KD, Pickett CB, Mantle TJ, Mannervik B, Hayes JD (editors), *Proceedings of the International Meeting on Structure and Function of Glutathione Transferases*, Boca Raton, FL: CRC Press, pp 87–99
- Bartosiewicz M, Penn S, Buckpitt A (2001) Applications of gene arrays in environmental toxicology: fingerprints of gene regulation associated with cadmium chloride, benzo(a)pyrene, and trichloroethylene. *Environmental Health Perspectives* 109:71–74
- Bickham JW, Rowe GT, Palatnikov G, Mekhtiev A, Mekhtiev M, Kasimov RY, Hauschultz DW, Wickliffe JK, Rogers WJ (1998) Acute and genotoxic effects of Baku Harbor sediment on Russian sturgeon, *Acipenser gueldensteidti*. *Bull Environ Contam Toxicol* 61:512–518
- Bodkin JL, Ballachey BE, Dean TA, Fukuyama AK, Jewett SC, McDonald L, et al. (2002) Sea otter population status and the process of recovery from the 1989 'Exxon Valdez' oil spill. *Marine Ecology Progress* 241:237–253
- Bommer UA, Thiele BJ (2004) The translationally controlled tumour protein (TCTP). *Int J Biochem Cell Biol* 36:379–385
- Bowen L, Aldridge B, Beckmen K, Gelatt T, Rea L, Burek K, et al. (2006) Differential expression of immune response genes in Steller sea lions: an indicator of ecosystem health?. *EcoHealth* 3:109–113

- Burchiel SW, Luster MI (2001) Signaling by environmental polycyclic aromatic hydrocarbons in human lymphocytes. *Clin Immunol* 98:2–10
- Burchiel SW, Lauer FT, McDonald JD, Reed MD (2004) Systemic immunotoxicity in AJ mice following 6-month whole body inhalation exposure to diesel exhaust. *Toxicology and Applied Pharmacology* 196:337–345
- Burczynski ME, McMillian M, Ciervo J, Li L, Parker JB, Dunn RT II, et al. (2000) Toxicogenomics-based discrimination of toxic mechanism in HepG2 human hepatoma cells. *Toxicological Science* 58:399–415
- Dahlman-Wright K, Cayailles V, Fuqua SA, Jordan VC, Katzenellenbogen JA, Korach KS, et al. (2006) International Union of Pharmacology. LXIV. Estrogen receptors. *Pharmacological Reviews* 58:773–781
- Daidoji T, Gozu K, Iwano H, Inoue H, Yokota H (2005) UDP-glucuronosyltransferase isoforms catalyzing glucuronidation of hydroxypolychlorinated biphenyls in rat. *Drug Metabolism and Disposition* 33:1466–1476
- Geraci JR, Williams TD (1990) Physiologic and toxic effects on sea otters. In: Geraci JR, St. Aubin DJ (editors), *Sea Mammals and Oil. Confronting the Risks* San Diego, CA: Academic Press, pp 211–221
- Ghanem MM, Batelli LA, Mercer RR, Scabilloni JF, Kashon ML, Ma JY, et al. (2006) Apoptosis and Bax expression are increased by coal dust in the polycyclic aromatic hydrocarbon-exposed lung. *Environmental Health Perspectives* 114:1367–1373
- Harvell CD, Kim K, Burkholder JM, Colwell RR, Epstein PR, Grimes DJ, Hofmann EE, Lipp EK, Osterhaus ADME, Overstreet RM, Porter JW, Smith GW, Vasta GR (1999) Emerging marine diseases-climate links and anthropogenic factors. *Science* 285:1505–1510
- Jenne DE, Tschopp J (1989) Molecular structure and functional characterization of a human complement cytotoxicity inhibitor found in blood and seminal plasma: identity to sulfated glycoprotein 2, a constituent of rat testis fluid. *Proc Natl Acad Sci* 86:7123–7127
- Kindt TJ, Goldsby RA, Osborne BA, Kuby J (2007) *Immunology*. 6th ed. New York: W.H. Freeman and Company
- Marrack P, Mitchell T, Hildeman D, Kedl R, Teague TK, Bender J, et al. (2000) Genomic-scale analysis of gene expression in resting and activated T cells. *Current Opinions in Immunology* 12:206–209
- Mazet JK, Gardner IA, Jessup DA, Lowenstine LJ, Boyce WM (2000) Evaluation of changes in hematologic and clinical biochemical values after exposure to petroleum products in mink (*Mustela vison*) as a model for assessment of sea otters (*Enhydra lutris*). *American Journal of Veterinary Research* 61:1197–1203
- Mazet JA, Gardner IA, Jessup DA, Lowenstine LJ (2001) Effects of petroleum on mink applied as a model for reproductive success in sea otters. *Journal of Wildlife Diseases* 37:686–692
- Monson DH, Doak DF, Ballachey BE, Johnson A, Bodkin JL (2000) Long-term impacts of the Exxon Valdez oil spill on sea otters, assessed through age-dependent mortality patterns. *Proceedings of the National Academy of Sciences of the United States of America* 97:6562–6567
- Nishiyama H, Higashitsuji H, Yokoi H, Itoh K, Danno S, Matsuda T, Fujita J (1997) Cloning and characterization of human CIRP (cold-inducible RNA-binding protein) cDNA and chromosomal assignment of the gene. *Gene* 204:115–120
- Oesch-Bartlomowicz B, Oesch F (2005) Phosphorylation of cytochromes P450: first discovery of a posttranslational modification of a drug-metabolizing enzyme. *Biochemical and Biophysical Research Communications* 338:446–449
- Peterson CH, Rice SD, Short JW, Esler D, Bodkin JL, Ballachey BE, Irons DB (2003) Long-term ecosystem response to the Exxon Valdez oil spill. *Science* 302:2082–2086
- Ramesh A, Walker SA, Hood DB, Guillen MD, Schneider K, Weyand EH (2004) Bioavailability and risk assessment of orally ingested polycyclic aromatic hydrocarbons. *International Journal of Toxicology* 23:301–333
- Rebar AH, Lipscomb TP, Harris RK, Ballachey BE (1995) Clinical and laboratory correlates in sea otters dying unexpectedly in rehabilitation centers following the Exxon Valdez oil spill. *Veterinary Pathology* 32:346–350
- Robinson J, Waller M, Parham P, Bodmer JG, Marsh SGE (2001) IMGT/HLA Database—a sequence database for the human major histocompatibility complex. *Nucleic Acids Research* 29:210–213
- Schwartz JA, Aldridge BM, Lasley BL, Snyder PW, Stott JL, Mohr FC (2004) Chronic fuel oil toxicity in American mink (*Mustela vison*): systemic and hematological effects of ingestion of a low-concentration of bunker C fuel oil. *Toxicology and Applied Pharmacology* 200:146–158
- Schwartz JA, Aldridge BM, Stott JL, Mohr FC (2004) Immunophenotypic and functional effects of bunker C fuel oil on the immune system of American mink (*Mustela vison*). *Veterinary Immunology and Immunopathology* 101:179–190
- Tsai MJ, O'Malley BW (1994) Molecular mechanisms of action of steroid/thyroid receptor superfamily members. *Annu Rev Biochem* 63:451–486
- Tsan M, Gao B (2004) Cytokine function of heat shock proteins. *American Journal of Physiology—Cell Physiology* 286:C739–C744
- U.S. Fish, Wildlife Service (USFWS) (2003) *Final Revised Recovery Plan for the Southern Sea Otter (Enhydra lutris nereis)*. Portland, OR: USFWS
- VanBlaricom G, Jameson RJ (1982) Lumber spill in central California waters: implications for oil spills and sea otters. *Science* 215:1503–1505
- Williams TM, McBain JF, Tuomi PA, Wilson RK (1995) Initial clinical evaluation, emergency treatments, and assessment of oil exposure. In: Williams TM, Davis RW (editors), *Emergency Care and Rehabilitation of Oiled Sea Otters: A Guide for Oil Spills Involving Fur-bearing Marine Mammals* Fairbanks, AK: University of Alaska Press, pp 45–57
- Ziegler SF (2007) FOXP3: not just for regulatory T cells anymore. *European Journal of Immunology* 37:129–138