EcoHealth 4, 298–309, 2007
DOI: 10.1007/s10393-007-0113-5

ECOHEALTH



*Original Contribution*

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

Lizabeth Bowen,[1,4] Federica Riva,[1] Chuck Mohr,[2] Brian Aldridge,[3] Julie Schwartz,[2] A. Keith Miles,[4] and Jeffrey L. Stott[1]

[1]*Laboratory for Marine Mammal Immunology, School of Veterinary Medicine, Department of Pathology, Microbiology and Immunology, University of California, One Shields Avenue, Davis, CA, 95616, USA*
[2]*School of Veterinary Medicine, Department of Pathology, Microbiology and Immunology, University of California, Davis, CA, 95616, USA*
[3]*Department of Veterinary Clinical Sciences, The Royal Veterinary College, The University of London, Hertfordshire, AL9 7TA, UK*
[4]*Western Ecological Research Center, Davis Field Station, U.S. Geological Survey, Davis, CA, USA*

**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 neresis)* 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-

Published online: August 24, 2007
*Correspondence to:* Lizabeth Bowen, e-mail: lbowen@ucdavis.edu

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

### 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 hyrdrocarbon 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 1800*g* for 20 minutes. Isolated mononuclear cells were suspended in sterile PBS (phosphate buffered saline) containing 0.5 M EDTA (pH 7.4), centrifuged (250*g*, 8 minutes), resuspended in cryopreservation media (10% Dulbecco's modified Eagle's medium, 10% DMSO, and 80% fetal bovine serum), rate frozen at -80°C, and then transferred to liquid nitrogen.

Animals were euthanized by $CO_2$ 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°C. A standard cDNA synthesis was performed on 2 (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°C, followed by an enzyme inactivation step of 5 minutes at 93°C, and stored at -20°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 µl volumes containing 20–60 pmol of each primer, 40 mM Tris-KOH (pH 8.3), 15 mM KOAc, 3.5 mM $Mg(OAc)_2$, 3.75 µg/ml bovine serum albumin (BSA), 0.005% Tween-20, 0.005% Nonidet-P40, 200 µM 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°C for 3 minutes, 40 cycles at 94°C for 30 seconds, 60°C for 30 seconds, and 72°C for 2 minutes, with a final extension step of 72°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 DH5α 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 | GGACAGAAMAAGAAAGGGAAAGATG | AHRR1 | GGTCTCTGAGTTRCAATGATATAATC |
| Estrogen receptor beta | ERBF1 | GGATATCACTATGGAGTCTGGTCG | ERBR1 | CATCATCATGGAGGCGTCGGTG |
| Glutathione-S-transferase | GLUF1 | CCTGAATGCCAAGGGAATCCGG | GLUR1 | GCCAGATGAGGTAATCAATCATAG |
| UDP-glucuronyltransferase | UDPF2 | GAGGACTCCACTGCAAACCTGC | UDPR2 | GGGATCCCATGGTAGATTGCCTC |
| Heat shock protein 90 | HSP90F1 | GCCTGAGGAAGTGCACCATGGA | HSP90R1 | GATCACAACCACTTTCTCTGCCAC |
| Heat shock protein 70 | HSP70F1 | ACCTGGGCACCACCTACTCCTG | HSP70R1 | GCTTGTTCTGGCTGATGTCCTTCT |
| Interleukin-2 | IL-2F1 | CAAGTGCAGTCATTGCTGCAGGAT | IL-2R1 | GTAATCCATTTGTTCAGAANTTCTACAG |
| Interleukin-12 | IL-12F1 | CMTCRTGGCCATRTGGGAACTGGAG | IL-12R1 | CACTGAATTTCARATCAGTACTGATTGC |
| Interleukin-18 | IL-18F1 | GATGAARACCTGGAATCRGATYACT | IL-18R1 | CATGTCCWGGRACACTTCTYTGAA |
| Interleukin-10 | IL-10F2 | GACTTTAAGGGTTACCTGGGTTGC | IL-10R2 | TCCACCGCCTTGCTCTTGTTTTC |
| iNOS | INOSF1 | CAGGAACCTACCAGCTGACGG | INOSR1 | GTGATGGCCGACCTGATGTTGC |
| TGF$\beta$ | TGFBF1 | CAGTACAGCAAGGTCCTGGCCC | TGFBR1 | CTGCTCCACCTTGGGTTGCG |
| COX-2 | COX-2F1 | GAGCTCTTCCTCCTGTGSCTGA | COX-2R1 | CTTTGRCTGTSGGMGGATACAYCT |
| S9 | S9F1 | GTGGCCCGGARCTGGGTTTG | S9R1 | GGCGYCTCTCYAARAAATCC |
| Metallothionein | metF1 | AGCCTTCCACGTGCGCCTTATAG | metR1 | GCACAGCAGCTGCACTTSTCYG |
| Complement cytolysis inhibitor | CYTF1 | ACGAGCTGCTAAAGTCCTACCAGTG | CYTR1 | ACTGAGGTGGTCGTGAAGCTCTTTG |
| HDCMB21P | HDCF1 | ATGTTCTCCGACATCTACAAGATCC | HDCR1 | CATGAACCATCACCTGCAGGAAAC |
| DQA | DQAUD | CCAGTACACCCATGAATTTGATGG | DQALD | GAAACACAGTCACCTCAGGAACC |
| DRA | DRAU102 | ATAAGTGGAGTCCCTGTGCTA | DRAL | CCCAGTGCTCCACCTTGCAGTCATA |
| DRB | DRBU71 | CGGGACSGAGCGGGTKC | ZcDRB4L | CCACTTGGCAGGTGTAGACCTCTCC |
| Interleukin-6 | IL-6F1 | CYCTGGGRCTGCTYCTGGTG | IL-6R1 | CTGACCAGARRARGGAATGCC |
| Thyroid hormone receptor | THRBF1 | GGACAAACCGAAGCACTGTCCAG | THRBR1 | GGAATATYGAGCTAAGTCCAAGTGG |
| Cold inducible RNA-binding protein | CIRBPF1 | TTCTGAGTGTAGTGTGGTAGGACCC | CIRBPR1 | AGTGGCTGAGGAATTCTTGTACGCA |
| FoxP3 | FOXP3F1 | CAGGCACTCCTCCAGGACAG | FOXP3R1 | CTCCCTGGACACCCATTCCAG |

2006). Each reaction contained 500 ng DNA in 25 µl volumes with 20 pmol SSP, Tris-Cl, KCl, $(NH_4)_2SO_4$, 2.5mM $MgCl_2$ (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 pleotrophic and often redundant

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

| Gene of interest | *Mustela vison* gene sequence 5′–3′ |
|---|---|
| Aryl hydrocarbon receptor | CGATAAGTGTCTGTAGTATTCAGGCTTCTTACCAAAGATAATCGATGGGCCTGGGTTCAGTCTAATGCACGCTTAGTGA |
| Estrogen receptor beta | AATCAGTGTACAATAGATAAGAATCGGCGCAAGAGCTGCCAGGCCTGCCGGCTCCGGAAGTGCTATGAAGTGG<br>GGATGGTGAAGT |
| Glutathione-S-transferase | TGATCTACGAATCTGCCATCACCTGTGAGTACCTGGATGATGTATATCCAGGAAAGAAGCTATT<br>GCCAGATGACCCCTATGAGAAAGCTCGTCAGAAGATGGTGTTTGAGTTATTTT |
| UDP-glucuronyltransferase | AGAGTTTGTCCAGAGCTCTGGAGAAAATGGTATTGTGGTGTTTACACTAGGGTCTATGATCACTAACCT<br>GCCAGAGGAAAAAGCCAATGTGATTGCATCAGCCCTTGCACAGATTCCACAAAAGGTTCTATGGAGATTTGCTGGC<br>AAGAAACCAGACAACTTAGGACCAAATACTCGGCTGTATGACTGGATCCCCCAGAATGACCTTCTTGGTCATCCAAAAACCAAA<br>GCCTTTGTAACTCATGGTGGAACCAATGGCATCTATGAGGCAATCTACCATGGG |
| Heat shock protein 90 | CCCAGGAACGCACCTTGACTCTAGTGGACACAGGCATTGGCATGACCAAAGCCGATCTCATAAATAATTTGGGAACAATTGCCAA<br>GTCGGGAACTAAAGCATTCAT |
| Heat shock protein 70 | TCGAGCGTAGCGGTGTCGGCCGCAGTTCGGCGACCCGGTGGTGCAGTCGGACATGAAGCACTGGCCCTTCCAGG<br>TGATCAGCGATGGCGATAAGCCCAAGGTGCAGGTGAGCTACAAGGA |
| Interleukin-2 | CCATAGTACTCAACTTTGGAGGAGTGCTATATTTAGCTCAAGCAAAAACTTTCACTTGACAGACATCAAGGAACTAAT<br>GAGCAATATCAATGTAACACTTCTGAAACTAAAGGGATCTGAAACAAGA |
| Interleukin-12 | TGGTGGTCCTCACCTGCCAAACCCCTGAAGAAGATGACATCACCTGGACCTCGGACCAGAGCAGTGACGTCTTAGGC<br>TCCGGTAAAACTCTGACCATTCAAGTCAAAGAATTTGGAGATGCTGGCCGGTATACCTGTCATAAAGGAGGCAAG<br>GTTCTGAGCCATTCGCTCCTGCTGATTCACAAAAAGGAAGATGGAATTTGGTCCACTGATATCTTA<br>AAGGAACAGAAAGAATCCAAAAATAAGATCTTTCTAAAATGTGAGGCAAAGAATTATTCTGGACGTTTCACCTGC<br>TGGTGGCTGACAGCAATCAGTACTGATC |
| Interleukin-18 | ATCATACGAAATTTGACGACCAAGTTCTCTTCGTTAACGAGGGCAATCAACCAGTGTTCGAGGATATGCCT<br>GATTCTGACTGTACAGAAAACGCATCCCATACCATATTTATCATAAATATGTATAAAGATAGCCTCACCAGAGGTCTGG<br>CGGTAACCATCTCTGTGATGTGTAACAGAATGTCTACTCTGTCCTGTAAGAACAAAACTATTTCCTTTAAGGAAATGAGACCTC<br>CAGACAGTATCAGCGACGAAGGGAATGACATCATATTCTTTCAAAGAAGTGT |
| Interleukin-10 | CAGTTTTACTTGGAGGAGGTGATGCCCCAGGCCGAGAACCACGACCCTGAAGTCAAGGAGCTCGTGAACT<br>CGCTGGGGGAAAAGCTGAAGACCCTGCGGCTGAGGCTGCGGCGCTGTCATCGATTTCTGCCCTGTGAGAACAA<br>GAGCAAGGCCGTGGAA |
| iNOS | CCCCGCTGCATCGGGAGGATCCAGTGGTCCAACCTGCAGGTCTTCGACGCCCGGAGCTGTTCCACTGCCAAGGAA<br>ATGTTCGAGCACATCTGCAGACACCTGCGTTATGCCACCAACAATGGCAACATCAGGTCGGCCATCAC |
| TGF+$\beta$ | AGACATCAGCTGTCTGCTTTCCGGGCAGAGTTGCGCCTGCTGAGGCTCAAGTTAAAAGCGGAGCAGCACGTGGAGCTGTAC<br>CAGAAATA |
| COX-2 | TTGCATTCTTTGCCCAGCACTTCACCCATCAATTTTTCAAGACAGATCATAAGCGAGGGCCAGGTTTCACCAAAG |

**Table 2.** Continued

| Gene of interest | *Mustela vison* gene sequence 5′–3′ |
|---|---|
| S9 | GACTCCATCCTTCTCGTCCGCCTGGATTCCCAGAAGCACATTGACTTCTCCTTGCGGTCTCCGCTATGGGGGTGGCCGCCC<br>AGGCCGCGTGAAGAGGAAGAATGCCAAGAAGGGCCAGGGA |
| Metallothionein | AGAGCTGCTGCTCCTGCTGCCCAGTGGGCTGTGCCAAGTGTGCCCAGGGCTGTGTTTGCAAAGGGA |
| Complement cytolysis inhibitor | CGTCTCCTGCTGAGCAGCTGGACGAGCAGTTTAGCTGGGTGTCCCAGCTGGCGAATCTCACTCA<br>GACTGAGGACCCGTTCTATCTCCAGGTCACGACGGTGAGTTCCCAGACTTCTGACTCCAGTGCTCCCTCTGGCGTC<br>ACTGAGGTGGTCGTGAAGCTCTTTGA |
| HDCMB21P | CTGTGTCTGGAGGTGGAGGGGAGATGGTCAGTAGGACAGAGGGTAACATTGATGACTC<br>GCTCATTGGTGGAAATGCCTCCGCTGAAGGCCCGGAGGGCGAAGGTACCGAAAGCACAGTCATCACTGGTGTT<br>GATATTGTCATGAACCATCACCTGCAGGAAACA |
| DQA | CAGCTTGATCACAGGTTCACTGAGAACTTGGCAATAGCGAAACAAAACTTGAACATCCTGACTAAACGGCTCCAA<br>CTATACCGCTGCTA |
| DRA | ATCACGCCATGTGGACTGGGAGAGCCCAACACCCTCATCTGCTTCATTGATAAGTTCTCCCCACCACGTGATCAATGTCACGTGG<br>CTTCGAAATGGAAACCCTGTCACCACA |
| DRB | GGATGACAGCTCTGACATTGATTTTGATGGTGCTGAGTCCCCTCTTGGCTTGGGCCAGGGACACCCCACCAC<br>ATTTCCTGKTYCTGRYGAMGTCGGAATGCTACTTCACCAACGGCACGGAGCGGGTGCGGTTCCTGGAKAGGTATTTCTATAAC<br>GGCGAGGAGTTC<br>STGCGCTTCGACAGCGACGTGGGGGAGTACCKGSCGGTGACCGAGCTGGGGCGGCCGGACGCTCCGTACTGGAACAGCC<br>AGAAGGACTTYGTGGAGCRGAMGCGGGCCGAGGTGGACACGTACTGCAGACACAACTACGGGGTGGGTGAGAGCTTCACG<br>GTGCAGCGGCGAGTGGAGCCTATAGTGACWGTGTATCCTKYGAAGACCCAGCCCCTGAAGCACCACAACCTCCTGGTCTG<br>CTCTGTGAATGGTTTCTATCCAGGCCACATTGAAGTCAGRTGGTTCCGGAATGGCCAGGAAGAGGAGTCTGGGGTCGTGTC<br>CACAGGTCTGATCCATAATGGAGACTGGACCTTCCAGACCCTGGTGATG<br>CTGGAGACAGTTCCTCAGAGTGGAGAGGTCTACACCT |
| Thyroid hormone receptor | ACTGGTAGCTAGTAGGAATGTCTGAAGCCTGCCTCCATAGGAAGAGCCATGCGGAGAGGCGCAGCGCATTGAAAAATGA<br>ACAGTCGTCACCACATCTCATCCAGGCCACTTGGACTTAGCTCAATATTCC |
| Cold inducible RNA-binding protein | AGGCGCTCCGGCTGCCCTGGGCCTGGCGTGAACGGCCCTTCTATGCTATGTATGACCCGCAGTAGATC<br>GCAGACGTCCCTCGAGAGGTTCTTGAAAATGTTTATTTATATTGTCCTTTTTTACTGGAAGACGTATGCATATCTTATTGATGTTG<br>TATTTGAAGTGGCTGAGGAATTCTTGTACGCAAA |
| FoxP3 | CCGCTGGGCCATCCTGGAAGCTCCCGAGAAGCAGCGGACACTCAACGAGATCTACCACTGGTTCACGCGCATGTT<br>TGCCTTCTTCAGAAACCACCCTGCCACCTGGAAAAACGCCATCCGCCACAACCTGA |

**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 | GTGCTGAGTACCATATACGGATGA | MvAHRR1 | GTTCAGTCTAATGCACGCTTAGTG |
| Estrogen receptor beta | Alignment | MvERBF1 | GCATTCAAGGACATAACGATTACAT | MvERBR1 | GTGGCTCCCGGAGGGAAAGGT |
| Glutathione-S-transferase | Alignment | MvGLUF1 | CTGGTGCCAGTTCTGGAAAACAG | MvGLUR1 | CTAAGGTCCCATCTTTGGTAAC |
| UDP-glucuronyltransferase | Microarray | MvUDPF2 | GAGCTCTGGAGAAAATGGTATTGT GGTGTTTACA | MvUDPR2 | CCGAGTATTTGGTCCTAAGTTGTCTG GTTTCTTG |
| Heat shock protein 90 | Alignment | MvHSP90F1 | CCAAGTTGGACAGCGGTAAAGAG | MvHSP90R1 | GGAGGCTCTTCAGGCTGGTGC |
| Heat shock protein 70 | Alignment | MvHSP70F1 | CCAGGTGGCGCTGAACCCGC | MvHSP70R1 | CAAGGTGCAGGTGAGCTACAAGG |
| Interleukin-2 | Alignment | MvIL-2F1 | GGCCACAGAATTGACTCATCTTCA | MvIL-2R1 | CTGAAACTAAAGGGATCTGAAACAAG |
| Interleukin-12 | Alignment | MvIL-12F1 | GCCATTCGCTCCTGCTGATTCAC | MvIL-12R1 | GTTTCACCTGCTGGTGGCTGAC |
| Interleukin-18 | Alignment | MvIL-18F1 | GTACAGAAAACGCATCCCATACC | MvIL-18R1 | CCTTTAAGGAAATGAGACCTCCAG |
| Interleukin-10 | Alignment | MvIL-10F2 | GACTTTAAGRGTTACCTGGGTTGC | MvIL-10R2 | TCCACSGCCTTGCTCTTRTTYTC |
| iNOS | Alignment | MvINOSF1 | CCGCTGCATCGGGAGGATCC | MvINOSR1 | GCAACATCAGGTCGGCCATCAC |
| TGF$\beta$ | Alignment | MvTGFBF1 | GCTCTTCAACACATCGGAGCTC | MvTGFBR1 | CAGCAACGATTCCTGGCGCTAC |
| COX-2 | Alignment | MvCOX-2F1 | CATTCCTGATCCCCAGGGCAC | MvCOX-2R1 | GACTGGGCCATGGGGTGGAC |
| S9 | Alignment | MvS9F1 | CCAGCGCCACATCAGGGTCCG | MvS9R1 | GAATGCCAAGAAGGGCCAGGG |
| Metallothionein | Microarray | MvmetF1 | GAGCTGCTGCTCCTGCTGCCC | MvmetR1 | CCAGGGCTGTGTTTGCAAAGGG |
| Complement cytolysis inhibitor | Microarray | MvCYTF1 | GCTGGACGAGCAGTTTAGCTGG | MvCYTR1 | CCAGTGCTCCCTCTGGCGTC |
| HDCMB21P | Microarray | MvHDCF1 | CTGTGTCTGGAGGTGGAGGGG | MvHDCR1 | CAGTCATCACTGGTGTTGATATTG |
| DQA | Alignment | MvDQAF1 | CTGTCTGGCAGCTGCCTGTGTT | MvDQAR1 | CCAATGAGGTTCCTGAGGTGAC |
| DRA | Alignment | MvDRAF1 | CACCAATGTACCTCCGGAGGTG | MvDRAR1 | GGAGTGTCGGAGACAGTCTTCC |
| DRB | Alignment | MvDRBF1 | CGGCGAGTGGAGCCTATAGTG | MvDRBR1 | CGGAATGGCCAGGAAGAGGAG |
| Interleukin-6 | Alignment | MvIL-6F1 | CCTGCAGTTCAGCCTGAGGGC | MvIL-6R1 | CATAAGTTATGTGCCCAATGGACAG |
| Thyroid hormone receptor | Alignment | MvTHRBF1 | GGACAAACCGAAGCACTGTCCAG | MvTHRBR1 | GGAATATYGAGCTAAGTCCAAGTGG |
| Cold inducible RNA-binding protein | Microarray | MvCIRBPF1 | TTCTGAGTGTAGTGTGGTAGGACCC | MvCIRBPR1 | TGCGTACAAGAATTCCTCAGCCACT |
| FoxP3 | Alignment | MvFOXP3F1 | CCGCTGGGCCATCCTGG | MvFOXP3R1 | CAGGTTGTGGCGGATGGC |

**Table 4.** Differential Gene Expression in Oil-fed Mink[a]

| Gene of interest | Spleen | | | CPT | | | Gene function |
|---|---|---|---|---|---|---|---|
| | Average normalized $C_T$ values | | | Average normalized $C_T$ values | | | |
| | Oiled mink | Control mink | *P*-value | Oiled mink | Control mink | *P*-value | |
| Aryl hydrocarbon receptor | 15.78 | 14.88 | 0.17 | 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 | 0.53 | 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 | 0.1 | 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 | 0.35 | NA | NA | | Catalyzes the glucoronidation of xenobiotic compounds (Daidoji et al., 2005) |
| Heat shock protein 90 | 2.81 | 3.12 | 0.26 | 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 | 0.02* | 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 | 0.04* | 17.67 | 18.4 | 0.16 | T cell growth factor (Kindt et al., 2007) |
| Interleukin-12 | 12.7 | 13.3 | 0.15 | 17.93 | 18.37 | 0.43 | Proinflammatory cytokine (Kindt et al., 2007) |
| Interleukin-18 | 5.13 | 4.08 | 0.002* | 6.91 | 6.54 | 0.29 | Proinflammatory cytokine (Kindt et al., 2007) |
| Interleukin-10 | 16.22 | 15.26 | 0.02* | 21.08 | 20.52 | 0.34 | Antiinflammatory cytokine (Kindt et al., 2007) |
| iNOS | 16.91 | 15.08 | 0.01* | 18.60 | 20.00 | 0.22 | Induced upon macrophage activation (Kindt et al., 2007) |
| TGF$\beta$ | 5.46 | 5.22 | 0.5 | 5.66 | 5.8 | 0.5 | Antiinflammatory cytokine (Kindt et al., 2007) |
| COX-2 | 7.08 | 7.18 | 0.31 | 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 cytolysis inhibitor | 8.82 | 7.86 | 0.05* | 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 | *P*-value | Oiled mink | Control mink | *P*-value | |
| HDCMB21P | −0.49 | −1.06 | 0.07 | −0.36 | −0.54 | 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 | 4.60 | 4.56 | 0.28 | Binding of pathogens/initiation of immune response (Kindt et al., 2007) |
| DRA | 4.28 | 3.88 | 0.16 | 5.57 | 5.46 | 0.31 | Binding of pathogens/initiation of immune response (Kindt et al., 2007) |
| DRB | 0.79 | 1.16 | 0.45 | 1.88 | 1.82 | 0.27 | Binding of pathogens/initiation of immune response (Kindt et al., 2007) |
| Interleukin-6 | 9.36 | 9.26 | 0.42 | 12.72 | 13.16 | 0.5 | Proinflammatory cytokine (Kindt et al., 2007) |
| Thyroid hormone receptor | 15.39 | 15.02 | 0.16 | 17.01 | 15.10 | 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 | 9.54 | 9.74 | 0.44 | Cold-shock protein (Nishiyama et al., 1997) |
| Fox FP3 | 11.12 | 11.28 | 0.32 | 10.78 | 10.68 | 0.32 | Selectively expressed in a subpopulation of T cells with regulatory activity (Ziegler, 2007). |

[a]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 cytolysis 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.

## Acknowledgments

We are grateful to Divyen Patel of Genome Explorations for considerable help in data interpretation. This research was funded in part by the California Department of Fish and Game's Oil Spill Response Trust Fund, through the Oiled Wildlife Care Network at the Wildlife Health Center, School of Veterinary Medicine, University of California, Davis.

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