Exxon Valdez Oil Spill
Restoration Project Final Report

# Investigations of Disease Factors Affecting Declines of Pacific Herring Populations in Prince William Sound
## Section III: The effects of oil and disease on various aspects of herring fitness
## Manuscripts

Restoration Project 99162B
Final Report

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**Study History:** This project was initiated under Restoration Project 95320-S in response to a request for proposals to investigate disease factors affecting Pacific herring decline in Prince William Sound and continues research from projects 95320, 96162 and 97162. The proposal is a joint effort of Simon Fraser University, the University of Washington, University of California, Davis and the Alaska Department of Fish & Game.

**Abstract:** Pacific herring, *Clupea pallasi*, were exposed both acutely (96 h) and chronically (9 wk) to the water-soluble fraction (WSF) of North Slope crude oil. Initial mean total PAH concentrations were approximately 10, 40 and 100 µg/L. Biological availability was suggested by a significant induction of hepatic cytochrome P450 content, ethoxyresorufin O-deethylase and glutathione S-transferase activities. Acute exposure caused a transient organismal stress response. Chronic exposure resulted in prolonged ionoregulatory disturbance and an inability to mount a successful stress response. Acute and chronic exposure also affected the ability of herring to swim. The time courses and magnitudes of several key post-exercise parameters including plasma cortisol, lactate and muscle glycogen indicated that recovery from exhaustive exercise was also affected. Short-term exposure to WSF significantly affected respiratory burst activity (RBA) in macrophages and plasma lysozyme concentrations. With subchronic exposure, RBT activity continued to be affected. Fish in the high treatment group were less susceptible to the pathogen *Vibrio anguillarum* following acute hydrocarbon exposure; however, this group was the most susceptible by the end of the experiment. This study shows that hydrocarbon exposure can affect several physiological systems of herring at low ppb concentrations resulting in decreased performance and potentially, their fitness.

**Key Words:** *Clupea harengus pallasi*, herring, *Exxon Valdez*, oil, water-soluble fraction, stress response, swimming performance, exercise, immunotoxicology, fitness

**Project Data:** *Description of data*- several sets of data were gathered by laboratory experiments and include: effects of oil exposure on Pacific herring stress biochemistry, immunology and disease resistance, and swimming performance and exercise recovery. *Format*- Data regarding experimental data are stored in Microsoft Excel and text files in WordPerfect 6.1. *Custodian*- Contact Dr. Chris Kennedy at the Department of Biological Sciences, Simon Fraser University, Burnaby, BC, Canada, V5A 1S6. ) Phone: (604) 291-5640, fax: (604) 291-3496 or email at: ckennedy@sfu.ca). *Availability*- Copies of text in annual reports are available for the

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Kennedy, C.J. and A.P. Farrell. (2006). Immunological alterations in juvenile Pacific herring, *Clupea pallasi*, exposed to aqueous hydrocarbons derived from crude oil. Submitted to Env. Poll. Oct. 2006.

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**Executive Summary**

Although near-record spawning biomass returns of Pacific herring were predicted for 1993, the population crashed when less than half of the >100,000 tons of spawning herring returned to Prince William Sound. Spawning herring sampled from Prince William Sound (PWS), showed a high prevalence of two pathogens, namely Viral Hemorrhagic Septicemia Virus (VHSV) and *Ichthyophonus hoferi* (ITP). The presence of these pathogens has led to the suggestion that disease was the likely cause of morbidity of herring in PWS. It is also unclear whether the Exxon Valdez oil spill contributed to these population declines.

Stressors such as disease and pollution can affect the long-term survival of fish without being acutely lethal, through reductions in overall 'health' or 'fitness'. The long-term objective of this study, therefore, is to document cause-effect relationships for oil on herring fitness or health to determine probable causes of population declines. The categories of fitness chosen for this study include herring stress biochemistry, and performance in terms of the immune system and swimming.

In the first set of experiments, juvenile Pacific herring, *Clupea pallasi*, were exposed both acutely (96 h) and chronically (9 wk) to three concentrations of the water-soluble fraction (WSF) of North Slope crude oil. Mean (±SE) total PAH (TPAH) concentrations at the beginning of the acute exposure experiment were: 9.7±6.5, 37.9±8.6 and 99.3±5.6 µg/L. TPAH concentrations declined with time and the composition of the WSF shifted toward larger and more substituted PAHs. Significant induction of hepatic cytochrome P450 content, ethoxyresorufin O-deethylase and glutathione S-transferase activities in WSF-exposed fish indicated that hydrocarbons were biologically available to herring. Significant but temporary, elevations in plasma cortisol (4.9-fold and 8.5-fold increase over controls in the 40 and 100 µg/L groups, respectively), lactate (2.2-fold and 3.1-fold over controls in the 40 and 100 µg/L groups), and glucose (1.3-fold, 1.4-fold and 1.6-fold over controls in the 10, 40, and 100 µg/L groups) occurred in fish exposed acutely to WSF. All values returned to baseline levels by 96 h. Similar responses were seen with the first of several sequential WSF pulses in the chronic exposure study. Subsequent WSF pulses resulted in muted cortisol responses and fewer significant elevations in both plasma lactate and glucose concentrations. Hematocrit, leucocrit, hemoglobin concentration and liver glycogen content were not affected by acute or chronic WSF exposure. Plasma [$Cl^-$], [$Na^+$] and [$K^+$] were significantly higher in the 100 µg/L WSF-exposed group by 96 h compared to control fish, and continued to be elevated through the entire chronic exposure period. Unlike the measured stress parameters, ionoregulatory dysfunction was not modulated by WSF pulses. The results of this study suggest that chronic exposure to WSF affects at least two important physiological systems in herring: the ability of fish to maintain ion homeostasis and the interrenally-mediated organismal stress response.

In a second set of experiments, the swimming performance and recovery of juvenile herring from exercise were determined following exposure to the water-soluble fraction (WSF) of North Slope crude oil for over 8 weeks. Mean (±SE) total polycyclic aromatic hydrocarbon (TPAH) concentrations at the beginning of exposures were: 0.2±0.1 (control), 9.6±2.5 (low), 40.7±6.9 (medium), and 120.2±11.4 (high) µg/l. Biological availability of hydrocarbons was confirmed by a significant induction of hepatic cytochrome P450 content and ethoxyresorufin O-deethylase activity. Critical swimming speed (Ucrit) was significantly reduced in fish exposed to the

highest concentration of WSF for 96 h (11±3.7% reduction), and at the two highest concentrations at four weeks (16±3.6% and 29±5.4% reductions) and eight weeks (11±3.8% and 40±5.7% reductions). Mortality occurred in all groups of herring 24 h following Ucrit swim trials, with significantly higher mortalities in fish exposed to WSF in a concentration- and time-dependent manner (maximum mortality [72.2±5.5%] in the 8 week, high oil exposure group). Burst swimming alone resulted in increases in plasma cortisol, lactate, $Na^+$ and $Cl^-$ concentrations, and decreases in muscle glycogen that returned to baseline values by 24 h. An interpretation of WSF-exposure on post-exercise metabolic recovery was complicated by pre-exercise alterations in several parameters. The time courses and magnitudes of several key post-exercise parameters including plasma cortisol, lactate and muscle glycogen were significantly altered by WSF-exposure. This study clearly shows that hydrocarbon exposure can reduce the swimming ability of fish and their ability to recovery from exhaustive exercise

In a third set of experiments, Pacific herring were exposed acutely and subchronically to 3 concentrations (control: 0.15±0.10, low: 10.5±3.7, medium: 35.7±7.7 and high: 127.3±11.3 µg/L total PAH [TPAH]) of aqueous hydrocarbons derived from North Slope crude oil in order to assess their impact on immune defense mechanisms. TPAH concentrations declined and composition shifted toward larger and more substituted PAHs as the time of exposure increased. Hydrocarbons were bioavailable to herring, as indicated by induction of hepatic cytochrome P450 and ethoxyresorufin O-deethylase activity. Hydrocarbon exposure did not affect hematocrit, leucocrit or differential white blood cell counts. Acute (24 and 96 h) exposures resulted in a transient stress response as shown by increases in plasma cortisol, lactate and glucose. At 96 h, a persistent ionoregulatory dysfunction resulted in elevated concentrations of plasma $Na^+$, $Cl^-$ and $K^+$, which persisted through the entire exposure period (8 wk). Short-term exposure in the high treatment group significantly enhanced respiratory burst activity (RBA) in macrophages and decreases in plasma lysozyme concentrations. With subchronic exposure (4 to 8 wk), RBT activity was significantly reduced. Fish in the high treatment group were less susceptible to the pathogen *Vibrio anguillarum* following acute hydrocarbon exposure; however, this group was the most susceptible by the end of the experiment. This study shows that hydrocarbon exposure can variably affect teleost immune systems and depends on chemical concentration and composition, exposure duration and pathogen challenge.

These results suggest a possible role of oil exposure in herring population declines, given that juvenile fish in these experiments were affected at concentrations of TPAH much lower than reported in the literature. Exposures of herring to oil affected several important physiological systems leading to evidence of stress, endocrine disruption, ionoregulatory dysfunction, and immunotoxicity. This suggests many potential performance disruptions, however, this study documented reduced disease resistance and swimming performance. Understanding how herring respond to hydrocarbon exposure and their recovery from exposure has important implications to herring management strategies and herring fisheries practices and will aid in the recovery of this and similar resources, as well as in successful monitoring of fish health in the future.

## Introduction

The Pacific herring (*Clupea harengus pallasi*) spawning population in 1989 was the largest in many years when the *Exxon Valdez* oil spill occurred in Prince William Sound (PWS). Although

near-record spawning biomass returns were predicted for 1993, the population crashed when less than half of the >100,000 tons of spawning herring returned to PWS. Several hypotheses have been put forward to explain the population decline that include the direct or indirect effects of oil or its components on herring habitat, food resources or their survival and fitness. Pearson et al. (1995) concluded that the levels of hydrocarbons measured in various matrices in PWS were too low to pose a serious risk to either adult or juvenile herring. This conclusion appears to be premature and relies mainly on results of acute toxicity tests. A more comprehensive examination of both lethal and sublethal toxic effects of hydrocarbons on several life stages of Pacific herring is needed to realistically assess the impact of such events on fish populations.

Approximately 15 to 43% of the returning fish were observed to have external lesions including ulcerations and hemorrhaging beneath the skin. Meyers et al. (1993) reported isolation of a rhabdovirus, identified as the North American strain of viral hemorrhagic septicemia virus (VHSV), by serum neutralization and cDNA probe methods. Therefore, it has been suggested that VHSV may have played a role in the population decline of the herring populations in Prince William Sound. One suggestion is that mortality may occur during these epizootics from progressive ulcerating skin lesions resulting in possible osmoregulatory failure and/or entry points for other pathogens (Meyers et al. 1993). These authors suggest that the virus may manifest its effects following stress from various factors including viral erythrocytic necrosis virus (VENV), spawning, commercial fishing or nutritional deficiency through lack of forage. More recent studies have indicated that VHSV was present in about 5% of herring tested in 1994, but lesions associated with infection from another pathogen, *Ichthyophonus hoferi* (ITP), were present in about 29% of herring sampled. It has been suggested that ITP may also have been a major cause of herring morbidity between the 1992 and 1993 spawning seasons (Marty et al. 1994).

Stress due to anthropogenic contamination, i.e. the *Exxon Valdez* oil spill, may have affected fish health or performance leading to the observed high mortalities and infection rates in surviving fish. Other studies have shown that stress from exposure to polycyclic aromatic hydrocarbons (PAHs) can impair immunological responses, possibly resulting in reduced survival or fitness (Garrett, 1993). It has been shown that VHSV expression in carrier fish appears to be enhanced under stress of exposure to oil (Meyers, unpublished report). Furthermore, it is suggested that even if VHSV is not the primary pathogen, the high level of ITP incidence is indicative of a much weaker immune system in the herring. In addition, the extent of ITP infection and tissues infected (heart, skeletal muscle and brain) suggest life-threatening effects (Freiberg and Farver, 1995; Marty et al., 1994).

From the information that had existed prior to 1995, there had been no definitive evidence on whether VHSV, ITP or oil exposure through the *Exxon Valdez* oil spill, or some combination of these stressors had caused a decline in herring populations. In this project, Section I had as its objectives to determine the prevalence and severity of VHSV, ITP and other lesions in surviving spawning Pacific herring in PWS through several years. Sections II and III of this proposal have as their combined objectives to determine definitive links and relationships between diseases and hydrocarbon exposure and morbidity, mortality, pathogenicity, and overall fitness and 'health' of Pacific herring. In 1996 and 1997, results from these studies showed that fish exposed to low levels of hydrocarbons experienced acute lethality as well as very significant sublethal effects on several aspects of herring fitness that included effects on the herring immune system. These

results begin to link oil exposure to a reduced immunocompetence in herring that may have contributed to increased disease prevalence observed in the field section of this proposal.

The results of this project in the examination of the effects of hydrocarbons on herring health are aimed at answering several questions regarding herring population dynamics such as: 'Are herring that survive exposure to hydrocarbons 'healthy' or are they surviving at a reduced fitness level? What are the differences in acute vs. chronic exposures? In the absence of such information, sound management of the herring stock in PWS will be a difficult task.

**Objectives**

From all of the information that has been made available through laboratory and field studies investigating the decline of herring stocks in Prince William Sound (PWS), there is no definitive evidence on whether viral hemorrhagic septicemia virus (VHSV), *Ichthyophonus hoferi* (ITP) or oil exposure *via* the *Exxon Valdez* oil spill, or some combination of these stressors has caused a decline in herring survival, performance or reproductive fitness. It is also unclear if the fish that survived exposure to one or more of these stressors are 'healthy' or are surviving at a reduced fitness level.

The laboratory component of this proposal addresses important information needs. The objectives of the proposed study will contribute directly towards discovering why herring populations are recovering at their present rate in PWS. The long-term objectives of Section III of this project, therefore, were to document the effects of oil on herring survival and performance.

The overall hypothesis being tested in this project was:

'The exposure of herring to oil reduces herring fitness in one or more of the following categories: 1) immunology, 2) stress biochemistry, and 3) swimming performance.

<u>Objectives:</u>

1) To supply analytical support for Section I (the field component: Dr. G. Marty) of this research project.

2) To determine the effects of oil exposure on the stress biochemistry of Pacific herring.

3) To determine the effects of oil exposure on the swimming performance and exercise recovery of Pacific herring.

3). To determine the effects of oil exposure on various components of the immune system and disease resistance in herring.

Manuscript 1:

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# Ion homeostasis and interrenal stress responses in juvenile Pacific herring, *Clupea pallasi,* exposed to the water-soluble fraction of crude oil

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Running head: effects of oil on herring ion regulation and stress response

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**Abstract**

Juvenile Pacific herring, *Clupea pallasi*, were exposed both acutely (96 h) and chronically (9 wk) to three concentrations of the water-soluble fraction (WSF) of North Slope crude oil. Mean (±SE) total PAH (TPAH) concentrations at the beginning of the acute exposure experiment were: 9.7±6.5, 37.9±8.6 and 99.3±5.6 µg/L. TPAH concentrations declined with time and the composition of the WSF shifted toward larger and more substituted PAHs. Significant induction of hepatic cytochrome P450 content, ethoxyresorufin O-deethylase and glutathione S-transferase activities in WSF-exposed fish indicated that hydrocarbons were biologically available to herring. Significant but temporary, elevations in plasma cortisol (4.9-fold and 8.5-fold increase over controls in the 40 and 100 µg/L groups, respectively), lactate (2.2-fold and 3.1-fold over controls in the 40 and 100 µg/L groups), and glucose (1.3-fold, 1.4-fold and 1.6-fold over controls in the 10, 40, and 100 µg/L groups) occurred in fish exposed acutely to WSF. All values returned to baseline levels by 96 h. Similar responses were seen with the first of several sequential WSF pulses in the chronic exposure study. Subsequent WSF pulses resulted in muted cortisol responses and fewer significant elevations in both plasma lactate and glucose concentrations. Hematocrit, leucocrit, hemoglobin concentration and liver glycogen content were not affected by acute or chronic WSF exposure. Plasma [$Cl^-$], [$Na^+$] and [$K^+$] were significantly higher in the 100 µg/L WSF-exposed group by 96 h compared to control fish, and continued to be elevated through the entire chronic exposure period. Unlike the measured stress parameters, ionoregulatory dysfunction was not modulated by WSF pulses. The results of this study suggest that chronic exposure to WSF affects at least two important physiological systems in herring: the ability of fish to maintain ion homeostasis and the interrenally-mediated organismal stress response.

## Introduction

Petroleum-derived hydrocarbons are a major contributor to the contamination of aquatic environments. Approximately 5 million tons of crude oil from a variety of sources enters the marine environment each year (Neff 1990). Typical concentrations of total hydrocarbons in contaminated marine coastal waters can be as high as 80 µg/L, with occasional reports of up to 500 µg/L in the Arabian Gulf (Badawy and Al-Harthy 1991; Madany et al. 1994; Alkindi et al. 1996). Much attention has been paid to large crude petroleum spills and their visible surface effects, however, of more recent concern are the potential effects of dissolved hydrocarbons, which are the most available to marine biota (Neff and Anderson 1981). Of particular interest are the polycyclic aromatic hydrocarbons (PAHs), which are known to produce a myriad of lethal and sublethal effects in a wide range of biota.

The potential effects of hydrocarbons on marine benthic and intertidal organisms have been the primary focus of research to date. Notwithstanding, organisms that spend some or most of their lifecycle in the pelagic environment, such as the Pacific herring (*Clupea pallasi*), may also be negatively impacted by exposure. The acute toxicity of oil and its components have been well documented for several teleosts (Anderson et al. 1974; Rice et al. 1987) and reported effects in larval and juvenile stages include morphological, histopathological, and genetic damage (Brown et al., 1996; Hose et al. 1996; Kocan et. al. 1996; McGurk and Brown, 1996; Norcross et al. 1996; Carls et al. 1987, 1999; Heintz et al. 1999). Recently, work on the potential mechanisms underlying the common suite of PAH-induced developmental abnormalities in fish have been undertaken (Incardona et al. 2004). Still, more information is certainly needed on sublethal effects to further predictions regarding the risks of exposure to pelagic populations.

The exposure of fish to sublethal concentrations of contaminants can disturb homeostasis and impose considerable stress on physiological systems. The stress responses in teleosts is well documented and involves a series of cellular (e.g. heat shock protein production), neuroendocrine (e.g. catecholamines and corticosteroid release), biochemical (hyperlacticemia and hyperglycemia), and organismal responses (e.g. reduced growth, predisposition to disease, impaired reproduction, and a reduced capacity to tolerate subsequent stress [Adams 1990]), depending on the stressor and duration of its imposition.

Several reasons prompted an examination of the neuroendocrine and biochemical stress responses of juvenile Pacific herring exposed acutely and chronically to the WSF of crude oil. First, the paradigm of the neuroendocrine stress response is well documented in teleosts, and generally yields a consistent pattern for xenobiotic stressors. Second, fish are exposed to dissolved pollutants *via* an extensive respiratory surface and, in seawater, also by drinking. The high bioavailability of many chemicals in water, in combination with a variety of highly sensitive perceptive mechanisms in the integument, typically generate an integrated stress response in fish in addition to toxic effects. The ability of fish to mount an appropriate stress response, and the negative consequences associated with chronic stress, give its measurement both evolutionary and ecological significance.

## Materials and Methods

### *Fish*

Juvenile Pacific herring (8.2 to 13.8 g) were obtained through a local supplier in West Vancouver, BC. Fish were transported to facilities at the Fisheries and Oceans Canada, West Vancouver Laboratory, BC, with a minimal use of nets to reduce trauma to the young fish. Fish

were held in 500-L fiberglass tanks supplied with flowing filtered seawater, salinity 31 ppt, water temperature 11.0±0.5°C and dissolved $O_2$ content above 95% saturation. Following transfer, mortality in the first week was approximately 5.2%, which declined to less than 0.25% per week. When the mortality rate had stabilized, fish were acclimated for a further 4 wk in experimental tanks before an experiment was performed. Fish were fed twice daily *ad libitum* with frozen krill until one day before an experiment.

***Chemicals and Exposure***

Herring were exposed to 3 concentrations of the water-soluble fraction (WSF) of North Slope crude oil in 500-L fiberglass tanks. All exposures were performed in duplicate. WSFs were generated by seawater continuously passing through a modified apparatus developed by Carls et al. (1995, 1998), which consisted of a 15 cm diameter x 80 cm length PVC column containing Siporax® ceramic beads (Aquatic Eco-Systems Inc., Apopka, FL) that had been pre-soaked in North Slope Crude oil for 2 d. Preliminary experiments indicated that oil absorption to the beads is saturated by 48 h. Dilution water up wells through the column and over the oil soaked beads and then flows into the bottom of an individual treatment tank containing herring. A trap inside the column prevents slick overflow. Appropriate levels of hydrocarbons are generated by varying the amount of beads in each column. This method has previously generated water containing initial total polycyclic aromatic hydrocarbon (TPAH) concentrations of 100 µg/L, which declined to approximately 30 µg/L 16 days following the initiation of water flow (Carls et al. 1995, 1998). In control tanks, water flowed through columns with beads that were not soaked in oil. Hydrocarbon analysis of water samples taken at days 1, 2, 4, 7, 14 and 21 by gas chromatography-mass spectroscopy was performed by the National Oceanic and Atmospheric Administration, National Marine Fisheries Service, Auke Bay Laboratory, AK, according to Short et al. (1996). In the first set of experiments, fish were acutely exposed to WSF for 96 h using one column to generate WSF. For the 9-wk chronic WSF exposure, additional new WSF columns were brought on line at 4 and 8 wk to maintain consistent TPAH concentrations with time.

***Sampling and Measurements***

Biological sampling was done at the same time of day to avoid diurnal variations in plasma cortisol or other parameters (Peter et al. 1978). Fish were rapidly netted from tanks and immediately sacrificed by anesthetization in 0.5 mg/L buffered MS 222 (Iwama et al. 1989). Extreme care was taken to minimize any disturbance to fish in other treatment tanks. After weighing, blood samples were obtained from the caudal vasculature using heparinized microcapillary tubes. A sub sample of whole blood was immediately assayed for hemoglobin concentration (Sigma Chemical Co., St. Louis, MO) and the remaining blood was centrifuged at 13,000 x*g*. Following hematocrit and leucocrit determinations, the plasma was separated and frozen in liquid nitrogen. Fish were then dissected, the livers weighed and immediately frozen in liquid nitrogen for subsequent analysis of either biotransformation enzyme activities or glycogen content. All samples were eventually stored at –86°C for no longer than 3 wk before analysis. Plasma cortisol concentrations were determined using radioimmunoassays (IncStar Corp., Stillwater, MN). Plasma was spectrophotometrically analyzed for lactate and glucose using standard colorimetric kits (Sigma). Liver glycogen content was measured in liver samples according to standard procedures (Sweeting 1989). All assays were performed in duplicate.

Plasma was analyzed for ion concentrations using a Model 8015 Benchtop ISE meter (VWR Scientific, Toronto, ON) equipped with specific ion selective electrodes (ISE) for $Na^+$ (Model 84-11, Orion Research Inc., Beverly, MA), $K^+$ (Model K001503-003B, Phoenix Electrode Co.,

Houston, TX), and $Cl^-$ (Model 27502-12, Cole-Parmer Instrument Co., Vernon Hills, IL.). All plasma samples were analyzed in triplicate. Measurements were repeated if the disagreement between triplicates was >3%. Electrodes were calibrated before use and checked against a standard following the measurement of 5 triplicate sets. For quality control, random plasma samples were analyzed for $Na^+$ and $K^+$ using a Pye Unicam SP131 Atomic Absorption Spectrophotometer (Unicam Ltd., Cambridge, UK) for direct comparison to ISE determined values.

Liver microsomes were prepared according to the method of Kennedy (1995). Hepatic microsomal and cytosolic protein content was determined using the method of Bradford (1976). Cytochrome P-450 (P450) content was determined by the method of Omura and Sato (1964), which measures the CO-difference spectra of dithionite-reduced microsomes. The activity of cytochrome P4501A1 (CYP1A) proteins was assessed by the EROD assay according to Burke and Mayer (1974). Glutathione-S-transferase (GST) in the supernatant was measured spectrophotometrically as described by Habig et al. (1974). All assays were performed in duplicate.

***Calculations and Statistics***

Two-way ANOVA and a Holm-Sidak post hoc test were used to determine whether the measured biological parameters differed between replicate trials at the each measurement time. As no differences were detected between trials, data were pooled and differences between treatment groups and exposure times were analyzed using two-way ANOVA and a Holm-Sidak post hoc test. All data analyses were conducted with a fiducial limit significance at $p<0.05$, using SigmaStat version 3.0 (SPSS Scientific, Chicago, IL).

**Results**

*Chemical analysis*

Initial aqueous TPAH concentrations in exposure tanks were: control (0.07 to 0.24 µg/L), low (7.3 to 12.1 µg/L), medium (26.2 to 49.6 µg/L) and high (78.3 to 120.2 µg/L). TPAH concentrations declined with time, and since declines were similar across treatments, each exposure treatment remained distinct (Fig. 1). Comparable aqueous concentrations were reported by Carls et al. (1995) using a similar design. In that study, alkane concentrations ranged from 1.28 µg/L (control) to 119 µg/L with concentration declines similar to those of TPAH. Figure 2 shows the relative contributions of aqueous individual PAH with time. PAHs were predominantly smaller and less substituted (e.g. naphthalenes) at the start of column operation, and progressively shifted to larger and more substituted PAH (e.g. phenanthrenes) with time. This was attributed to exhaustion of smaller PAH from the oiled beads (Carls et al. 1995).

*Bioavailability*

At time 0, there were no significant differences among groups for concentrations and activities of total P450, EROD and GST (Table 1) in the chronic trial. Significant induction of cytochrome P450 was evident in the 40 and 100 µg/L groups by 96 h. Maximum induction occurred at 8 wk, with cytochrome P450 content reaching 2.8-fold higher in fish exposed to the highest concentration of WSF compared to controls. EROD induction was seen in the 10, 40 and 100 µg/L groups by 96 h, with maximum induction in the 100 µg/L group at 4 wk (3-fold increase). GST activity was similar in control, 10 and 40 µg/L groups, and was only significantly elevated in the 100 µg/L group at 8 and 8.3 wk.

*Sublethal effects*

Acute WSF-exposure resulted in a significant stress response in herring while chronic exposure resulted in an apparent abolition of the stress response. Control plasma cortisol, lactate and glucose concentrations did not change significantly over time in either the acute or chronic trials. Plasma cortisol concentrations (Fig. 3) were significantly higher than controls and reached maximum concentrations between 4 and 12 h in the 40 and 100 µg/L groups (62.7±4.0 and 133±6.6 ng/ml, respectively). By 96 h, cortisol concentrations in all groups were at baseline levels and were not significantly different from each other. Plasma lactate concentrations in the 40 and 100 µg/L WSF-exposed fish reached maximum concentrations (8.5±0.4 and 9.4±0.2 mmol/L, respectively) by 6 to 12 h. Plasma glucose concentrations were significantly higher than controls in all WSF-exposed groups and reached maximum concentrations in the 10, 40 and 100 µg/L groups (8.0±0.3, 9.4±0.3 and 10.9±0.3 mmol/L, respectively) between 24 and 48 h. In all groups, glucose concentrations returned to baseline by 96 h. There were no significant effects of short-term WSF exposure on hematocrit (range 22 to 45%), leucocrit (0 to 2.1%), hemoglobin concentration (65.4 to 82.3 g/L), or liver glycogen content (4.4 to 9.7 mg/g). No significant differences were seen in mean plasma $Cl^-$ (control 154±2 mmol/L), $Na^+$ (control 175±4 mmol/L) or $K^+$ (control 4.0±0.3 mmol/L) between 0 and 72 h. A 96-h exposure to the 100 µg/L concentration of WSF led to significant increases over controls in plasma $Cl^-$ (168±3 mmol/L), $Na^+$ (187±4 mmol/L) and $K^+$ (5.2±0.3 mmol/L).

In the chronic 9-wk exposure experiments, fish were exposed to pulses of hydrocarbons and lower molecular weight PAHs at 0, 4 and 8 wk. Significant increases occurred in all parameters in fish exposed to the highest concentration of WSF during the first introduction of aqueous hydrocarbons to the tanks (Fig. 4). Concentrations of cortisol, lactate and glucose were similar to those seen in the 96-h acute exposure. Responses of cortisol and glucose returned to baseline values by 96 h. However, unlike the acute exposure (Fig. 3), lactate values remained elevated at 96 h. When a new column was brought online at 4 wk exposure, a second significant cortisol increase occurred, although it was 52% lower than the initial cortisol peak. No cortisol response occurred with a further column replacement at 8 wk. Similarly, fish exposed to WSF initially responded with increases in both plasma lactate and glucose, however, as new pulses of hydrocarbons were initiated, these responses were muted at 4 wk and not apparent at 8 wk. As positive controls, hydrocarbon naïve groups of herring were kept in parallel conditions as experimental fish, and challenged with hydrocarbons at either 4 or 8 wk to WSF. These fish responded with increases in all of the measured parameters to levels similar to exposed fish at the start of the experiment (Fig. 4), indicating that time in the tanks alone did not contribute to the lack of a stress response in fish exposed to several pulses of hydrocarbons. In the chronic exposure, no significant differences were seen between the WSF group and controls in hematocrit, leucocrit, hemoglobin or liver glycogen concentration.

Subacute (>96 h) and chronic exposure of herring to WSF also caused a significant ionoregulatory disturbance. Significant increases in plasma $Cl^-$, $Na^+$ and $K^+$ were seen by 96 h, which continued to be higher than controls through the majority of the exposure period (Fig. 5). Concentrations of plasma $Cl^-$ in the 100 µg/L group were not significantly different from controls at 8.3 wk.

## Discussion

This study focused on the impacts of aqueous hydrocarbon exposure on an economically and ecologically important teleost, the Pacific herring, and successfully utilized a previous method for exposing fish to aqueous hydrocarbons generated from crude oil (Carls et al. 1995) in which

smaller and more volatile hydrocarbons (e.g. naphthalenes) predominate initially, with larger PAHs (e.g. phenanthrenes) becoming relatively more abundant with time. This study documented the induction of biotransformation enzymes, an initial transient stress response with acute exposure, and an apparent loss of the ability to mount a stress response and a prolonged ionoregulatory disturbance with chronic exposure.

The uptake of hydrocarbons was indirectly assessed by the determination of hepatic cytochrome P450 (P450) levels, EROD and GST activities. Many hydrocarbons in oil, including benzo[a]pyrene are known inducers of P450-associated and other xenobiotic biotransforming enzymes (Kennedy 1995). Induction of P450 and EROD indicated that hydrocarbons were bioavailable to herring in this exposure system. Maximal induction occurred at approximately day 3 and continued for up to 8 wk of exposure. Other studies have shown induction to similar levels with PAHs and water accommodated fractions of crude oil (Gagnon and Holdway 2000; Barron et al. 2003). Maximum hepatic EROD activity induction occurred 2 days following exposure of juvenile Atlantic salmon to the water accommodated fraction of Bass Strait crude oil (Gagnon and Holdway 2000). GST was only induced in the 40 and 100 µg/L WSF concentrations by 4 wk. GST can be induced by PAH exposure, but is typically achieved 1 to 3 wk following exposure (Andersson et al. 1985).

Crude oil, oil in water dispersions, and water-soluble fractions of differing composition have been shown to be acutely toxic to several fish species. For example, adult herring had the lowest reported 96-h $LC_{50}$ to crude oil (1 mg/L) of 39 Alaskan marine species (Rice et al. 1987). No acute mortality occurred in the present study at any WSF concentration. Adult herring mortalities resulting from exposure to TPAH concentrations as low as 28 ppb for 8 days were reported by Carls et al. (1995). However, of the fish that died in the study, 97% had lesions consistent with published descriptions of viral hemorrhagic septicemia virus (VHSV) in Pacific herring. Previous studies have demonstrated that exposure to North Slope crude oil at 0.4 to 5 µg/L TPAH causes malformations, genetic damage, mortality, decreased size, and impaired swimming in larval herring and reduced marine survival in pink salmon (Marty et. al. 1997; Carls et al. 1999; Heintz et al. 2000; Barron et al. 2003). This study demonstrates significant sublethal effects at low TPAH concentrations in juvenile fish.

Teleosts, like other vertebrates, can activate a neuroendocrine response to stressors, which results in increases in circulating concentrations of cortisol, the major corticosteroid in fish plasma. Although the precise mechanisms in fish are unclear, it is generally agreed that important metabolic roles of cortisol during stress include glucose-regulation and glycogen-repletion processes, both of which are important pathways for the recovery from stress (Mommsen et al. 1999). Cortisol may also play a role in the peripheral mobilization of substrates, providing precursors for hepatic gluconeogenesis (Mommsen et al. 1999).

Cortisol values in herring (12 to 21 ng/ml) were well within the baseline range reported for other unstressed teleosts (Morrow et al. 2004). Herring exposed acutely to WSF exhibited a classical stress-related increase in plasma cortisol concentrations within the first hour of exposure, followed by a slow rise in plasma lactate and glucose concentrations. Cortisol, lactate and glucose dynamics were concentration-dependent, results that are similar to those reported for other teleosts (Thomas et al. 1980; Thomas and Rice 1987; Alkindi et al. 1996).

Thomas et al. (1980) suggested that the stress response to WSF is due to components of oil that were acutely toxic to fish, namely the volatile aromatic hydrocarbon fraction that includes naphthalenes, benzene, toluene, ethylbenzene and trimethylbenzene. Naphthalene exposure has been shown to increase plasma cortisol levels in other species (DiMichelle and Taylor 1978; Thomas et al. 1980). Interrenal activation in some species by some pollutants has been attributed to their irritant properties (Schreck and Lorz, 1978). Naphthalene causes gill

hyperplasia in *F. heteroclitus*, a condition that has been demonstrated for most irritants (Gardner 1975). This is further evidence that the noxious volatile components of WSF may be responsible for stimulating the ascending neural pathways of the HPI axis. WSF composition, therefore, may explain the transience of the stress response in herring. Although the relative contribution of naphthalenes to TPAH are still substantial by day 4, the decrease in their relative abundance (Fig. 2) and the decrease in absolute concentrations of TPAH (Fig. 1), may place naphthalene and other volatile chemical concentrations below the threshold necessary for HPI activation.

While the neuroendocrine response to an acute WSF exposure is consistent with other work, the response to chronic pulses was unexpected and novel. A different temporal pattern for cortisol, lactate and glucose concentrations was seen when herring were exposed chronically to WSF, with pulses occurring when new columns were brought on line every 4 wk. Analysis of WSF indicates that with each new column, WSF composition should include proportionately more volatile hydrocarbons at higher concentrations than in the latter stages of WSF generation by replaced columns. If naphthalene and other volatiles are responsible for stimulating the HPI axis, increased cortisol concentrations and a hyperglycemic stress response should result when columns are changed. Interestingly, there was a significant increase in plasma cortisol at 4 wk that was approximately 52% of the initial cortisol peak, and no response at 8 wk.

Several possibilities exist to explain the lack of a stress response with repeated, pulse exposures to the more acutely toxic hydrocarbons at their highest concentrations. First, it is possible that adaptation of the corticosteroid response to hydrocarbons may have partially taken place by 4 wk, and completely at 8 wk. However, the finding for *F. heteroclitus* that continuous exposure to naphthalene for 15 d resulted in a constantly elevated plasma cortisol concentration (DiMichelle and Taylor 1978) suggests that the pulse regimen may be the significant factor in any adaptation. Alternatively, induction of P450-associated enzymes by PAH in WSF may have resulted in increased cortisol metabolism and clearance from blood (Hansson and Lidman 1978). In support of this, biotransformation enzymes including P450 stayed elevated despite oscillating exposure concentrations. Thirdly, some compounds may act directly as endocrine disruptors, targeting the pituitary or adrenocortical tissues (Dorval et al. 2003). Multiple sites in the HPI axis may be affected including the perception of stimuli, synthesis and secretion of the main secretagogue for cortisol (ACTH), the synthesis (e.g. disruption of the steroidogenic acute regulatory [StAR] protein expression [Walsh et al. 2000; Stocco, 2000]) and the secretion of cortisol. For example, the sensitivity of interrenal cells to ACTH was abolished in trout exposed to ß-naphthoflavone (Wilson et al. 1998). More recently, Neelakanteswar and Vijayan (2004) have shown that aryl hydrocarbon receptor (AhR) agonists may disrupt interrenal corticosteroidogenesis and target tissue responsiveness to glucocorticoid stimulation. The WSF in this experiment contains AhR agonists, and it is possible that disruption of the cortisol response process involves several mechanisms. Finally, it has been shown that exposure of fish to PAHs such as naphthalene can result in histopathological effects in a variety of tissues. Mummichogs exposed to naphthalene show major necrosis in interrenal tissues (DiMichelle and Taylor 1978) that could result in a reduced ability to respond to secretagogues or to produce the hormone.

Many toxic substances including petroleum hydrocarbons cause osmoregulatory disturbances in teleosts (Englehardt et al. 1981). In the present study, initial disturbances in plasma ions were expected to coincide with stress-related physiological changes seen at the beginning of hydrocarbon exposure, however, this did not occur. Significant increases in plasma ion concentrations only occurred after 4 days of WSF exposure in the chronic trial and continued beyond the primary stress response to WSF. The long-term osmoregulatory dysfunction caused by hydrocarbon exposure may have several explanations. First, it has been shown that lower

molecular weight aromatic hydrocarbons can cause a decrease in the activity of ion-specific ATPases (Englehardt et al. 1981). Secondly, lipid-soluble hydrocarbons can directly affect membrane permeability, particularly in the gills, which in a hypertonic environment, would lead to increased uptake of monovalent ions (Zbanyszek and Smith, 1984). Thirdly, a preliminary examination of herring gill structure in this study following fixation by standard histological techniques (Speare and Ferguson 1989), embedding, sectioning (4 to 6 µm), and staining (hematoxylin and eosin) of the third gill arch, and examination of lamellae by light microscopy showed that herring gills exposed to WSF generally exhibited epithelial hyperplasia which was more pronounced toward the distal tip of the filament than near the base. The only other morphological alteration in gill tissue was a minor epithelial lifting which also occurred to varying degrees. Other studies have shown similar gill damage and an influx of ions (McKeown and Marsh, 1977; Englehardt et al. 1981). Others have shown much more extensive gill damage upon exposure to oil components. For example, juvenile flounder gills exhibited dose-dependent hyperplasia in the distal one-third of secondary filaments, increases in the thickness of the basal interlamellar troughs, and fusion of adjacent filaments when exposed to crude oil contaminated sediments. Englehardt et al. (1981) have suggested that damage or changes in chloride cells likely account for the electrolyte imbalance.

The lack of a cortisol response with continued WSF exposure may also play a role in the continued hydromineral imbalance observed. Cortisol, in addition to growth hormone and insulin-like growth factor (Mommsen et al. 1999), are considered seawater–adapting hormones (Madsen 1990). Cortisol can increase the cellular differentiation of chloride cells and stimulate branchial $Na^+/K^+$-ATPase activity (McCormick 1995). Glucocorticoid receptor gene expression in chum salmon (*Oncorhynchus keta*) chloride cells (Uchida et al. 1998), support a direct effect of cortisol on chloride cell function in fish (McCormick 1995). Elevated plasma cortisol may be necessary to compensate for the hydromineral imbalance caused by WSF. This hypothesis is further supported by Englehardt et al. (1981) who reported that cortisol supplementation during an oil emulsion exposure was effective in normalizing the hydromineral imbalance in freshwater immature rainbow trout.

The significance of WSF-induced ion imbalances in herring are unknown, however, it is possible that increased ion flux may increase energy costs associated with osmoregulation. It is believed that cortisol is involved in the production of glucose, the preferred oxidative substrate for gill metabolism, which provides energy for protein synthesis and sodium pump activation in seawater. The potential adrenocortical effect of WSF may preclude an appreciable ability for fish to recover from ion disturbances. A disruption of osmoregulation in marine fish and a net gain of ions may impair numerous physiological systems and even lead to mortality. For example, the acutely lethal effect of both copper and silver appears to be an increase in plasma $Cl^-$ and $Na^+$ concentrations along with a pronounced, but not lethal, hyperammoniaemia (Stagg and Shuttleworth 1982; Wilson and Taylor 1993; Hogstrand et al. 1999). In starry flounder (*Platichthys stellatus*) exposed to 1000 µg Ag/L, plasma [$Cl^-$] and [$Na^+$] concentrations taken at the point of death were 245 mM and 224 mM, respectively, in comparison to pre-exposure values of 149 ($Cl^-$) and 172 mM ($Na^+$) (Hogstrand et al. 1999). In the present study, [$Cl^-$] and [$Na^+$] concentrations did rise substantially from control values of 154 and 178 mM, respectively, to 172 ($Cl^-$) and 202 mM ($Na^+$), and although these increases did not result in acute mortality, they represent a significant ionoregulatory disruption.

The results provide clear evidence that endocrine and ionoregulatory effects occur in juveniles at concentrations which have been shown only to affect herring at the embryo/larval stages. Moreover, the pulsatile nature of exposures used in this study have not typically been examined by other investigators, and may represent a realistic and important exposure scenario.

The ecological implications of a decrease in the ionoregulatory capability of fish are perhaps more clear than those of an altered endocrine function. However, it is likely that a diminished stress response and homeostatic ability will result in reduced fitness when fish are presented with biotic or abiotic challenges. Moreover, cortisol's putative role as a hypo-osmoregulatory hormone, and its role in reproduction, growth, and the immune response in fish (Maule et al. 1987; Donaldson 1990; Hontela et al. 1995; Mommsen et al. 1999) further suggest that chronic exposure to low sublethal levels of pollutants such as PAHs which affect adrenocortical function may affect a variety of physiological systems.

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**Table 1.** Cytochrome P450 content (nmol/mg microsomal protein), EROD activity (pmol/min/mg microsomal protein) and GST activity (nmol/min/mg cytosolic protein) of control and exposed herring at three WSF concentrations in the chronic trial. Time of exposure is given as hours (h) and days (d). Values are means ± SE. n=7 * indicates a significant difference ($p<0.05$) between an exposed fish group and the control.

| Parameter | Time | | | | | |
|---|---|---|---|---|---|---|
| | 0h | 96h | 28d | 30d | 56d | 58d |
| Cyt P450 | | | | | | |
| Control | 0.123±0.01 | 0.124±0.012 | 0.123±0.009 | 0.126±0.012 | 0.133±0.011 | 0.112±0.012 |
| Low | 0.126±0.014 | 0.120±0.014 | 0.147±0.015 | 0.160±0.015 | 0.124±0.016 | 0.140±0.015 |
| Medium | 0.125±0.016 | 0.260±0.030* | 0.246±0.016* | 0.255±0.021* | 0.392±0.037* | 0.341±0.032* |
| High | 0.111±0.016 | 0.274±0.030* | 0.351±0.023* | 0.351±0.021* | 0.373±0.023* | 0.348±0.020* |
| EROD | | | | | | |
| Control | 24.7±2.7 | 24.4±2.4 | 24.2±1.9 | 26.2±2.1 | 29.7±3.6 | 24.7±2.6 |
| Low | 25.7±2.5 | 24.5±3.0 | 27.3±2.5 | 33.9±2.4* | 32.6±2.8* | 37.7±2.1* |
| Medium | 25.7±1.8 | 26.2±2.4 | 44.6±4.1* | 37.6±3.1* | 47.2±3.0* | 45.3±4.0* |
| High | 21.8±2.5 | 73.6±8.6* | 114.5±7.8* | 114.6±7.7* | 110.0±7.7* | 113.1±64* |
| GST | | | | | | |
| Control | 105.2±6.2 | 103.8±3.6 | 98.5±3.8 | 101.4±3.3 | 104.1±3.9 | 101.1±8.5 |
| Low | 98.7±3.9 | 98.6±3.1 | 105.7±3.0 | 96.2±2.8 | 95.2±3.2 | 97.3±4.2 |
| Medium | 100.6±3.6 | 96.6±3.1 | 97.6±3.1 | 95.1±4.1 | 101.3±4.2 | 98.3±3.2 |
| High | 102.8±3.8 | 101.0±2.8 | 105.6±5.4 | 100.9±4.1 | 174.6±5.8* | 171.1±7.8* |

**Figure Legend**

Figure 1. Total Polycyclic Aromatic Hydrocarbon (TPAH) concentrations in treatment tanks as a function of time after initiation of water flow through a WSF column. Data points are single composite values for replicate tanks at each time period. (○) Control, (▲) 10 µg/L, (■) 40 µg/L , (●) 100 µg/L.

Figure 2. Individual PAHs as a function of percent TPAH in treatment tanks as a function of time following initiation of water flow through a WSF column.

Figure 3. Plasma cortisol, lactate and glucose concentrations in control fish (○) and those exposed to 10 (■), 40 (□), and100 µg/L(●) concentrations. Values are means ± SE of n=14 fish. * denotes a significant difference ($p<0.05$) from controls.

Figure 4. Plasma cortisol, lactate and glucose concentrations in control fish (○) and those exposed to a 100 µg/L(●) concentration of WSF over 8.3 wk. New pulses of WSF were brought on line at 0, 4 and 8 wk. Single symbols denote control values for fish which had been exposed to WSF at 4 (□) and 8 wk (■) only (naïve fish). Time of exposure is given as hours (h) and days (d). Values are means ± SE of n=14 fish. * denotes a significant difference ($p<0.05$) from controls.

Figure 5. Plasma $Cl^-$, $Na^+$ and $K^+$ concentrations in control fish (○) and those exposed to a 100 µg/L (●) concentration of WSF over 8 wk. New pulses of WSF were brought on line at 0, 4 and 8 wk. Time of exposure is given as hours (h) and days (d). Values are means ± SE of n=14 fish. * denotes a significant difference ($p<0.05$) from controls.

Figure 1.

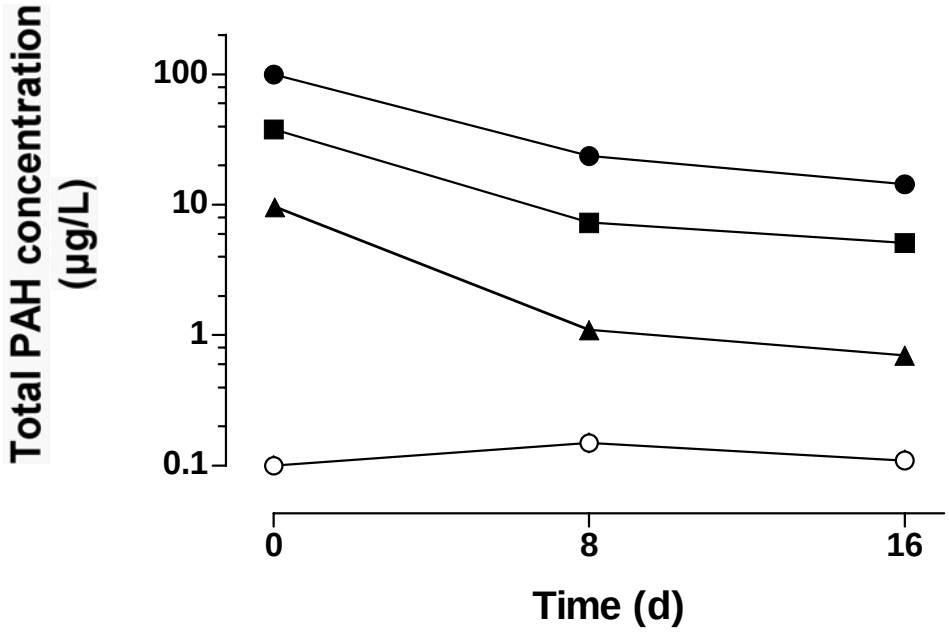
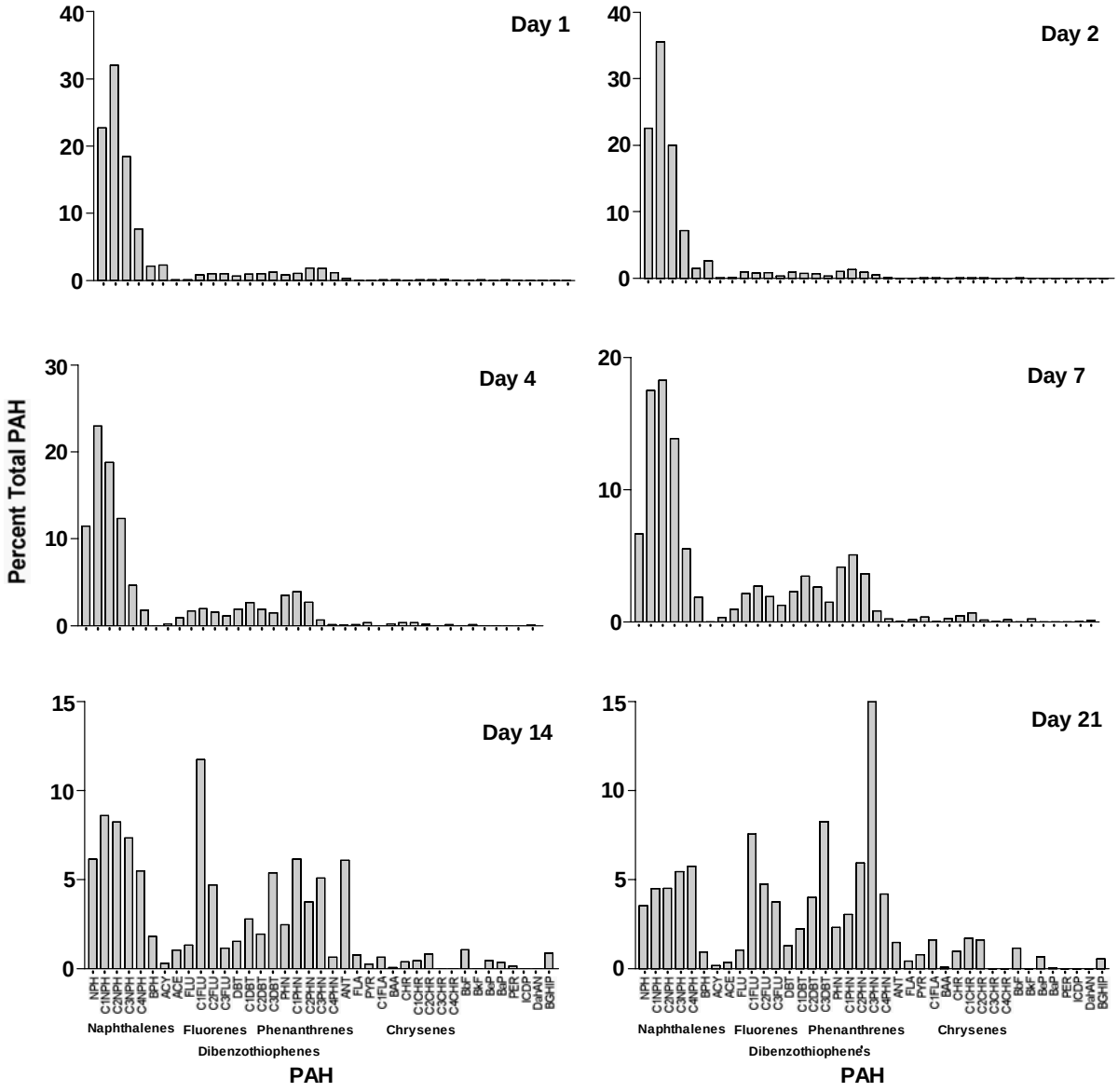


Figure 2.


Day 1
Day 2
Day 4
Day 7
Day 14
Day 21
Percent Total PAH
PAH
Naphthalenes
Fluorenes
Phenanthrenes
Dibenzothiophenes
Chrysenes
NPH
C1NPH
C2NPH
C3NPH
C4NPH
BPH
ACY
ACE
FLU
C1FLU
C2FLU
C3FLU
DBT
C1DBT
C2DBT
C3DBT
PHN
C1PHN
C2PHN
C3PHN
C4PHN
ANT
FLA
PYR
C1FLA
BAA
CHR
C1CHR
C2CHR
C3CHR
C4CHR
BbF
BkF
BaP
BeP
PER
IDP
DahAN
BghiP

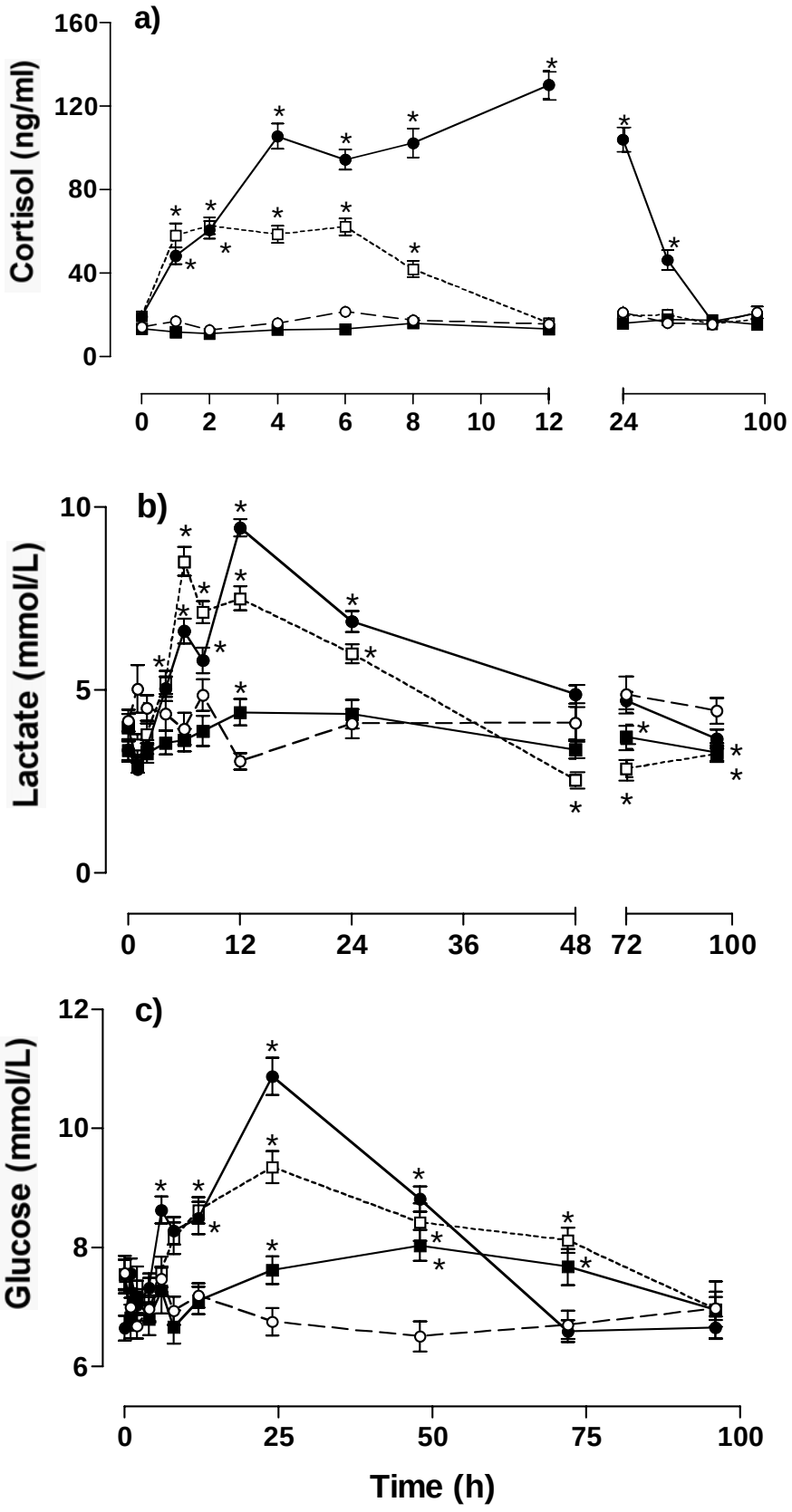

a)
Cortisol (ng/ml)
160
120
80
40
0
0
2
4
6
8
10
12
24
100
b)
Lactate (mmol/L)
10
5
0
0
12
24
36
48
72
100
c)
Glucose (mmol/L)
12
10
8
6
0
25
50
75
100
Time (h)

Figure 3.

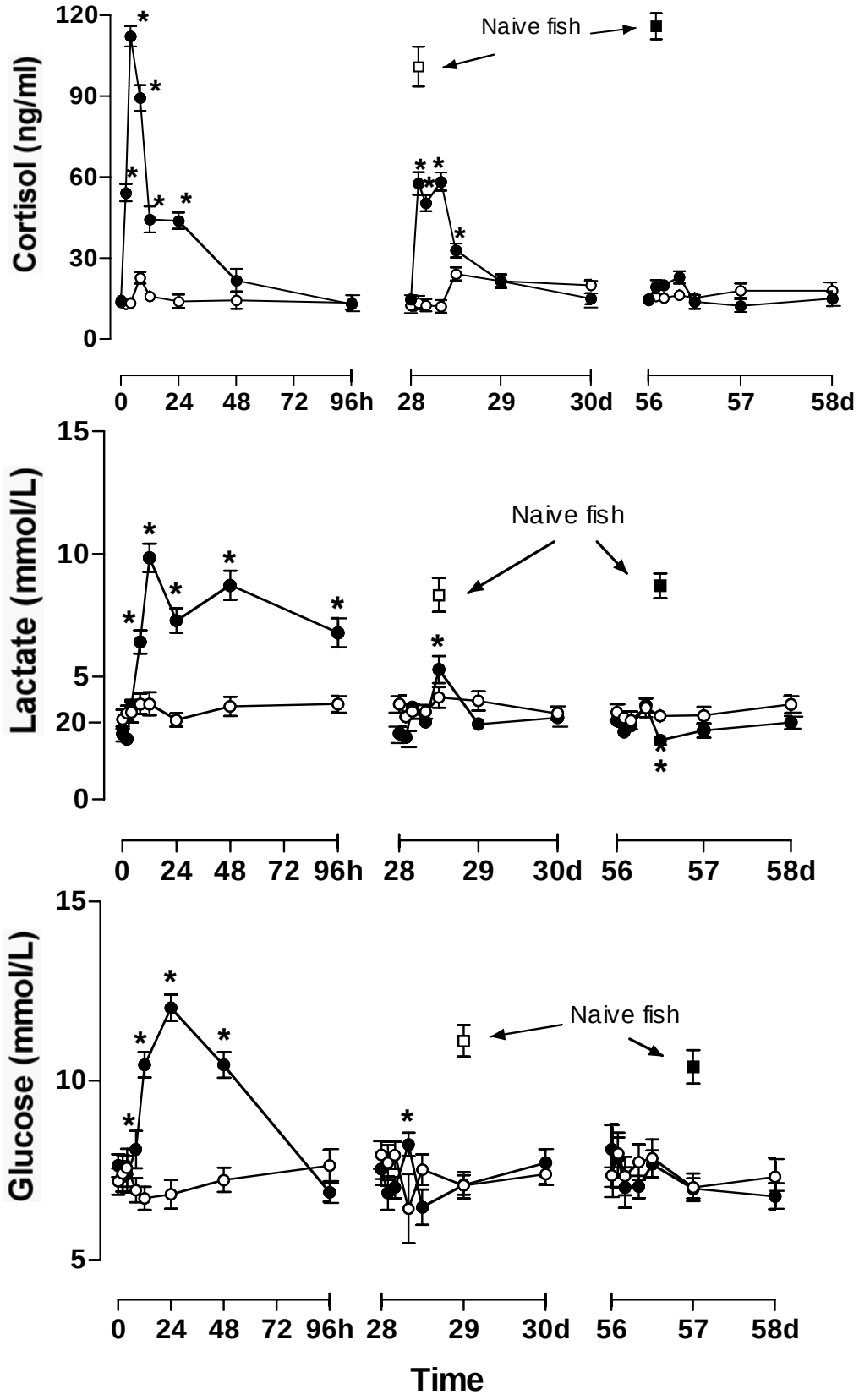

Cortisol (ng/ml)
120
90
60
30
0
Naive fish
0 24 48 72 96h 28 29 30d 56 57 58d
Lactate (mmol/L)
15
10
5
20
0
Naive fish
0 24 48 72 96h 28 29 30d 56 57 58d
Glucose (mmol/L)
15
10
5
Naive fish
0 24 48 72 96h 28 29 30d 56 57 58d
Time

Figure 4.

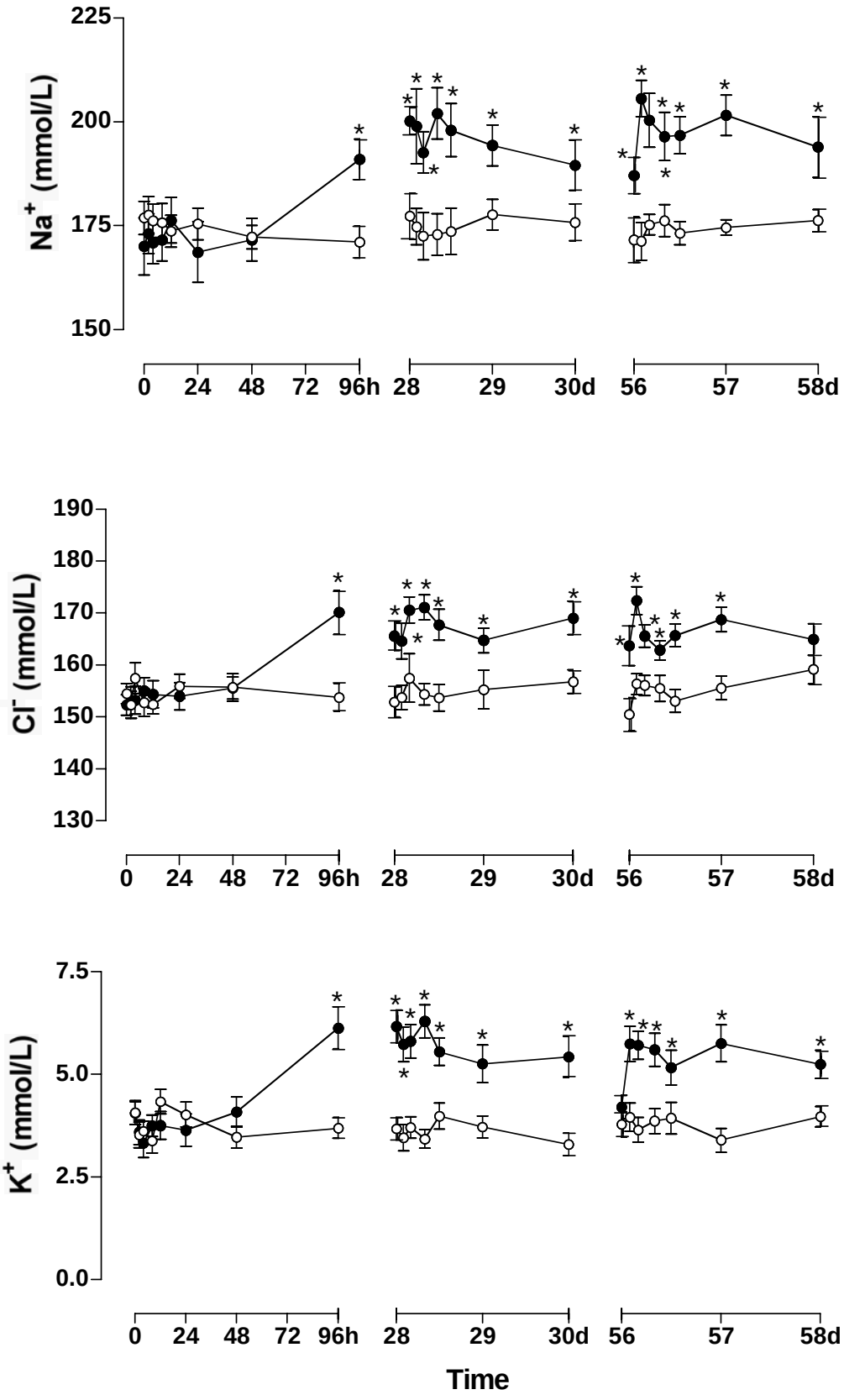

225
200
175
150
Na+ (mmol/L)
190
180
170
160
150
140
130
Cl- (mmol/L)
7.5
5.0
2.5
0.0
K+ (mmol/L)
0 24 48 72 96h 28 29 30d 56 57 58d
Time

Figure 5.

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# Effects of exposure to the water-soluble fraction of crude oil on the swimming performance and the metabolic and ionic recovery post-exercise in Pacific herring

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**Keywords** - Fish, Oil, Polycyclic Aromatic Hydrocarbons, Swimming Performance, Exercise Recovery

**Abstract**-The swimming performance and recovery of juvenile herring from exercise were determined following exposure to the water-soluble fraction (WSF) of North Slope crude oil for over 8 weeks. Mean (±SE) total polycyclic aromatic hydrocarbon (TPAH) concentrations at the beginning of exposures were: 0.2±0.1 (control), 9.6±2.5 (low), 40.7±6.9 (medium), and 120.2±11.4 (high) µg/l. Biological availability of hydrocarbons was confirmed by a significant induction of hepatic cytochrome P450 content and ethoxyresorufin O-deethylase activity. Critical swimming speed (Ucrit) was significantly reduced in fish exposed to the highest concentration of WSF for 96 h (11±3.7% reduction), and at the two highest concentrations at four weeks (16±3.6% and 29±5.4% reductions) and eight weeks (11±3.8% and 40±5.7% reductions). Mortality occurred in all groups of herring 24 h following Ucrit swim trials, with significantly higher mortalities in fish exposed to WSF in a concentration- and time-dependent manner (maximum mortality [72.2±5.5%] in the 8 week, high oil exposure group). Burst swimming alone resulted in increases in plasma cortisol, lactate, $Na^+$ and $Cl^-$ concentrations, and decreases in muscle glycogen that returned to baseline values by 24 h. An interpretation of WSF-exposure on post-exercise metabolic recovery was complicated by pre-exercise alterations in several parameters. The time courses and magnitudes of several key post-exercise parameters including plasma cortisol, lactate and muscle glycogen were significantly altered by WSF-exposure. This study clearly shows that hydrocarbon exposure can reduce the swimming ability of fish and their ability to recovery from exhaustive exercise.

## Introduction

The ubiquitous and pervasive nature of petroleum-derived hydrocarbons and the magnitude of their input to aquatic ecosystems are the two main impetuses for research focused on their toxicity to aquatic organisms. Spills of crude oil are a large contributor to global hydrocarbon pollution. The most bioavailable fraction of oil to marine biota such as teleosts are the dissolved hydrocarbons which include the polycyclic aromatic hydrocarbons (PAHs). The general importance of PAHs as a toxic component of petroleum-derived hydrocarbons is well established. Polycyclic aromatic hydrocarbons are acutely toxic to fish and have several sites of action, including those at the genetic level [1] through to those affecting organism bioenergetics and ecology [2]. Predicting the toxicity of these compounds is fraught with challenges including dynamic chemical profiles, nonspecific and chemical-specific mechanisms of action, and variable intra- and interspecies sensitivities. Moreover, because hydrocarbons from oil spills can persist in near shore sediments for decades or longer [3], investigations into toxicity need to incorporate long-term exposure regimes.

An extensive literature on the acute toxicity of oil and its components exists for various teleost species. For example, Pacific herring (*Clupea pallasi*), the dominant ichthyofaunal species in Prince William Sound during the 1989 spill from the Exxon Valdez, have the lowest 96-h LC50 value to crude oil (1 mg/L) among 39 Alaskan marine species [4]. Larval and juvenile stages of herring exposed to hydrocarbons also show genetic damage, histopathological and morphological effects [5].

PAHs are also implicated as endocrine disruptors in fish, specifically as modulators of steroidogenesis [6]. Immunotoxicity and carcinogenicity are both hallmarks of PAH toxicity in teleosts. Moreover, individual PAHs have distinct and specific developmental consequences when fish are exposed at early life history stages [7]. For example, defects in zebra fish embryos induced by dibenzothiophene or phenanthrene appear to have direct effects on cardiac conduction, leading to secondary consequences in cardiac morphogenesis, kidney and neural tube development. However, the etiology for pyrene exposure, is completely different, consisting of anemia, peripheral vascular defects, and neuronal cell death, effects similar to those described for potent aryl hydrocarbon receptor ligands [7]. Consequently, the multifaceted acute and chronic toxicities presented by organisms exposed to petroleum hydrocarbons presents a unique challenge in assessing sublethal toxicity with performance indicators that rely on the optimum functioning and integration of several key physiological systems, any or all of which may be targets for toxicity.

We selected locomotor capacity and exercise recovery ability as integrated measures to examine the effects of PAH exposures in Pacific herring. Locomotor capability is generally cited as a potential fitness parameter because of its direct impact on foraging success, predator-prey interactions and dominance hierarchy encounters. A less commonly stated, but potentially important, constraint on fitness is the ability to move between habitat patches of varying quality, or among patches required for successful completion of different biological functions such as reproduction or sheltering. Interestingly, recovery from bouts of intense locomotor activity can be equally important, if several movements are needed in a relatively short time period and this may be particularly important for repeated attacks by predators.

The measurement of locomotory performance has been suggested as an important criterion in the determination of sublethal effects of toxicants on fish. Sublethal contaminant exposure can impose substantial stress on physiological systems, resulting in decreased performance, especially of integrated systems such as locomotion. As a result, locomotion (e.g. critical

swimming speed, Ucrit) has previously been used to define tolerance limits to a wide variety of pollutants and environmental parameters, including bleached Kraft pulp mill effluent [8], metals [9], and pesticides [10]. In fish, Ucrit is a commonly used measure of prolonged swimming performance. Ucrit has been used as a physiological endpoint to assess the impact of environmental stress on fish, including challenges such as unfavorable temperatures, hypoxia and disease and toxins [10]. The ecological relevance of Ucrit is that it appears to be a good indicator of the ability of a fish to swim through stretches of strong current, for example, when salmon migrate upstream because maximum aerobic capacity is measured during these tests [11]. In addition, the repeatability of Ucrit assessment is very high at the level of individual fish both in the short and the longer term [11]. Coupled with the utility of Ucrit and recovery measurements as integrated physiological endpoints, is the well-established sensitivity of Ucrit with respect to alterations in both biotic and environmental factors [12]. Therefore, given the difficulty of extrapolating between fish species and the absence of Ucrit data for Pacific herring, the objective of this study was to determine if exposure to the water soluble fraction (WSF) of oil (specifically concentrations in the µg/l range) affects herring swimming performance and their recovery from exercise.

**Materials and methods**

*Fish*

Juvenile Pacific herring, (*Clupea pallasi*; 9.2 to 14.2 g) were obtained through a local supplier in West Vancouver, BC. Fish were transported to facilities at the Department of Fisheries and Oceans Canada, West Vancouver Laboratory, BC. Fish were held in 500-L fiberglass tanks supplied with filtered flowing seawater, with a salinity of 31 ppt, a water temperature of 10±0.5°C and a dissolved $O_2$ content >95% saturation. Following transfer, mortality in the first week was approximately 4.1%, which then declined to < 0.25% per week. When the mortality rate had stabilized, fish were acclimated for a further four weeks in experimental tanks before an experiment was performed. Fish were fed twice daily ad libitum with frozen krill until one day before an experiment.

*Chemicals and Exposure*

Herring were exposed to three concentrations of the water-soluble fraction of (WSF) of North Slope crude oil (which was a gift from Dr. R. Kocan) in 500-L fiberglass tanks for up to 8.3 weeks. Water samples were taken in triplicate and analyzed as in [14] by the National Oceanic and Atmospheric Administration, National Marine Fisheries Service, Auke Bay Laboratory, AK. WSFs were generated by seawater continuously passing through a modified apparatus developed by [13], as described thoroughly in [14]. Briefly, the apparatus consisted of a polyvinyl chloride (PVC) cylinder containing ceramic beads that had been soaked in North Slope Crude oil. Dilution saltwater up wells through the cylinder and over the oil soaked beads and into treatment tanks. This method generates seawater containing initial total polycyclic aromatic hydrocarbon (TPAH) concentrations of up to 100 µg/l [14].

*Swimming performance*

Following WSF exposure, a modified swim chamber as described by [10] was used to determine the swimming performance of juvenile herring ($n$=480). A Valeport current meter (Valeport Marine Scientific Ltd., Dartmouth, UK) was used to determine water velocity within the test chamber. After exposure to WSF (in triplicate for each concentration), fish ($n$=6-8) were removed from exposure tanks using buckets to reduce trauma and scale loss and placed in a swim chamber supplied with flowing fresh uncontaminated seawater [10]. Fish were allowed 3

h to acclimate to the chamber before a swim trial began. The fatigue speed, or critical swimming speed (Ucrit), is a measure of prolonged swimming performance and was determined by a modified procedure of [10]. The initial water velocity was approximately 0.5 body lengths (bl)/s based on the average body length of the group based on a subsample of fish from a control tank. Water velocity was increased by 0.75 bl/s every 30 min for this incremental velocity test. A fish was considered exhausted when it did not respond to gentle prodding whenever it rested at the screen at the back of the chamber. The time of exhaustion and length of the fatigued fish were taken to determine Ucrit. Fish from each treatment group were then housed in separate tanks and monitored for 24 h.

*Recovery from swimming*

High post-exercise mortality occurred in fish that were swum in the Ucrit swim trials described above. Therefore, beyond an assessment of post-exercise mortality, the effects of WSFs on exercise recovery could not be readily determined from Ucrit tests. In a separate set of experiments, fish ($n$=384) were exposed to three concentrations of WSF as described above (in triplicate), and fish were subsequently exercised by chasing them in a 250 L oval tank [15,16] during which they swam in bursts for 5 min. This method of exercise has been well established to exhaust fish, as indicated by the near total depletion of white muscle glycogen, adenosine triphosphate, and phosphocreatine stores [15,17]. Accordingly, we sampled blood and muscle tissues from fish ($n$=6-8) before exercise (controls) and then at 0, 1,2,4,6,8,12 and 24 h following 'burst' swimming. To minimize the physiological effects of handling stress, fish were immediately anesthetized in 0.5 mg/L MS222. Each fish was quickly blotted dry, and its weight and length recorded. After weighing, blood samples were obtained from the caudal vasculature using heparinized microcapillary tubes and centrifuged at 13,000 x $g$ for 1 min. Following hematocrit and leucocrit determinations, the plasma was separated and frozen in liquid nitrogen. Livers were excised from the fish and a sub-sample of muscle from the left lateral side of the fish was removed and tissues were frozen until analysis. All samples were eventually stored at –86°C for not longer than three weeks before analysis.

Plasma cortisol concentrations were determined using a radioimmunoassay (IncStar Corp., Stillwater, MN). Plasma lactate and glucose were analyzed spectrophotometrically using standard colorimetric kits (Sigma). All assays were performed in duplicate except where indicated that triplicates were performed. Plasma was analyzed for ion concentrations using a Model 8015 Benchtop ISE meter (VWR Scientific, Toronto, ON) equipped with specific ion selective electrodes (ISE) for $Na^+$ (Model 84-11, Orion Research Inc., Beverly, MA), and $Cl^-$ (Model 27502-12, Cole-Parmer Instrument Co., Vernon Hills, IL.). All plasma samples were analyzed for ions in triplicate. Measurements were repeated if the disagreement between triplicates was >3%. Electrodes were calibrated before use and checked against a standard following the measurement of five triplicate sets. For quality control, random plasma samples were analyzed for $Na^+$ using a Pye Unicam SP131 Atomic Absorption Spectrophotometer (Unicam Ltd., Cambridge, UK) for direct comparison to ISE determined values. Muscle glycogen was assayed as in [15]. Livers were analyzed for cytochrome P450 or ethoxyresorufin O-deethylase (EROD) activity as estimates of hydrocarbon bioavailability. Liver microsomes were prepared and enzyme activities assayed according to [18]. Briefly, selected livers were homogenized in ice-cold buffer: 150 mM KCl, 0.2 M HEPES, 5 mM EDTA, pH 7.4. The homogenate was centrifuged for 20 min at 10,000 x $g$ at 4°C. The supernatant was recentrifuged for 65 min at 100,000 x $g$ at 4°C. The microsomal pellet was resuspended in a 150 mM KCl, 100 mM Tris-HCl, 20% glycerol v/v buffer, pH 7.4. Hepatic microsomal and cytosolic protein

content, cytochrome P-450 (P450) content and EROD activity were measured [18]. All assays were performed in duplicate.

*Calculations and Statistics*

Two-way analysis of variance (ANOVA) and a Holm-Sidak post-hoc test were used to determine whether the swim speed and measured biological parameters differed between replicate trials at the each measurement time. As no differences were detected between trials, data were pooled and differences between treatment groups and exposure times were analyzed using two-way ANOVA and a Holm-Sidak post-hoc test. All data analyses were conducted with the fiducial limit significance set at $p<0.05$ using SigmaStat version 3.0 (SPSS Scientific, Chicago, IL).

## Results

*Chemical analysis of water*

Aqueous TPAH concentrations in exposure tanks at time T=0 following the start up of a cylinder were: control (0.12 to 0.20 µg/L), low (8.3 to 11.7 µg/L), medium (28.2 to 50.9 µg/L) and high (87.5 to 145.2 µg/L). TPAH concentrations declined with time and since the percentage declines were similar across treatments, each exposure treatment remained distinct (Fig. 1). Due to this decline, and the total length of exposures (up to 8.3 weeks) we decided that to maintain relatively constant TPAH concentrations, new cylinders would be brought on line at 0, 4 and 8 weeks. Therefore, fish were subjected to 'pulses' of hydrocarbons when new cylinders were employed, followed by declines in concentrations with time.

*Bioavailability*

The induction of hepatic biotransformation enzymes was used as a bioindicator of hydrocarbon exposure and uptake in this experimental system. The content of cytochrome P450 and the activities of EROD in fish livers from each of the experimental groups are shown in Table 1. At T=0, there were no significant differences among groups. Significant induction of cytochrome P450 was evident in the medium and high WSF exposure groups 96 h after exposure. Cytochrome P450 content was approximately 5.4-fold higher in fish exposed to the highest concentration of WSF compared to controls. Although EROD was also significantly elevated after a 96-h exposure, the time course to maximal EROD induction was slower than that seen for cytochrome P450. In the medium and high WSF exposure groups, maximum (an approx. 5.3-fold increase) EROD occurred after 8.3 weeks of exposure. The low WSF exposure concentration did not result in increases in either EROD or cytochrome P450 (Table 1).

*Swimming performance and post-swim mortality*

The Ucrit values of control fish ranged from 5.2 to 5.4 bl/s and did not significantly change over the course of the experiment (Fig. 2). Ucrit values for fish exposed to the highest WSF concentration for 24 h were significantly lower compared with control fish. For the medium and high WSF exposure groups, Ucrit was significantly reduced through the 96 h to 8.3 week exposure period. The decreases in Ucrit were dependent on both exposure time and exposure concentration, with maximum depression of Ucrit (approx. 40%) occurring after 8.3 weeks in the high WSF exposure group. Concurrent with the lack of hepatic enzyme induction, there was no significant effect on Ucrit of the low WSF exposure group.

The original intent of this study was to examine the effect of WSF exposure on recovery of herring from exhaustive exercise in a Ucrit test. However, this was not possible because there were significant post-swim mortalities in all treatment groups at 24 h post-swim (Fig. 3). Mortalities in control fish ranged from 22 to 28% and as expected, there was no difference in this mortality for the low WSF exposure group. Exposure to the medium and high WSF concentration increased post-swim mortality over controls. Mortalities increased to approximately 50% for the medium WSF exposure group and remained constant through the exposure period. For the high WSF exposure group, mortality was also approximately 50% after a 96-h exposure period but increased with exposure time and reached 72±6% after 8.3 weeks exposure.

*Exercise recovery*

In order to determine the physiological effects of WSF exposure on the ability of herring to recover from exercise, fish were forced to burst swim for 5 min, and then assayed for various parameters during recovery.

In control fish, hematocrit (Hct), leucocrit (Lct), plasma cortisol, lactate, $Na^+$, $Cl^-$, and muscle glycogen concentrations were measured before and for 24 h after burst exercise. Burst exercise did not significantly affect either Hct (range 25% to 47%) or Lct (range 0% to 2.3%). Plasma cortisol concentrations prior to exercise ranged from 14 to 25 ng/ml and as expected, increased significantly post-exercise and had returned to the pre-exercise level by 8 h (Fig. 4). Similarly, plasma $Na^+$ and $Cl^-$ concentrations peaked post-exercise and had recovered by 8 h. The post-exercise increase in plasma lactate had subsided by 4 h, whereas the recovery of the decrease in muscle glycogen concentrations took 12 h. Consequently, by 24 h post-exercise, all of the measured variables had recovered to pre-exercise levels.

Consistent with the other results, the low WSF exposure group did not differ significantly in its response to burst exercise compared with control fish. Consequently the following descriptions focus only on the comparison between the control fish and the medium and high WSF exposure groups.

In the 24 h WSF exposures, the medium and high groups showed significant pre-exercise physiological disturbances. In WSF-exposed fish, pre-exercise plasma cortisol and lactate concentrations were significantly higher compared to the control group (Fig. 4). In addition, the peak plasma cortisol and lactate concentrations were significantly higher in the high WSF exposure group. Plasma cortisol had not recovered by 24 h post-exercise, but plasma lactate had returned to pre-exercise levels. Peak plasma cortisol concentrations were also significantly higher in the medium WSF exposure group compared to controls, but lower than the high WSF exposure group and had recovered by 8 h post-exercise which were similar to controls. In contrast to plasma cortisol, medium and high WSF exposure did not significantly affect the pre- and post-exercise levels for plasma $Na^+$ and $Cl^-$ concentrations or muscle glycogen concentrations (Fig. 4).

In 96-h WSF exposures, the stimulatory effects of WSF exposure on pre-exercise concentrations of plasma cortisol and lactate were not evident and concentrations were similar to control fish pre-exercise (Fig. 5). However, plasma $Na^+$ and $Cl^-$ concentrations were significantly elevated in the high WSF exposure group pre-exercise (Fig. 5). Post-exercise, the pattern of increased plasma cortisol and lactate concentrations and reduced muscle glycogen were identical to that of the control fish. However, the peak responses of the plasma $Na^+$ and $Cl^-$ concentrations were exacerbated for the high (but not the medium) WSF exposure group and had not returned to control levels by 24 h post-exercise (Fig. 5). Thus, the higher pre-exercise concentrations persisted beyond the recovery of other parameters from burst activity.

In 4.3 week WSF exposures, pre-exercise baseline status was similar to that observed for the 96-h exposure period with plasma cortisol and lactate and muscle glycogen concentrations being identical to those of control fish, while the pre-exercise plasma $Na^+$ and $Cl^-$ concentrations were significantly higher than control fish in both the medium and high WSF groups (Fig. 6). Post-exercise, there was a significant suppression of the exercise-induced peak response of plasma cortisol concentrations in both the medium and high WSF exposure groups compared with control fish. In addition, plasma cortisol returned to pre-exercise values more rapidly. Concurrent with the suppression of the cortisol response to exercise, and in contrast with the 24 h exposure group the post-exercise response of plasma lactate was essentially the same as that of control fish. In contrast, the pre-and post-exercise plasma $Na^+$ and Cl- concentrations were very similar to the response seen at 96 h exposure and remained elevated compared with the control baseline condition (Fig.6).

In 8.3 week WSF exposures, pre-exposure concentrations were not different from the 4.3 week exposure group, which showed significant elevations in plasma $Na^+$ and $Cl^-$ concentrations. Consistent with the progressive changes that occurred with longer WSF exposure, by 8.3 weeks the typical exercise-induced peak responses of plasma cortisol and lactate were completely suppressed in the medium and high WSF exposure groups. Post-exercise there was no significant increase in either of these parameters (Fig. 7). The post-exercise depression of muscle glycogen recovered significantly faster in WSF-exposed fish than in controls (Fig. 7). Similar to the 4.3 week WSF exposure group, pre and post-exercise plasma $Na^+$ and $Cl^-$ concentrations remained significantly higher in the both the medium and high WSF exposure groups compared with control fish (Fig. 7).

**Discussion**

Limited information on the impacts of short term aqueous hydrocarbons exposure on pelagic teleost species was the motive for the present study on swimming performance and exercise recovery in Pacific herring. This study used a WSF-generating apparatus to produce different concentrations of aqueous hydrocarbon dispersions in exposure tanks. Consistent with the known changes in the chemical composition of oil slicks over time, these WSF exposures consisted of predominantly smaller and more volatile hydrocarbons (e.g. naphthalenes) in the early part of the exposure period, with larger PAHs (e.g. phenanthrenes) becoming relatively more abundant over time as the overall TPAH concentration decreased with time [14]. This allowed us to study concentration-dependent as well as time-dependent effects. In fact, we discovered important time-dependent as well as the expected concentration-dependent effects of WSF exposure on Ucrit, Ucrit post-exercise mortality, pre-and post-exercise levels of plasma cortisol, lactate, $Na^+$ and $Cl^-$, and post-exercise recovery of muscle glycogen. Moreover, there was a clear threshold for the sub-lethal responses we measured for WSF exposure. Exposure to the lowest WSF concentration had no significant effect compared with control fish for any of the parameters that were measured, whereas the medium and high WSF exposure groups showed significant effects as early as 24 h and for as long as 8.3 weeks.

In order to determine if hydrocarbons in exposure tanks were bioavailable to herring, the induction of biotransformation enzymes including hepatic cytochrome P450 (P450) content and EROD activities were measured. Several components in the oil water dispersion created by the apparatus, including benzo[a]pyrene, are known inducers of P450-associated and other xenobiotic biotransforming enzymes. Increased levels of P450 and EROD activities in WSF-exposed fish indicated that hydrocarbons were bioavailable to herring in this exposure system.

Maximal induction of EROD in this study occurred at approximately week eight of this study, whereas the maximal response of cytochrome P450 occurred sooner at 96 h that persisted for the 8.3 week exposure period. Exposure to hydrocarbons such as PAHs, water-accommodated fractions of crude oil, and WSFs will induce enzymes to similar levels.

The Ucrit test was chosen because it was deemed to be an appropriate swim test for a rover-predator teleost species, and therefore would have relevance towards determining Darwinian fitness of fish in the field [19]. In this study, reductions in Ucrit occurred in a WSF concentration-dependent and time-dependent manner, and became significant at sublethal concentrations as low as 41 µg/l PAH by 96 h. In another study, exposure of coho to the WSF of Cook Inlet crude oil only affected swimming performance at >2.5 mg/l in 48-h exposures and at 0.66 mg/l in longer exposures of 5 to 13 d [20]. These results show that hydrocarbon exposure in the µg/l range can influence swimming performance when exposures are of a longer duration.

Impaired Ucrit is often related to an inability to supply enough oxygen to the gills, deliver enough oxygen to the tissues, remove metabolic products, provide adequate substrates, or to activate enzymatic processes. For example, a concentration-dependent impairment of gill structure as a result of 2-(thiocyanatomethylthio)-1,3-benzothiazole (TCMTB) exposure was correlated with a decrease in Ucrit in juvenile salmon [10]. It is likely that WSF impairs respiratory gas exchange based on observations of gill hyperplasia that was more pronounced toward the distal tip of the filament than near the base [14]. These observations are consistent with previous studies documenting gill damage with exposure to hydrocarbons [21,22] with much more extensive gill damage upon exposure to oil components. Such gill changes could also adversely affect extra-renal excretion and ionic exchange at the gills and explain some of the ionic effects observed in the present study, which are discussed below.

Understanding the effects of WSF on post-exercise recovery is complicated because of its effects on pre-exposure levels of recovery indicators including plasma cortisol, lactate, and plasma $Na^+$ and $Cl^-$ concentrations. The elevated level of plasma cortisol and lactate levels in WSF-exposed fish at 24 h before exercise indicate that WSF causes a classical stress response. Higher cortisol and lactate concentrations were concentration-dependent, results which are similar to those reported for striped mullet (*Mugil cephalus*) exposed to the WSF of No. 2 fuel oil [23], flounder (*Pleuronectes flesus*) exposed to WSF of Omani crude oil [24], and juvenile coho salmon (*Oncorhynchus kisutch*) exposed to the WSF of Cook Inlet crude oil [20]. The stress response in the present study is transient as cortisol and lactate concentrations returned to baseline values by 96 h.

Interestingly, baseline plasma cortisol concentrations were higher in the group exposed to the highest concentration of WSF at the 4 week sampling point compared to the 96 h sampling period, which coincided with the installation of a new WSF-generating column. This is attributed to the high total PAH concentrations, and the higher proportion of lower molecular weight compounds such as naphthalenes that result when new generator operation is initiated [14]. A stress response to WSF is likely due to components of oil which are acutely toxic to fish, namely the volatile aromatic hydrocarbon fraction that includes naphthalenes, benzene, toluene, ethylbenzene and trimethybenzene [23]. Naphthalene exposure has been shown to increase plasma cortisol levels in *Fundulus heteroclitus* [25] and mullet [23]. However, [14] attributed a lack of a cortisol response with cylinder replacement at eight weeks as seen in the present study to adaptation of the corticosteroid response to hydrocarbons, exhaustion of the cortisol response, induction of P450-associated enzymes by PAH in WSF resulting in increased cortisol metabolism and clearance from blood [26], or some compound acting directly as endocrine disruptors, targeting the pituitary or adrenocortical tissues [27]. The results of this study are

strong evidence for the latter mechanism, since exercise did not result in an increase in plasma cortisol concentrations as is seen in control fish.

WSF exposure also affected pre-exercise plasma $Na^+$ and $Cl^-$ concentrations. Many toxic substances including petroleum hydrocarbons can cause osmoregulatory disturbances in teleosts [21,22,14]. Although rapid ionic disturbances are typically seen in stressful situations no significant change in plasma $Na^+$ or $Cl^-$ concentrations was noted until 96 h exposure to WSF, which continued for eight weeks of exposure. The long-term osmoregulatory dysfunction caused by hydrocarbon exposure may have several explanations: lower molecular weight aromatic hydrocarbons can cause a decrease in the activity of ion-specific ATPases [28.22], lipid-soluble hydrocarbons can directly affect membrane permeability, as well as affecting gill structure which would modulate ion flux across the gill epithelium.

In addition to the possible effects of WSF on gills and oxygen transport, the high levels of plasma ions may also affect swimming ability in herring. It has been suggested that as environmental ion composition deviates from the regulatory set point of animals, basal osmoregulatory costs increase, reducing an animal's scope for activity [29]. For example, exposure of more sensitive species of perch to acid water potentially increase osmoregulatory costs which result in reduced swimming ability [30]. Although difficult to determine, it is possible that increased osmoregulatory costs reduced the ability of fish to swim in the present study.

The method used to exercise fish in this study exhausts them, as indicated by near depletion of muscle glycogen stores [31]. This technique is reproducible in that fish are exhausted to the same degree, again as indicated by muscle metabolite status [15]. This technique also results in the same type of metabolic and endocrine disturbances as is seen in studies where fish were exercised to exhaustion in a swim tunnel [17]. This type of high-intensity, burst-type exercise is itself stressful to the fish [31]. Typically, exhaustive exercise causes an elevation in circulating levels of cortisol and catecholamines [32,33]. Cortisol peaks approximately 1-2 h post-exercise [32]. Currently, evidence suggests that the stimulus for cortisol release is not exercise *per se*, but rather post-exercise inactivity [16]. Muscle glycogen is broken down with an associated accumulation of lactic acid, which quickly dissociates into lactate and metabolic protons [32, 34,35], some of which leaks into the blood [35]. The resynthesis of glycogen often requires 4-6 h and often up to 12 h in some species [15]. Plasma lactate levels typically return to baseline levels by 6-12 h [31].

Exhaustive exercise stress also causes a large disturbance of ionic, osmotic and fluid volume homeostasis [34]. The hormonal responses in exercise lead to osmotic imbalances in fishes in hypotonic and hypertonic environments. A major component contributing to these imbalances is increased gill permeability that promotes passive movements of small ions and water along concentration gradients.

In control fish in this study, exercise resulted in typical increases in plasma cortisol and lactate, and $Na^+$ and $Cl^-$ concentrations and decreases in muscle glycogen values, which recovered within time frames reported for other teleosts. At 24 h, WSF-induced stress accompanied by swimming-induced stress resulted in the highest levels of cortisol and lactate, and a limited recovery of these parameters. [20] also showed that the stress from WSF and swimming were cumulative. Muscle glycogen and plasma ion levels showed typical exercise effects and recovery, and were not affected by higher levels of cortisol in swum WSF-exposed fish. At 96 h, WSF no longer induced a stress response, however, osmoregulatory dysfunction was evident by higher pre-exercise ion concentrations, which remained elevated following exercise. All other parameters responded similarly to control fish.

At 4.3 to 8.3 weeks exposure to WSF, two important effects of WSF were occurring in herring that affected both pre- and post-exercise responses, namely osmoregulatory dysfunction and an impaired stress (cortisol) response. Following exercise at 4 weeks, plasma cortisol increased in control fish, but was significantly lower in the high WSF-exposed groups. In fish exposed to WSF for 8 week, plasma cortisol levels did not increase above baseline levels in the medium and high exposure groups following exercise, indicating a severely impaired cortisol response. Plasma lactate concentrations in pre-exercised fish were similar in all groups, however, the response to exercise varied, a pattern that was similar to muscle glycogen levels; in fish that produced limited cortisol (high group), in response to exercise, both plasma lactate and muscle glycogen levels recovered faster than fish which showed a typical cortisol response to exercise. Studies have shown that the removal of cortisol elevations through post-exercise activity [16] or pharmacological inhibition [31] result in an enhancement of metabolic recovery in rainbow trout. It has been suggested that low cortisol levels promote lactate-based *in situ* glycogen synthesis and therefore blood lactate levels are lower due to a reduced transfer from muscle to blood as the lactate is rapidly channeled to glycogen synthesis. Alternatively, the lower blood lactate levels in swimming fish may reflect enhanced removal of lactate from the blood.

Typical increases in plasma ions occurred in control fish with exercise and decreased to baseline levels by 24 h. In fish exposed to WSF for 96 h or longer, plasma ions continued to be higher than in control fish at all time points both pre- and post-exercise, presumably due to effects discussed previously. Increases in plasma ions were not exacerbated by swimming, as was found with post-exercise cortisol levels. It is possible that the ionoregulatory dysfunction contributed to post-swim mortality in WSF-exposed fish, however, no clear relationship between WSF concentration, post-exercise ion concentrations and post-swim mortality could be discerned.

This study clearly shows that WSF exposure affects both the swimming performance of herring and their ability to recover following burst swimming at very low µg/l concentrations. WSF exposure for different time periods resulted in different effects, in the short-term, it induced stress, and in the long-term, an absence of cortisol secretion and osmoregulatory dysfunction, conditions which altered the pre-exercise condition of the fish and their response to exercise. Overall, these results indicate that exposure to oil can have both lethal and sublethal effects on herring fitness by significantly affecting locomotory performance of these feral animals, which is of considerable interest from management, physiological, environmental, ecological and evolutionary perspectives.

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Table 1. Cytochrome P450 content (nmol/mg microsomal protein) and ethoxyresorufin-*O*-deethylase (EROD) activity (pmol/min/mg microsomal protein) of control and exposed herring at three water soluble fraction concentrations. Values are means ± standard error. *n*=7. * indicates a significant difference ($p<0.05$) between an exposed fish group and the control

| Parameter | Time | | | | |
|---|---|---|---|---|---|
| | 0 | 24 h | 96 h | 4.3 weeks | 8.3 weeks |
| **Cytochrome P450** | | | | | |
| Control | 0.122±0.029 | 0.131±0.032 | 0.134±0.019 | 0.131±0.022 | 0.133±0.027 |
| Low | 0.144±0.027 | 0.128±0.028 | 0.140±0.021 | 0.136±0.020 | 0.131±0.029 |
| Medium | 0.120±0.023 | 0.146±0.034 | 0.300±0.028* | 0.312±0.046* | 0.306±0.044* |
| High | 0.117±0.011 | 0.118±0.031 | 0.327±0.056* | 0.321±0.038* | 0.299±0.041* |
| ***EROD*** | | | | | |
| Control | 33.3±4.6 | 25.6±6.2 | 37.7±5.7 | 31.8±4.7 | 29.5±4.7 |
| Low | 27.9±4.7 | 30.9±5.1 | 31.1±6.2 | 37.8±5.1 | 38.9±6.6 |
| Medium | 30.5±5.3 | 28.8±3.7 | 52.5±7.3* | 68.9±5.0* | 53.3±5.1* |
| High | 35.7±4.3 | 34.0±8.3 | 99.5±10.2* | 156.6±11.9* | 159.0±20.6* |

## FIGURE LEGENDS

Figure 1. Total Polycyclic Aromatic Hydrocarbon (TPAH) concentrations in treatment tanks as a function of time after initiation of water flow through a water soluble fraction (WSF) generating column. Data points are single composite values for replicate tanks at each time period. (○) Control, (▲) 10 µg/l, (■) 40 µg/l, (●) 100 µg/l.

Figure 2. Ucrit values for herring exposed to various concentrations of water soluble fraction (WSF) for 24 h, 96 h, 4 weeks and 8 weeks. Control fish (○) and those exposed to L (■), M (□), and H (●) concentrations. Values are means ± SE of $n$=7 fish. * denotes a significant difference ($p$<0.05) from controls. From Kennedy and Farrell 2005 (14).

Figure 3. Percent Mortality in herring exposed to various concentrations of water soluble fraction WSF for 24 h, 96 h, 4 weeks and 8 weeks. Control fish ( ) and those exposed to ▨ ( ), M ( ), and H ( ) concentrations. Values are means±SE of three tanks. * denotes a significant difference ($p$<0.05) from controls.

Figure 4. Plasma cortisol, lactate, $Na^+$ and $Cl^-$ concentrations, as well as muscle glycogen concentrations pre- and post-exercise from fish exposed to WSF for 24 h. Control fish (○) and those exposed to L (■), M (□), and H (●) concentrations before and following burst exercise. Values are means ± SE of $n$=6 fish. * denotes a significant difference ($p$<0.05) from controls.

Figure 5. Plasma cortisol, lactate, $Na^+$ and $Cl^-$ concentrations, as well as muscle glycogen concentrations pre- and post-exercise from fish exposed to WSF for 96 h. Control fish (○) and those exposed to L (■), M (□), and H (●) concentrations before and following burst exercise. Values are means ± SE of $n$=6 fish. * denotes a significant difference ($p$<0.05) from controls.

Figure 6. Plasma cortisol, lactate, $Na^+$ and $Cl^-$ concentrations, as well as muscle glycogen concentrations pre- and post-exercise from fish exposed to WSF for 4.3 weeks. Control fish (○) and those exposed to L (■), M (□), and H (●) concentrations before and following burst exercise. Values are means ± SE of $n$=6 fish. * denotes a significant difference ($p$<0.05) from controls.

Figure 7. Plasma cortisol, lactate, $Na^+$ and $Cl^-$ concentrations, as well as muscle glycogen concentrations pre- and post-exercise from fish exposed to WSF for 8.3 weeks. Control fish (○) and those exposed to L (■), M (□), and H (●) concentrations before and following burst exercise. Values are means ± SE of $n$=6 fish. * denotes a significant difference ($p$<0.05) from controls.

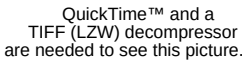
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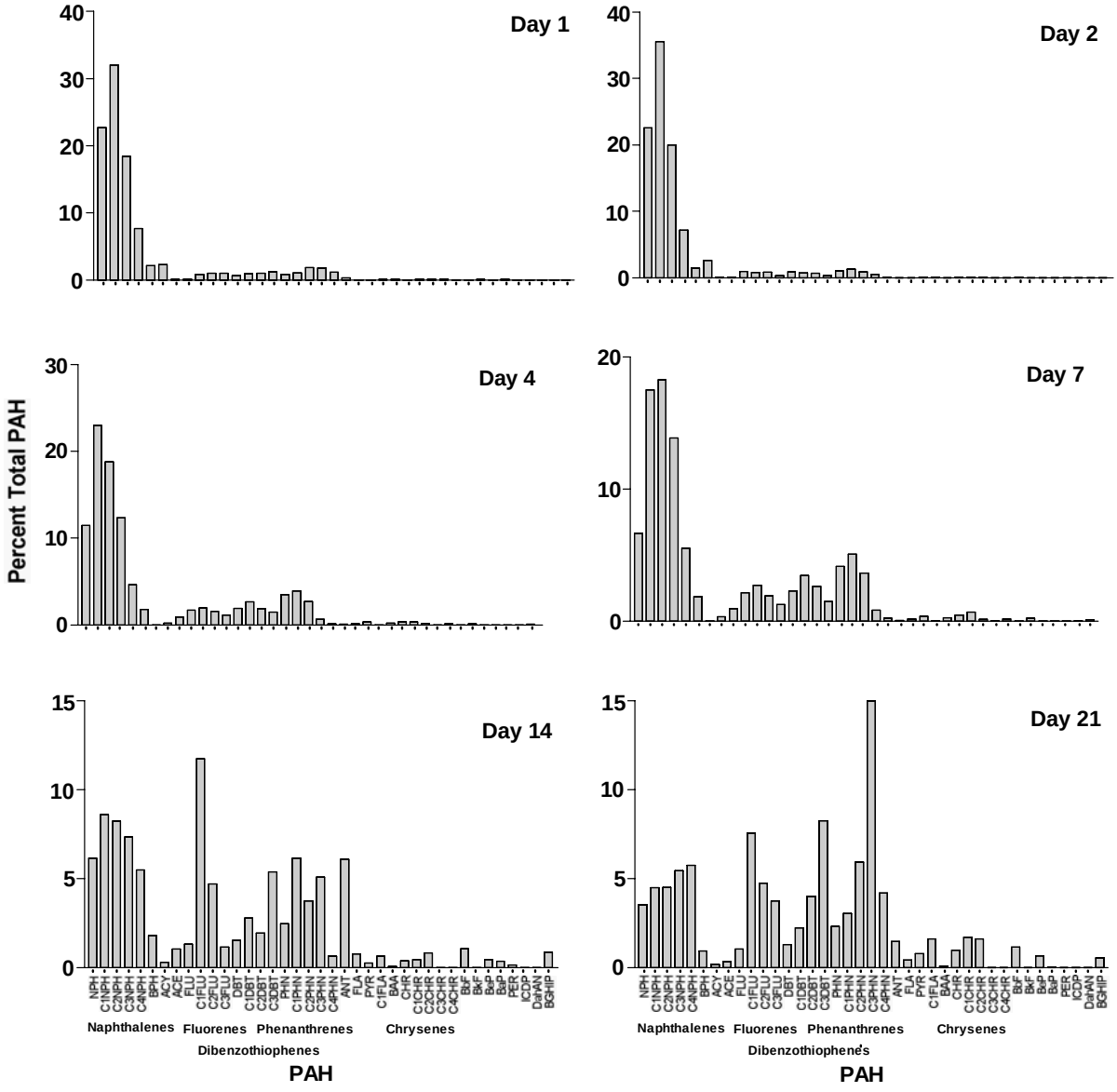
Figure 1.

Figure 2.


Day 1
Day 2
Day 4
Day 7
Day 14
Day 21
Percent Total PAH
NPH
C1NPH
C2NPH
C3NPH
C4NPH
BPH
ACY
ACE
FLU
C1FLU
C2FLU
C3FLU
DBT
C1DBT
C2DBT
C3DBT
PHN
C1PHN
C2PHN
C3PHN
C4PHN
ANT
FLA
PYR
C1FLA
BAA
CHR
C1CHR
C2CHR
C3CHR
C4CHR
BbF
BkF
BaP
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ICDP
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BGHIP
Naphthalenes
Fluorenes
Phenanthrenes
Dibenzothiophenes
Chrysenes
PAH

Figure 3.

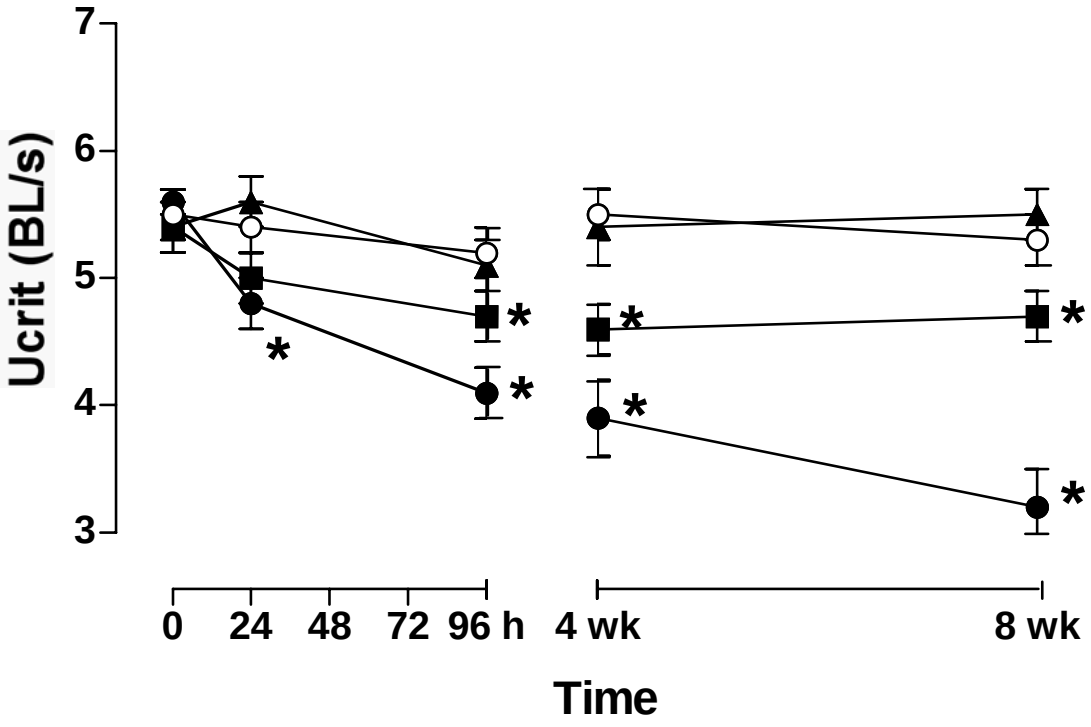


Figure 4.

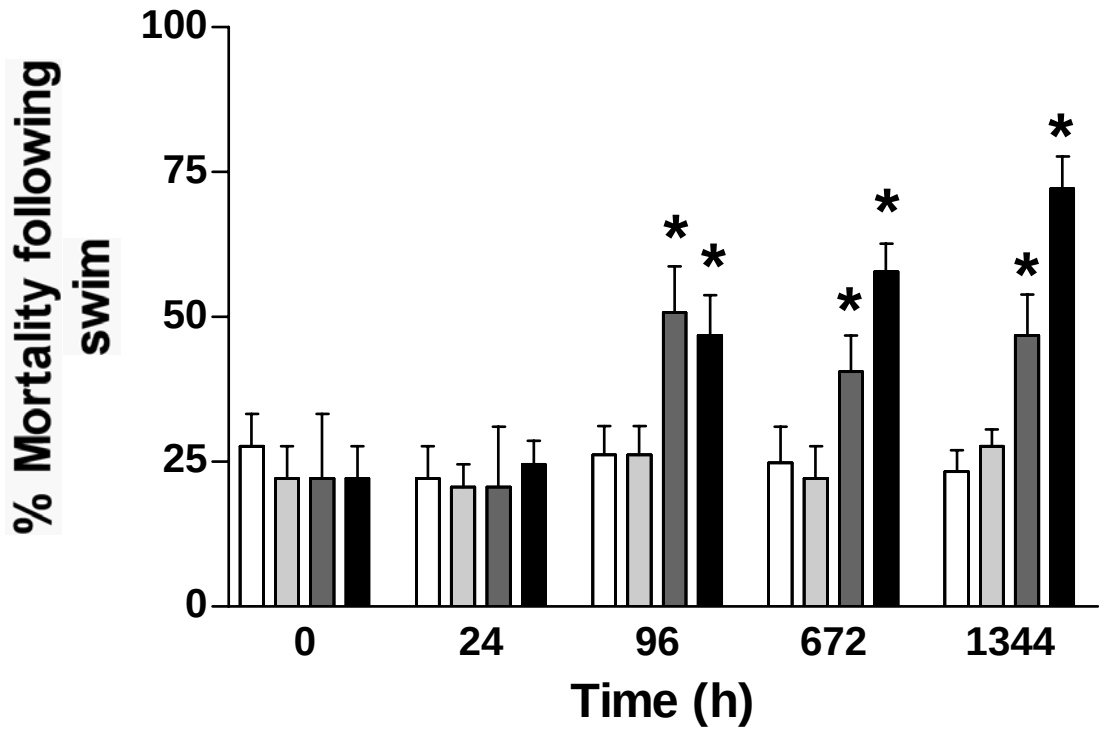


Figure 5.

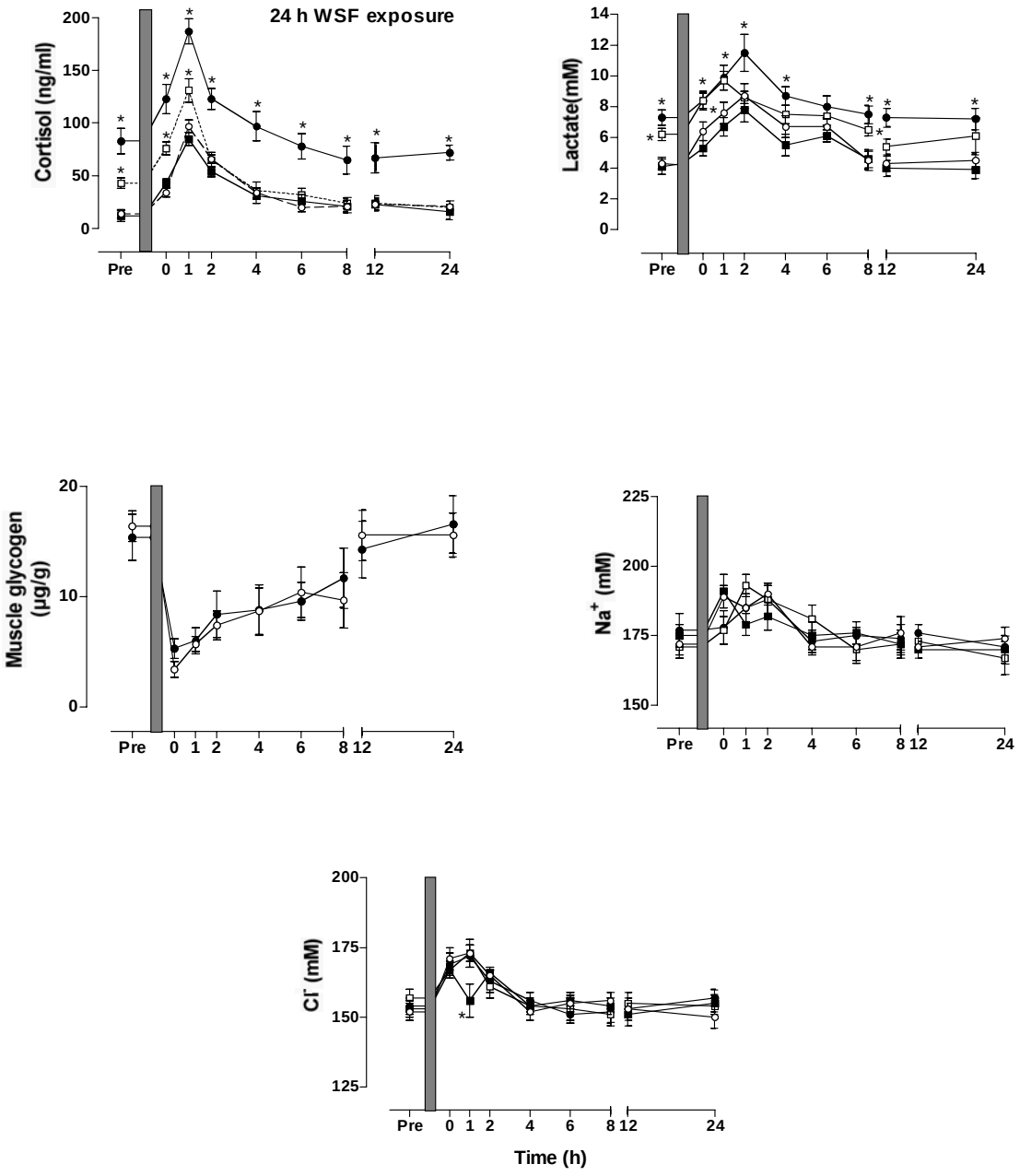
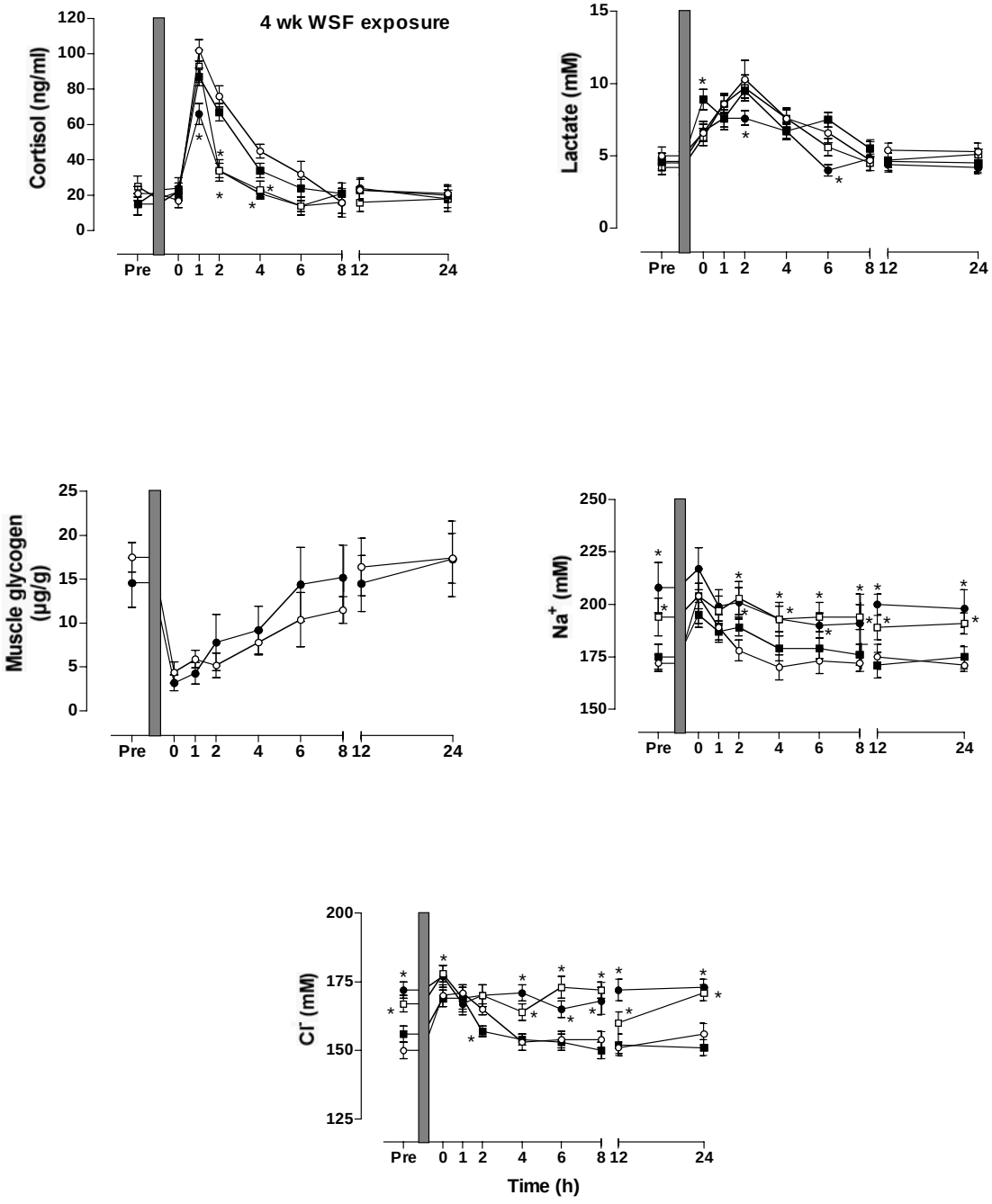
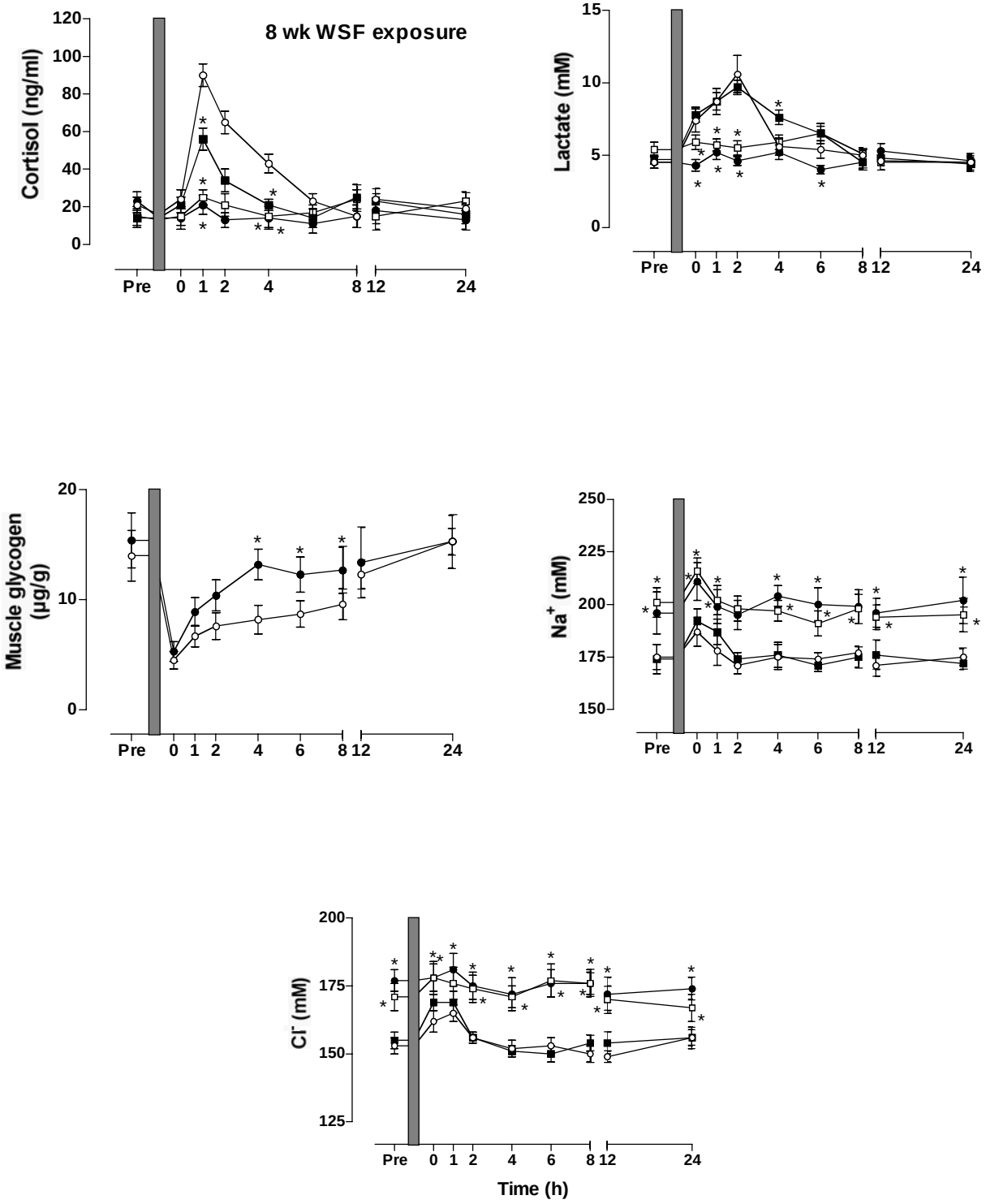

24 h WSF exposure
Cortisol (ng/ml)
Lactate(mM)
Muscle glycogen (µg/g)
Na+ (mM)
Cl- (mM)
Pre
0
1
2
4
6
8
12
24
Time (h)

Figure 6.


4 wk WSF exposure
Cortisol (ng/ml)
Lactate (mM)
Muscle glycogen (µg/g)
Na+ (mM)
Cl- (mM)
Pre 0 1 2 4 6 8 12 24
Time (h)


8 wk WSF exposure
Cortisol (ng/ml)
Lactate (mM)
Muscle glycogen (µg/g)
Na+ (mM)
Cl- (mM)
Pre
0
1
2
4
6
8
12
24
Time (h)

Manuscript 3:

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# Immunological alterations in juvenile Pacific herring, *Clupea pallasi*, exposed to aqueous hydrocarbons derived from crude oil

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Running head: effects of oil on herring immunology and disease resistance

**Keywords** - Pacific herring, *Clupea pallasi*, oil, hydrocarbons, immune systems, immunology, disease, toxicity, fish, stress

**Abstract-** Pacific herring were exposed acutely and subchronically to 3 concentrations (control: 0.15±0.10, low: 10.5±3.7, medium: 35.7±7.7 and high: 127.3±11.3 µg/L total PAH [TPAH]) of aqueous hydrocarbons derived from North Slope crude oil in order to assess their impact on immune defense mechanisms. TPAH concentrations declined and composition shifted toward larger and more substituted PAHs as the time of exposure increased. Hydrocarbons were bioavailable to herring, as indicated by induction of hepatic cytochrome P450 and ethoxyresorufin O-deethylase activity. Hydrocarbon exposure did not affect hematocrit, leucocrit or differential white blood cell counts. Acute (24 and 96 h) exposures resulted in a transient stress response as shown by increases in plasma cortisol, lactate and glucose. At 96 h, a persistent ionoregulatory dysfunction resulted in elevated concentrations of plasma $Na^+$, $Cl^-$ and $K^+$, which persisted through the entire exposure period (8 wk). Short-term exposure in the high treatment group significantly enhanced respiratory burst activity (RBA) in macrophages and decreases in plasma lysozyme concentrations. With subchronic exposure (4 to 8 wk), RBT activity was significantly reduced. Fish in the high treatment group were less susceptible to the pathogen *Vibrio anguillarum* following acute hydrocarbon exposure; however, this group was the most susceptible by the end of the experiment. This study shows that hydrocarbon exposure can variably affect teleost immune systems and depends on chemical concentration and composition, exposure duration and pathogen challenge.

**Introduction**

Disease agents in fish populations are believed to exert important effects on host population dynamics through enzootic or epizootic events. Enzootic diseases cause long-term impacts on physiological processes that can affect growth, reproduction, and survival. Epizootic diseases can affect population dynamics by reducing populations in short-term events, which if sufficient, may result in stochastic processes causing extinction (Gulland 1995). Immune suppression is the mechanism by which toxicants are believed to increase disease incidence (Zelikoff 1993).

Immune defense mechanisms are of obvious strategic importance to fish when faced with environmental challenges of a biological or chemical nature. Indeed, the measurement of immunological performance in fish has been suggested as an important criterion in the determination of stressor or toxicant exposure in fish. Although the immune systems of fish have not been studied as extensively as those of mammals, techniques for the assessment of immune function in fish have been well developed because they share with mammals a number of structural and functional characteristics important to the humoral, cell-mediated, and nonspecific aspects of the immune response (Ellis 1977). In fact, some of the same criteria used to determine the immunotoxic potential of xenobiotics in mammalian systems are also used to assess immunotoxicity in fish (Zelikoff 1994).

Environmental xenobiotics, including metals, insecticides, fungicides, halogenated aromatic hydrocarbons, and PAHs (reviewed in Zelikoff 1994), can suppress teleost immune responses. While this could lead to increased host susceptibility to infectious diseases and possibly cancer, the relationship between toxicant exposure and disease in aquatic organisms has not been clearly defined, and only a large body of circumstantial evidence exists linking various diseases in feral fish with pollutant discharges (Mearns and Sherwood, 1976; Waterman and Kranz 1992).

Petroleum-derived hydrocarbons are a major contributor to the contamination of aquatic environments and approximately 5 million tons of crude oil enters the marine environment each year from a variety of sources (Neff 1990). Two hallmarks of PAH toxicity are carcinogenicity and immunotoxicity, in addition to well-documented sublethal effects which include morphological, histopathological and genetic damage (Brown et al., 1996; Hose et al. 1996; Kocan et. al. 1996; McGurk and Brown, 1996; Norcross et al. 1996; Carls et al. 1987, 1999; Heintz et al. 1999), physiological and stress effects (Thomas and Rice 1987; Kennedy and Farrell 2005; Kennedy and Farrell 2006), endocrine disruption (Evanson and Van der Kraak 2001; Kennedy and Farrell 2006), and ecological effects (Reddy et al. 2002). Recent work has shown that PAHs can have both common mechanisms of action (e.g. nonpolar narcosis) and chemical-specific mechanisms (e.g. activation of the aryl hydrocarbon receptor pathways) underlying toxicity (Incardona et al. 2004).

The Exxon Valdez oil spill (EVOS) occurred in March 1989, three weeks prior to the peak of spawning of Pacific herring, *Clupea pallasi*, in Prince William Sound (PWS) and was subsequently followed by drastic declines in spawning biomass in following years. One hypothesis for this population decline was that oil exposure of herring resulted in immunosuppression and subsequent expression of viral hemorrhagic septicemia virus (VHSV). Corroborating evidence for this hypothesis is that, of the fish that returned to spawn, many had external hemorrhages and appeared lethargic (Marty et al. 1998). Also, the North American strain of VHSV has been isolated from pooled samples of Pacific herring (Meyers et al. 1994). Moreover, Carls et al. (1995) showed that 97% of adult herring that died after exposure to PAH concentrations as low as 28 µg/l had lesions consistent with published descriptions of VHSV. Thus, it is possible that herring are asymptomatic carriers of pathogens such as VHSV under

normal conditions, and that stressors such as hydrocarbon exposure may impair the immune systems ability to contain the lethal effects of the virus. In addition to VHSV, *Ichthyophonus hoferi* was also implicated as a possible pathogen contributing to population declines, as it was isolated in Pacific herring following the Exxon Valdez oil spill and earlier had been associated with declines in Atlantic herring populations (Patterson 1996; Marty et al. 1998). Although Marty et al. (1998) and Elston et al. (1997) suggest that the weight of evidence does not support a relationship between disease outbreak in PWS as a result of permanent immune suppression caused by hydrocarbon exposure when fish were larvae or yearlings, the literature clearly shows that exposures to compounds such as PAHs are immunomodulatory in teleosts. In fact, it has been suggested that exposure to PAHs in 1989 following the *EVOS* may have caused damage to developing cells destined to become functional immune cells and that abnormal function could be evident at a later stage in life if challenged with specific pathogens (Kocan *et al*., 1997). In view of these contradicting views with respect to the potential effects of low sublethal concentrations of petroleum-derived aqueous hydrocarbons on immune systems of pelagic marine organisms, this study utilized both acute and subchronic exposure regimes in its examination of hydrocarbon immunotoxicity in Pacific herring.

**Materials and Methods**

*Fish*

Juvenile Pacific herring, (10.3 to 17.8 g) were obtained through a local supplier in West Vancouver, BC. Fish were transported to facilities at the Fisheries and Oceans Canada, West Vancouver Laboratory, BC, with a minimal use of nets to reduce trauma to the young fish. Fish were held in 500-L fiberglass tanks supplied with flowing filtered seawater, salinity 31 ppt, water temperature 11.0±1.5°C and dissolved $O_2$ content >95% saturation. Following transfer, mortality in the first week was approximately 5.7%, which declined to less than 0.25% per wk. When the mortality rate had stabilized at this level, fish were acclimated for a further 4 wks in experimental tanks before an experiment was performed. Fish were fed twice daily *ad libitum* with frozen krill until one day before an experiment.

*Chemicals and exposure*

Herring were exposed to three concentrations of the water-soluble fraction (WSF) of North Slope crude oil in 500-L fiberglass tanks for 8.1 wks. All exposures were performed in triplicate. WSFs were generated by seawater continuously passing through a modified apparatus described in Kennedy and Farrell (2005). This method has previously generated water containing initial total polycyclic aromatic hydrocarbon (TPAH) concentrations of up to 120 µg/L, which declined to approximately 30 µg/L, 16 days following the initiation of water flow (Kennedy and Farrell 2005). Due to the results of water TPAH analyses in short-term experiments, fish were exposed to WSF subchronically for 8.1 wks by bringing on new cylinders at time 0, 4, and 8 wks to maintain relatively constant hydrocarbon concentrations.

*Tissue sampling*

Fish were sampled at 24 h, 96 h, 4.1 wk and 8.1 wk of exposure. To minimize handling stress, fish were immediately anesthetized in a solution of 0.5 mg/l MS222 in seawater. Each fish was quickly blotted dry, and its body mass and length recorded. One drop of blood from the severed caudal vasculature was used for blood smears. A further blood sample was obtained in small heparinized capillary tubes, which were then centrifuged at 13,000 x *g* and hematocrit (Hct) and leucocrit (Lct) were measured using digital calipers (Janz et al. 1991). Plasma was then separated and frozen in liquid nitrogen. Plasma, rather than serum, was used in this study

because comparisons of blood chemistry values from paired plasma and serum samples from rainbow trout, channel catfish, hybrid tilapias (*Oreochromis* spp.) and hybrid striped bass (*Morone* spp.) showed that levels determined from serum may not accurately reflect blood chemistry levels in the circulation because changes in fish serum can occur rapidly even when samples are handled properly (Hrubec and Smith, 1999). The livers were removed from the fish, weighed, and immediately frozen in liquid nitrogen for analysis of biotransformation enzyme activities. The head kidney tissue was obtained *via* sterile dissection and was placed immediately into cell culture medium (Hanks Balanced Salts Solution, HBSS) on ice in sterilized centrifugation tubes (Tierney et al. 2004). All plasma and liver tissue samples were eventually stored at -86°C for not longer than 3 wks before analysis.

*Biochemical measurements*

Liver microsomes were prepared according to the method of Kennedy (1995) to assess hydrocarbon bioavailability through measurements of cytochrome P450 and ethoxyresorufin O-deethylase (EROD) activity. Briefly, livers were homogenized in ice-cold buffer: 0.15 M KCl, 0.2 M HEPES, 5 mM EDTA, pH 7.4. The homogenate was centrifuged for 20 minutes at 10,000 x *g* at 4°C. The supernatant was recentrifuged for 65 minutes at 100,000 x *g*. The microsomal pellet was resuspended in a 0.15 M KCl, 100 mM Tris-HCl, 20% glycerol v/v buffer, pH 7.4. Hepatic microsomal and cytosolic protein content was determined using the method of Bradford (1975). Cytochrome P-450 (P450) content was determined by the method of Omura and Sato (1964). The activity of cytochrome P4501A1 (CYP1A) proteins was assessed by the EROD assay according to Burke and Mayer (1974). All assays were performed in duplicate.

Plasma cortisol concentrations were determined using radioimmunoassays (IncStar Corp., Stillwater, MN). Plasma was spectrophotometrically analyzed for lactate and glucose using standard colorimetric kits (Sigma). All assays were performed in duplicate. Plasma was analyzed for ion concentrations using a Model 8015 Benchtop ISE meter (VWR Scientific, Toronto, ON) equipped with specific ion selective electrodes (ISE) for $Na^+$ (Model 84-11, Orion Research Inc., Beverly, MA), $K^+$ (Model K001503-003B, Phoenix Electrode Co., Houston, TX), and Cl- (Model 27502-12, Cole-Parmer Instrument Co., Vernon Hills, IL.). All plasma samples were analyzed in triplicate. Measurements were repeated if the disagreement between triplicates was >3%. Electrodes were calibrated before use and checked against a standard following the measurement of 5 triplicate sets. For quality control, random plasma samples were analyzed for $Na^+$ and $K^+$ using a Pye Unicam SP131 Atomic Absorption Spectrophotometer (Unicam Ltd., Cambridge, UK) for direct comparison to ISE determined values.

*Immunological assays*

Blood films were air dried and stained with modified Wright-Giemsa (Sigma). The relative leukocyte proportions were estimated by counting approximately 100 white cells for each slide at 1000x power, providing a resolution of 0.5% (Tierney et al. 2004). Cells were identified according to previously published guidelines (Ellis 1977; Tierney et al. 2004).

The lysoplate assay was used to measure plasma lysozyme levels (Balfry and Iwama 2004). Lysoagar plates were prepared using a suspension of *Micrococcus lysodeikticus* in a 0.5% agarose mixture containing 0.06 M phosphate buffer (pH 6.0 and 0.02% NaCl). Three-millimeter holes were punched into the hardened agarose. Plasma samples were diluted 1:1 with 0.85% saline and 15 µl was placed into the wells in the lysoagar plates. Standards were prepared from hen egg-white lysozyme (Sigma). After 20-h incubation at room temperature, the diameters of the clearance zones were measured using precision digital calipers.

The number of NBT-positive neutrophils present in the head kidney was measured using the nitro-blue tetrazolium (NBT) method (Jeney and Anderson 1993). Head kidneys were

homogenized by mastication using the plunger of a syringe and aliquots of HBSS/kidney homogenate (60 µl) were placed into welled microscope slides. After a 30-min incubation in a sealed container, slides were rinsed with phosphate buffered saline and 50 µl of a 0.2% NBT solution was added to wells. Slides were incubated for a further 30 min, after which counts of activated (stained) versus non-activated (unstained) cells were made under 500x magnification.

*Disease challenges*

Disease challenges were performed following 24 h, 96 h, 4.1 wk and 8.1 wk exposures to the WSF of oil. For disease challenges, three replicates of 50 fish each were randomly selected from each treatment group and exposed to the bacterium, *Vibrio anguillarum*, a gram-negative bacterium and an opportunistic pathogen commonly encountered in the marine environment. It is the main causative agent of Vibriosis, a syndrome characterized by hemorrhagic septicemia (Egidius, 1987). The challenge dose of *V. anguillarum* was obtained from Fisheries and Oceans Canada, West Vancouver Laboratory. A sub-sample of the bacterial culture was used to make serial dilutions plates to confirm the concentration of the bacterial culture solution used for the bath challenge. Water in tanks was lowered slowly, and fish were exposed to $1 \times 10^4$ cfu/ml of *V. anguillarum*. All tanks were dosed simultaneously to prevent differences in bacterial numbers due to the culture changing with time and the concentration was again verified using serial dilution plates. Fish were observed twice daily for mortality and signs of disease for a 6-wk period following the challenge and a record of cumulative mortality was maintained. Each dead herring was evaluated for mass and length, and the head kidney was removed to test for the presence or absence of *V. anguillarum* using standard confirmatory microbiological techniques (Noga, 1996). These results, in combination with consistent clinical signs, were used to assign a positive or negative Vibriosis status to the herring and to determine proportional mortality due to *V. anguillarum.*

*Calculations and statistics*

Two-way ANOVA and a Holm-Sidak post-hoc test were used to determine whether the measured hematological, biochemical or immunological parameters differed between replicate trials at the each measurement time. As no differences were detected between trials, data were pooled and differences between treatment groups and exposure times were analyzed using two-way ANOVA and a Holm-Sidak post-hoc test. All percent data were arcsine transformed prior to analysis. Disease challenge mortality curves were compared to controls using the Mantel-Cox and Breslow test statistics All data analyses were conducted with a fiducial limit significance at $p<0.05$, using SigmaStat version 3.0 (SPSS Scientific, Chicago, IL).

**Results**

Aqueous TPAH concentrations in exposure tanks at time T=0 were: control (.10 to 0.18 µg/L), low (7.4 to 13.4 µg/L), medium (30.7 to 42.5 µg/L) and high (111.3 to 133.5 µg/L). TPAH concentrations declined with time (Fig. 1), and since declines were similar across treatments, each exposure treatment remained distinct (Carls et al. 1995). Using this design, alkane concentrations can range from 1.28 µg/L (control) to 119 µg/L (high treatment group) with concentration declines similar to those of TPAH (Carls et al. 1995). Figure 2 (from Kennedy and Farrell 2005 for reference) shows that in this system, PAHs are predominantly smaller and less substituted (e.g. naphthalenes) at the start of cylinder operation, and progressively shift to larger and more substituted PAH (e.g. phenanthrenes) with time.

Total cytochrome P450 levels and EROD activities were altered by exposure to aqueous hydrocarbons (Table 1) indicating that these compounds were bioavailable. At time 0, there

were no significant differences among groups in any measured parameter. Significant induction of cytochrome P450 was evident in the medium and high groups by 96 h and continued through the exposure period. Maximum induction occurred in the high treatment group, with cytochrome P450 content increasing 273% over controls. EROD induction was seen in all groups, with maximum induction in the high treatment group at 8.1 wks (5.4-fold increase over controls).

Control plasma cortisol, lactate and glucose concentrations did not change significantly over time during the experiment (Fig. 3). Plasma cortisol concentrations were significantly higher than controls in the high treatment group at 24 h, but returned to control levels by 96 h. When a new cylinder was brought online at 4 wks exposure, cortisol concentrations increased in the high treatment group, although it was 56% lower than at 24 h. No cortisol response occurred with a further cylinder replacement at 8 wks. Similarly, fish exposed to WSF initially responded with increases in both plasma lactate and glucose, however, as new pulses of hydrocarbons were initiated; these responses were either reduced or not evident (Fig. 3). Although the acute 24-h exposure to WSF did not affect plasma ion concentrations, generally WSF exposure at the medium and high exposure concentrations significantly increased all plasma ion concentrations from 96 h through 8.1 wk exposure (Fig. 3).

There were no significant effects of WSF exposure on Hct (range 19 to 52%), or Lct (0 to 3.1%). As well, no significant alterations were seen in the proportions of white blood cells in differential white blood cell counts due to a high variability between individuals regardless of treatment group. Ranges for percents of total white blood cells were thrombocytes (16 to 39%), lymphocytes (30 to 77%), neutrophils (11 to 20%), basophils (0 to 1%), eosinophils (0 to 1%), and monocytes (0 to 1%). Plasma lysozyme was significantly reduced in fish exposed to the highest concentration of WSF at 24 (28%) and 96 h (68%) compared to controls, but had returned to baseline values by 4 wk (Fig. 4). Figure 4 also shows that the proportion of NBT-positive neutrophils present in the head kidney was significantly higher in fish from the high treatment group with short-term exposure (145% and 187% over controls at 24 h and 96 h, respectively), and significantly lower than controls at 4.1 wk (73%) and 8.1 wk (74%).

Exposure of fish to aqueous hydrocarbons affected their susceptibility to the marine pathogen *V. anguillarum* (Fig. 5). There were no significant differences in the rate of mortality between control fish and those in the low and medium treatment groups, however, fish exposed to the highest concentration of hydrocarbons had a lower mortality rate and had less total fish die from the pathogen after a 24-h WSF exposure. At both 96 h and 4 wk exposure to WSF, there were no differences between any group in their susceptibility. However, at 8 wks exposure, there was a significant increase in the susceptibility of fish in the high treatment group compared to all other groups (Fig. 5).

**Discussion**

The exposure system used in this study has been successfully employed in several other studies to expose teleosts with low sublethal and environmentally relevant (Thomas et al. 1997) concentrations of the WSF of crude oil under both acute (Carls et al. 1998) and subchronic (Kennedy and Farrell 2005; Kennedy and Farrell 2006) scenarios. Although hydrocarbon concentrations declined and the hydrocarbon profiles changed with time, the employment of several WSF generating systems allowed for a relatively consistent hydrocarbon exposure that remained distinct between treatment groups. These low aqueous hydrocarbon concentrations were bioavailable to herring, as indicated by significant induction of both cytochrome P450 levels and EROD activity (Kennedy 1995; Gagnon and Holdway 2000). Using a similar design,

Thomas et al. (1997) found elevated tissue concentrations of axenic hydrocarbons as well as induction of mixed function oxidase enzymes.

A multitude of environmental toxicants can affect the immunological performance of teleosts (Anderson 1990). These effects can be brought about by chemical-specific mechanisms of action that target different components of the immune system or through induction of a physiological stress response, and the resultant downstream effects brought on by neuro-endocrine alterations. In this regard, activation of two hormonal axes, the sympathetico-chromaffin (SC) and hypothalmic-pituitary-interrenal (HPI), during stressful events culminates an increased concentration of circulating catecholamines and cortisol, respectively, which result in the mobilization of free energy and the reparation of homeostasis (Bonga, 1997; Mommsen et al. 1999). The present study confirms that acute exposure of teleosts to low sublethal concentrations of water-soluble hydrocarbons derived from crude oil initiates a transient activation of the HPI axis and temporarily increases plasma cortisol, lactate and glucose concentrations. The stress response to WSF is believed to be due to components of oil which are acutely toxic to fish, namely the volatile aromatic hydrocarbon fraction that includes naphthalenes, benzene, toluene, ethylbenzene and trimethybenzene (Thomas et al. 1997). As each new generator is employed, total PAH concentrations and the proportions of lower molecular weight compounds such as naphthalene are highest and should result in activation of the HPI axis and higher plasma cortisol levels. In this study, a muted cortisol response at 4.1 wk and no response at 8.1 wk with new generators is consistent with evidence that this is not an adaptation of the corticosteroid response to hydrocarbons but is the result of a compound(s) acting directly as an endocrine disruptor, targeting the pituitary or adrenocortical tissues (Dorval et al. 2003; Kennedy and Farrell 2006). PAH exposure has previously been shown to affect steroidogenesis (testosterone) in rainbow trout tissue (Evanson and Van Der Kraak 2001).

Osmoregulation can be compromised by catecholamine-induced gill and cardiac changes that are seen in stressed fish (Pickering 1998). No acute increase in plasma ions was associated with the cortisol stress response during the first 24 h of WSF exposure. instead, significant elevations in plasma $Na^+$, $Cl^-$, or $K^+$ concentrations were noted at 96 h, after the cortisol response had subsided. These disturbances continued for the duration of the experiment. $Cl^-$ and $K^+$ concentrations were more responsive than $Na^+$, and interestingly, they were the only disturbance observed with the lowest WSF exposure group, even though EROD and CP450 were not elevated. Many toxic substances including petroleum hydrocarbons can cause osmoregulatory disturbances in teleosts (Englehardt et al. 1981). Several explanations including effects on ion-specific ATPases by lower molecular weight aromatic hydrocarbons (Englehardt et al. 1981), affects on membrane permeability (Incardona et al. 2004), and the inability to increase cortisol concentrations may explain the continued ionoregulatory dysfunction. Cortisol has been shown to increase the cellular differentiation of chloride cells and stimulate branchial $Na^+/K^+$-ATPase activity (McCormick 1995). Elevated plasma cortisol may be necessary to compensate for the hydromineral imbalance caused by WSF, as cortisol supplementation during an oil emulsion exposure was effective in normalizing a hydromineral imbalance in freshwater immature rainbow trout (Englehardt et al. 1981). In addition to this possibilities, extensive gill damage upon exposure to oil components has been shown in fish and an influx of ions has been attributed to damage or changes in chloride cells (Englehardt et al. 1981). Specifically, herring gills exposed to WSF at similar concentrations exhibited epithelial hyperplasia and a minor epithelial lifting (Kennedy and Farrell 2005).

The selection of several hematological parameters was based on Anderson (1990) as potential indicators of stress and immunological dysfunction in teleosts. While Hct has been termed a primary tool for assessing fish health (Allen, 1993), there was no detectable effect of

WSF on Hct of herring. Likewise, the WBC differential count is a means of determining cell percentages of the cell types that comprise the WBCs and has been adopted by some researchers (McLeay and Gordon, 1977) in a manner comparable to mammalian hematology, although there is some controversy surrounding the significance of WBC differentials in interpreting the WBC status of fish. Again, no difference in the proportions of WBCs was found between WSF-exposed and control fish. Similarly, Lct is the percentage of blood by volume that is composed of WBCs and is an estimator of total WBC count and is considered an indicator of resistance to disease in fish (Wedemeyer 1996). The range of leucocrit values in this study was quite large (0 to 3.1%) and showed no effect of WSF exposure.

Constituents of crude oil have been shown to be immunosuppressive in a variety of fish species including flounder (Alkindi *et al*., 1996), striped mullet (Thomas *et al*., 1980), coho salmon (McKeown, 1981), cutthroat trout (Woodward *et al*., 1983), Atlantic cod and winter flounder (Dey *et al*., 1983). Consistent with these earlier studies on other teleost species, we saw immunosuppressive effects on more direct measures of immunocompetence.

Lysozyme (mucopeptide N-acetylmuramylhydrolase) is a stable hydrolytic antibacterial enzyme that is part of the first line of non-specific defenses against pathogens and other tissue insults. This enzyme attacks peptidoglycan in the cell walls of mostly Gram-positive bacteria, but also Gram-negative bacteria to some degree (Hutchinson and Manning, 1996). Lysozyme concentrations were significantly reduced in herring during the acute phase of WSF exposure (i.e., 24 and 96 h), but only at the highest concentration of WSF. However, by week 4 of WSF exposure, lysozyme concentration had returned to control levels.

The macrophage response to WSF exposure was more complex than that seen for lysozyme and also occurred only with the high exposure concentration. Macrophages are essential for phagocytosis, inflammation and antigen processing and also secrete lysozyme. The phagocytosis of foreign particles and damaged self-cells is a key function of the macrophage. The respiratory burst consists of a sequence of reactions that results in the production of reactive oxygen intermediates that are used to inactivate engulfed particles or those exterior to cells. In this study, the proportion of cells expressing RBA activity was increased significantly in fish exposed to high concentrations of WSF in the short-term, but was significantly reduced in fish exposed to hydrocarbons for extended periods. Therefore it appears that juvenile herring were mounting a macrophage defense that collapsed after 4 wks and persisted through the 8-wk exposure period. This conclusion is entirely consistent with the results for the pathogen challenge.

Susceptibility to a pathogen was assessed by infection and mortality to *V. anguillarum*. Pathogen challenge is considered the most important and comprehensive test in immunotoxicological screening (Wester *et al*., 1994) because it is a broad and direct test of an organism's response that has obvious biological significance at the individual and population levels. In the present study, herring were more tolerant to *V. anguillarum* following exposure to the highest concentration of WSF for 24 h, consistent with the elevated macrophage levels, but this enhanced tolerance was not maintained. By 8 wks of WSF exposure, herring were significantly more susceptible to this pathogen, which was coincident with suppressed macrophage activity.

The present results clearly conform to the general principle that the physiological ramifications of environmental stressors depend on the duration and on the intensity of stressor application (Ortuno et al. 2001). In all vertebrates, the activation of the HPI axis and resulting neuroendocrine and autonomic changes modulate both the innate (non-specific) and adaptive (acquired or specific) components of the immune system (Ruis and Bayne 1997). The existing paradigm suggests that this stressor-induced modification is the cause of a commonly observed

higher susceptibility of stressed individuals to diseases (Ruis and Bayne 1997). The stress-activated mechanism of immunodepression or immunosuppression in fish is not completely understood, but appears to be mediated through endocrine pathways. For example, several studies have shown that the acute stress brought on by short-term crowding of fish can result in depression in complement and phagocytic activities and the chemiluminescent response of fish pronephros cells (Scott and Klessius, 1981; Yin et al. 1995). High doses of cortisol in seabream (*Sparus aurata*) resulted in depressed respiratory burst activity of head-kidney leukocytes, the phagocytosis of yeast cells, and the total peroxidase content of leukocytes (Esteban et al. 2004).

Recent evidence, however, suggests that the type of stressor and its application may vary the response of the immune system and its function. The physiological events following SC and HPI axes activation can be suppressor or activators of the immune system (Tort et al. 1996). The enhancement of fish immune response, rather than its immunosuppression, has been observed to follow acute stress (e.g. increased concentrations of specific plasma proteins, such as lysozyme or complement, as well as enhanced yeast phagocytosis), leading to a better protection against any possible damage (Demers and Bayne 1997; Ruis and Bayne 1997; Ortuno et al. 2001). Maule et al. (1989) have specifically shown that an acute handling stress can decrease the susceptibility of juvenile spring Chinook salmon to *V. anguillarum* in spite of significantly depressed ability of lymphocytes to generate antibodies. The precise role of cortisol in either immunosuppression or immunostimulation is unclear, however, several studies have shown that cortisol administration *in vivo* and *in vitro* can result in immunosuppression and reduced disease resistance (Maule et al. 1987; Tripp et al. 1987).

In the present study, WSF exposure acted as an acute (24 and 96 h exposures) stressor as indicated by transiently increased cortisol values, suppressed lysozyme activity, elevated macrophage activity, enhanced pathogen tolerance, delayed plasma ionic disturbances. In contrast, with extended subchronic exposures (4 and 8 wk), when WSF inhibits cortisol release (Kennedy and Farrell 2005, 2006), macrophage activity and pathogen resistance are suppressed and ionic disturbances are sustained. These results suggest that cortisol may not be the main factor underlying either suppression or activation of the immune system. Dhabhar and McEwen (1996), for example, suggest that stress-induced changes in leukocyte distribution plays an important role in mediating a stress-induced increases in immune function by increasing immune surveillance, which may involve cortisol-independent alterations in blood flow patterns.

The successful avoidance of microbial infection depends to a great extent on the integrity of external barriers such as skin and the gills in teleosts. Stress-induced physiological modifications of immune function in herring exposed to WSF, likely act in concert with hydrocarbon-induced gill damage (as indicated by ionoregulatory dysfunction and histological results [Kennedy and Farrell 2005]) contributing to increased susceptibility to disease. Bath pathogen challenge was essential in herring to avoid the additional handling associated with an injection challenge and the likelihood of scale loss and skin damage. Based on the findings in other fish species, this challenge route should result in uptake both through the gills and by the gastro-intestinal tract in herring. In rainbow trout, uptake in gill tissue shortly after bath exposure to *V. anguillarum* suggests that the gills are also a route of entry (Baudin-Laurencin and Germon, 1987). WSF-induced alterations of gill structure and/or mucous lining of herring gills may lead to an enhanced penetration of pathogen in fish exposed to WSF for long periods.

An increase in the susceptibility of herring chronically exposed to WSF in the present study may also be due to the direct effects of hydrocarbons on immune system components. Field studies have correlated immunosuppression with tissue PAH concentration, although the exposure history of the fish is often unknown (Arkoosh *et al*., 1991; 1994). Suppression of phagocytic activity has been shown in fish from polluted rivers containing PAHs (Weeks *et al*.,

1986). If PAHs are immunosuppressive, then the potential for increased susceptibility to disease from opportunistic pathogens exists. In laboratory studies, PAHs have been indicated as mammalian immunotoxicants (Holladay *et al*., 1998). B(a)P and 7,12-dimethyl-benz(a)anthracene (DMBA) are two of the most commonly studied single PAHs and are immunotoxic and potent carcinogens (Anderson *et al*., 1995). Malmgren *et al*., (1952) first documented immunosuppression due to PAH exposure in mice. Mice exposed to B(a)P at 40 mg/kg for 7 days *via* subcutaneous injection exhibited a 50 to 66% reduction in polyclonal antibody response (Blanton *et al*., 1986). The immuno-suppressive effects of B(a)P have been shown to be multi-cellular in nature and are not due solely to cellular toxicity in mice (Blanton *et al*., 1986). Dunier and Siwicki (1993) suggested that chronic sublethal PAH challenge predisposes fish to disease. Both DMBA and B(a)P has been shown to decrease resistance to pathogens and neoplasia in fish (White *et al*., 1994). Exposure to DMBA by intraperitoneal (i.p.) injection at 1 to 100 mg/kg has been shown to be immunosuppressive in the oyster toadfish (*Opsanus tau;* Seeley and Weeks-Perkins, 1997). Macrophage phagocytosis activity was suppressed while nonspecific cytotoxic cell activity was almost completely inhibited in the toadfish. In contrast, tilapia exposed to DMBA by i.p. injection exhibited no alteration in phagocytosis until concentrations causing mortality were used (Hart *et al*., 1998). Similar to toadfish, juvenile European seabass *(Dicentrarchus labrax)* showed decreased phagocytosis and inhibited respiratory burst activity following injection with B(a)P at 20 mg/kg (Lemaire-Gony *et al*., 1995). There are several possible means by which PAHs may suppress the immune system, including 1) interaction with the Ah receptor by binding to this receptor and activating the Ah gene complex, 2) membrane perturbation effects, 3) altered interleukin (IL) production, 4) disruption of intracellular calcium mobilization, and 5) metabolic activation to reactive metabolites (White *et al*., 1994).

In conclusion, this study clearly shows that WSF exposure affects immune system components and pathogen resistance in a concentration- and time-dependent manner. We were able to show that low levels of WSF exposure could disrupt ionic regulation without affecting inducible liver enzymes, immunocompetence and pathogen resistance. Also, while medium levels of WSF exposure induced liver enzymes and caused ionic disturbances, there were no effects on measured immunological parameters and pathogen resistance. Only at the high concentration of WSF exposure was a stress response (elevated cortisol) effected and immunocompetence and pathogen resistance altered. It appeared that juvenile herring mounted a defense during the acute, stressful phase of high WSF exposure, with elevated lysozyme and macrophage activities and increased pathogen resistance. This defense was not sustained however and by the $8^{th}$ week of WSF exposure, macrophage activity was suppressed, gill structure and ionoregulation disturbed, and pathogen resistance reduced.

This study highlights the fact that predicting outcomes associated with combined pollutant and pathogen challenge can be complicated by opposing activating and suppressing factors in a time-dependent manner. Because of the complexity of the immune system and factors that can modify its function, much more research is needed in order to fully decipher the relationships between external challenges and organism responses. Immune-endocrine-toxicant interactions hold the key to understanding previously suspected interactions between toxicant exposure, stress, and disease.

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**Tables**

**Table 1.** Cytochrome P450 content (nmol/mg microsomal protein) and EROD activity (pmol/min/mg microsomal protein) of control and exposed herring at three WSF concentrations. Values are means ± SE. n=6 * indicates a significant difference ($p<0.05$) between an exposed group and controls.

| **Parameter** | **Time** | | | | |
|---|---|---|---|---|---|
| | 0 | 24 h | 96 h | 4.1 wk | 8.1 wk |
| Cytochrome P450 | | | | | |
| Control | 0.098±0.023 | 0.107±0.015 | 0.114±0.011 | 0.097±0.010 | 0.103±0.010 |
| Low | 0.133±0.022 | 0.118±0.013 | 0.125±0.018 | 0.113±0.024 | 0.145±0.022 |
| Medium | 0.120±0.019 | 0.134±0.023 | 0.200±0.020* | 0.277±0.025* | 0.299±0.037* |
| High | 0.122±0.015 | 0.121±0.025 | 0.271±0.031* | 0.333±0.056* | 0.295±0.029* |
| *EROD* | | | | | |
| Control | 33.1±4.2 | 29.3±3.7 | 27.7±4.5 | 30.1±3.9 | 30.5±3.5 |
| Low | 29.7±3.2 | 34.6±3.7 | 29.0±4.8 | 31.2±4.7 | 45.6±5.6* |
| Medium | 27.7±5.7 | 30.9±4.0 | 55.7±8.6* | 89.9±7.6* | 85.8±8.9* |
| High | 34.5±3.7 | 45.3±10.3* | 123.1±11.2* | 154.8±12.7* | 186.7±13.1* |

**Figure Legends**

Figure 1. Total Polycyclic Aromatic Hydrocarbon (TPAH) concentrations in treatment tanks as a function of time after initiation of water flow through a WSF column. Data points are single composite values for replicate tanks at each time period. ( ○ ) Control, ( ▲ ) 10 µg/L, ( ■ ) 40 µg/L , ( ● ) 100 µg/L.

Figure 2. Individual PAHs as a function of percent TPAH in treatment tanks as a function of time following initiation of water flow through a WSF column. From Kennedy and Farrell 2005.

Figure 3. Plasma cortisol, lactate, glucose, $Na^+$, $Cl^-$, and $K^+$ concentrations in herring exposed to various concentrations of WSF for 24 h, 96 h, 4.1 wk and 8.1 wk. Control fish ( □ ) and those exposed to L ( □ ), M ( ▩ ), and H ( ■ ) concentrations. Values are means ± SE of n=11 fish. * denotes a significant difference ($p<0.05$) from controls. Experiments were performed in triplicate.

Figure 4. Plasma lysozyme concentrations and respiratory burst activity in macrophages of herring exposed to various concentrations of WSF for 24 h, 96 h, 4.1 wk and 8.1 wk. Control fish (□) and those exposed to L (□), M (▩), and H (■) concentrations. Values are means±SE , n=11 * denotes a significant difference ($p<0.05$) from controls. Experiments were performed in triplicate

Figure 5. Percent mortality in herring exposed to WSF for 24 h, 96 h, 4 wk and 8 wk following a challenge with *V. anquillarum*. Control fish ( ○ ) , L ( Δ ), M (■), and H (●) concentrations. Values are averages of duplicate tanks.

Figure 1.

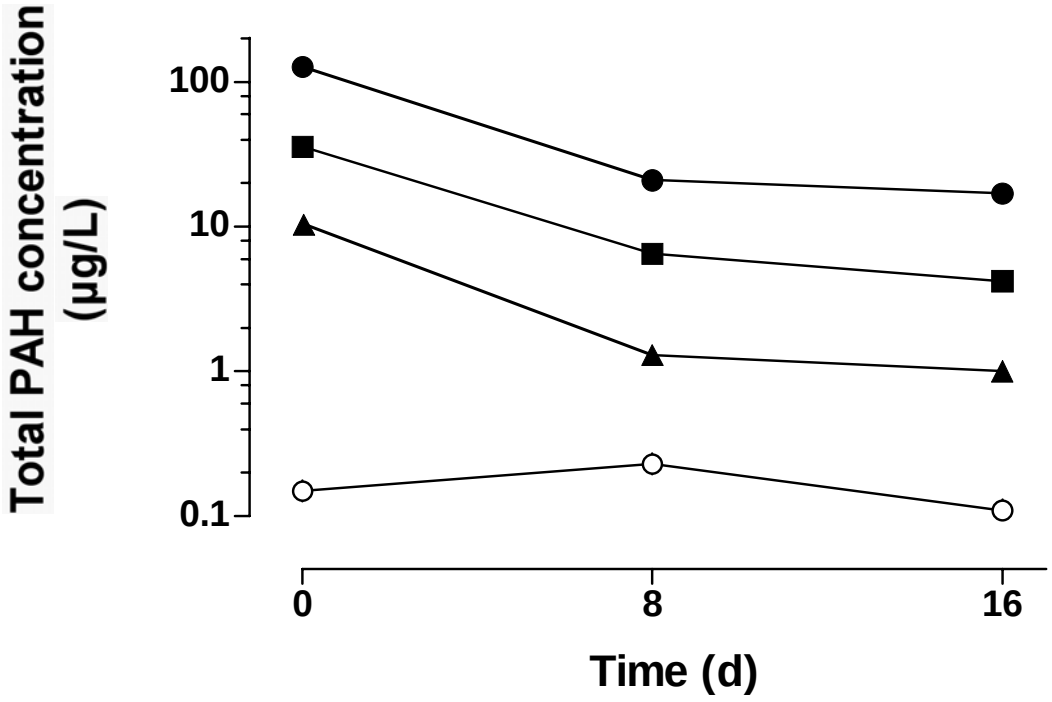


Figure 2.

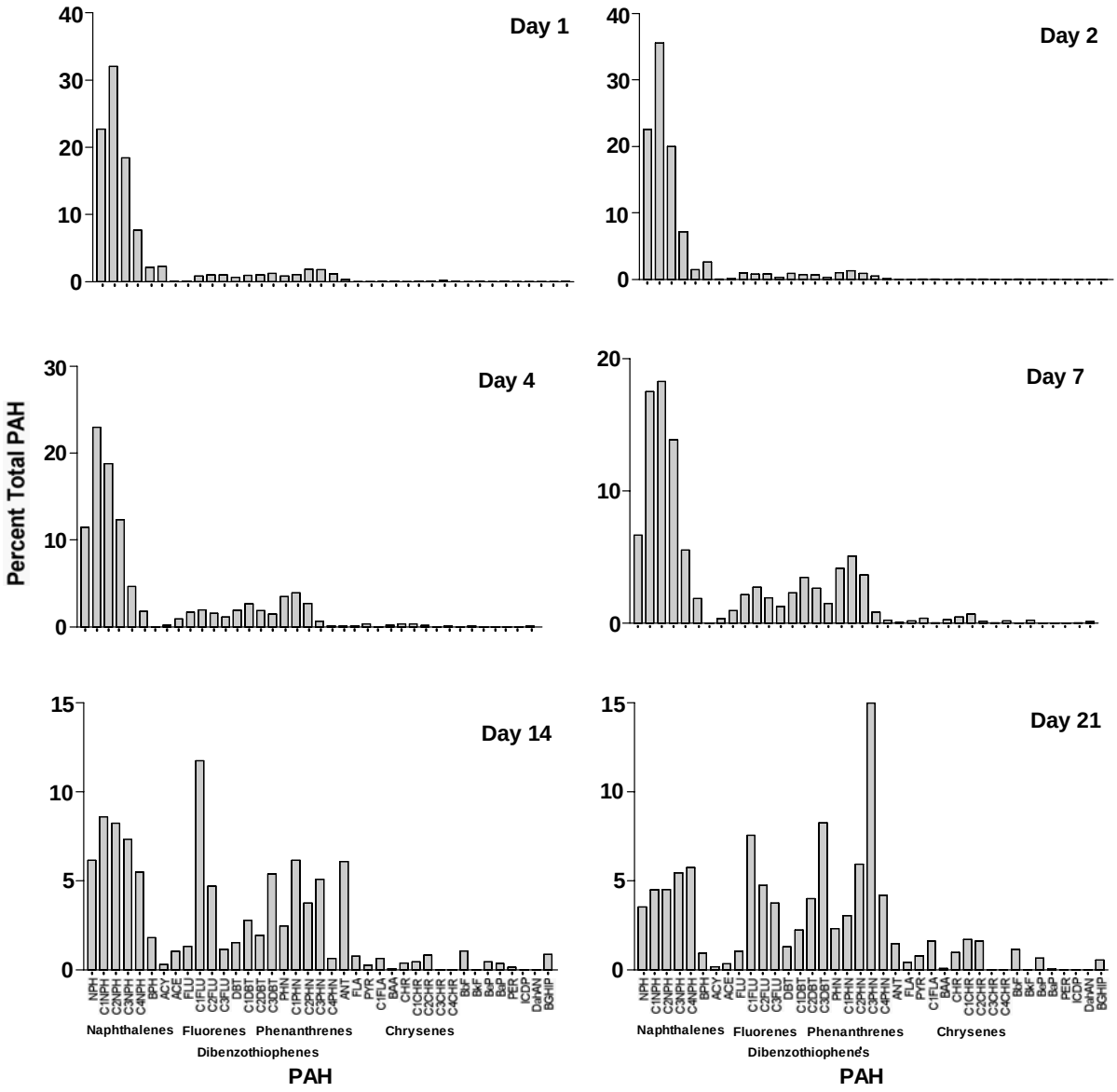



Figure 3.

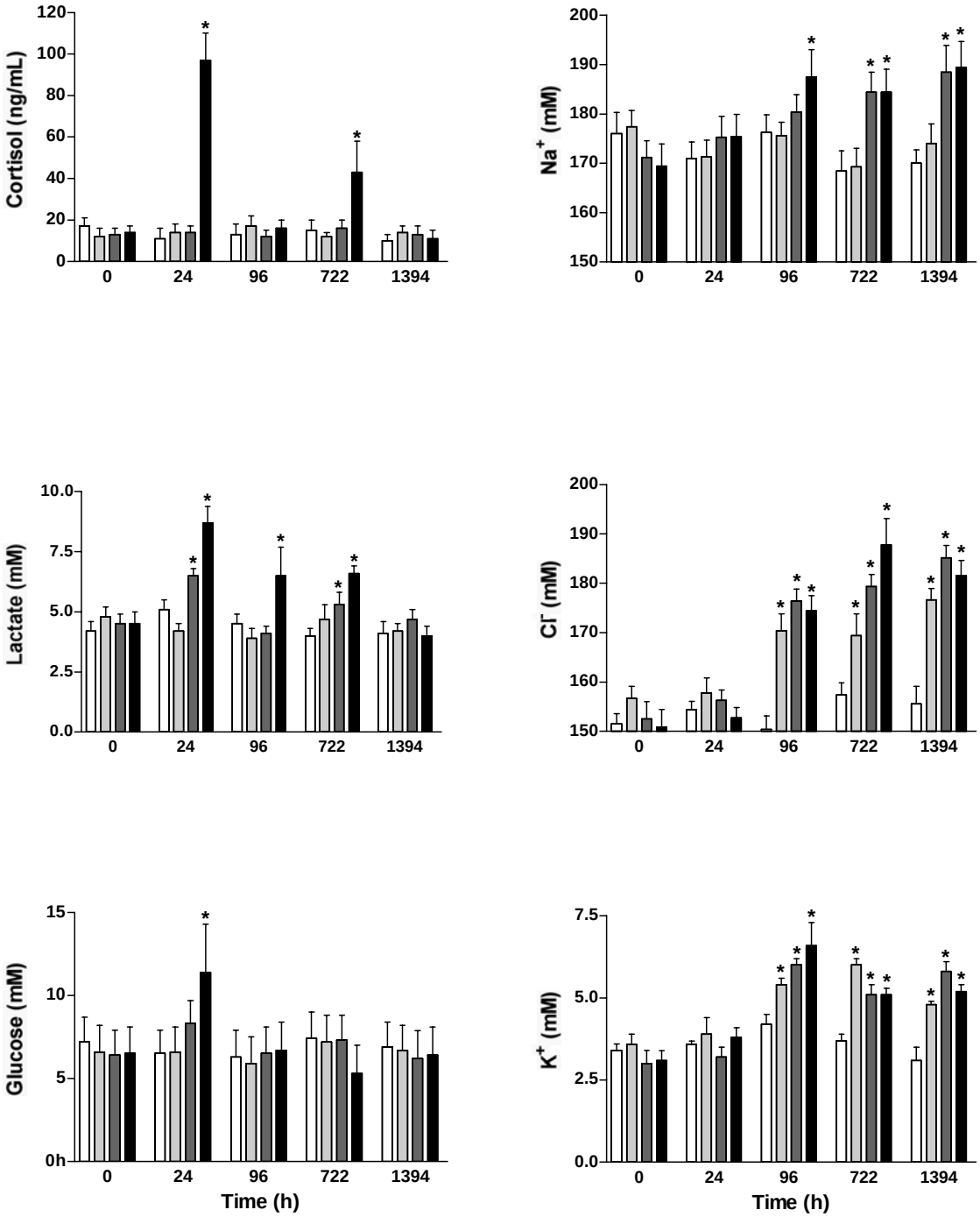
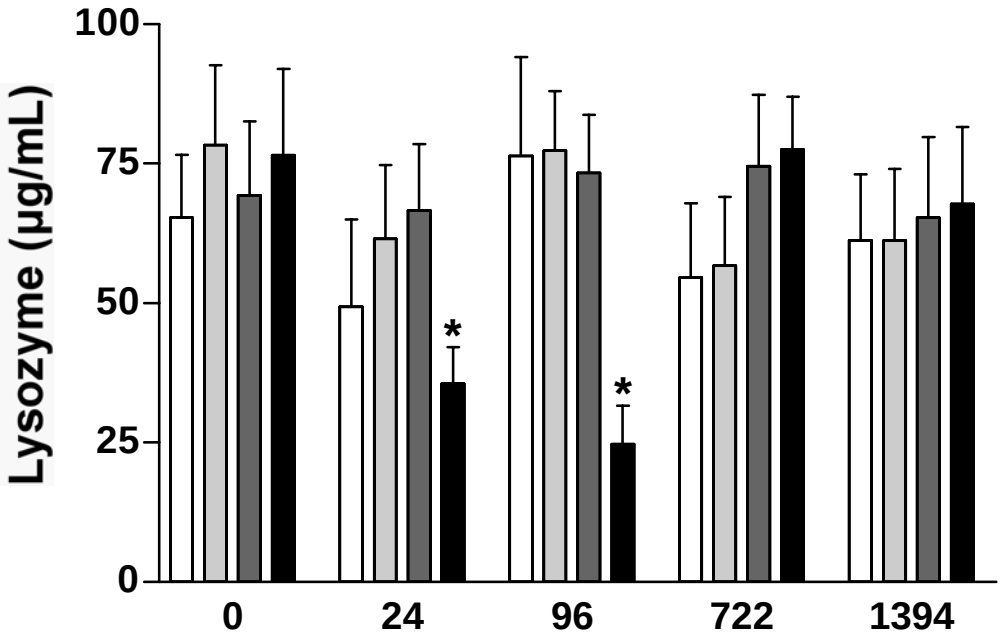
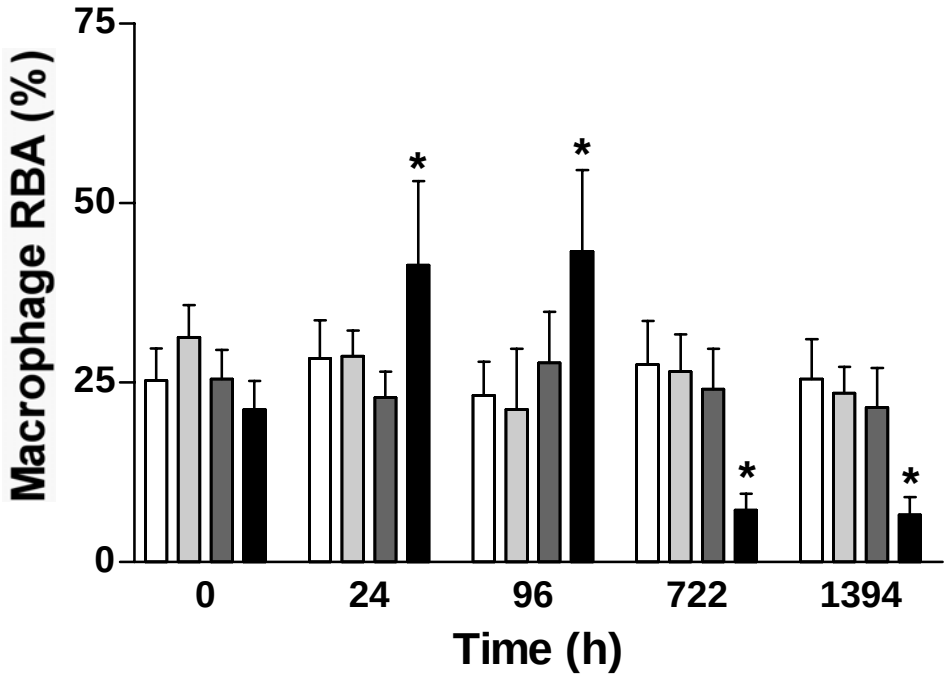

Cortisol (ng/mL)
120
100
80
60
40
20
0
0
24
96
722
1394
Na+ (mM)
200
190
180
170
160
150
Lactate (mM)
10.0
7.5
5.0
2.5
0.0
Cl- (mM)
Glucose (mM)
15
10
5
0h
K+ (mM)
7.5
5.0
2.5
0.0
Time (h)

Figure 4.


Lysozyme (µg/mL)
100
75
50
25
0
*
*
0
24
96
722
1394


Macrophage RBA (%)
75
50
25
0
*
*
*
*
0
24
96
722
1394
Time (h)

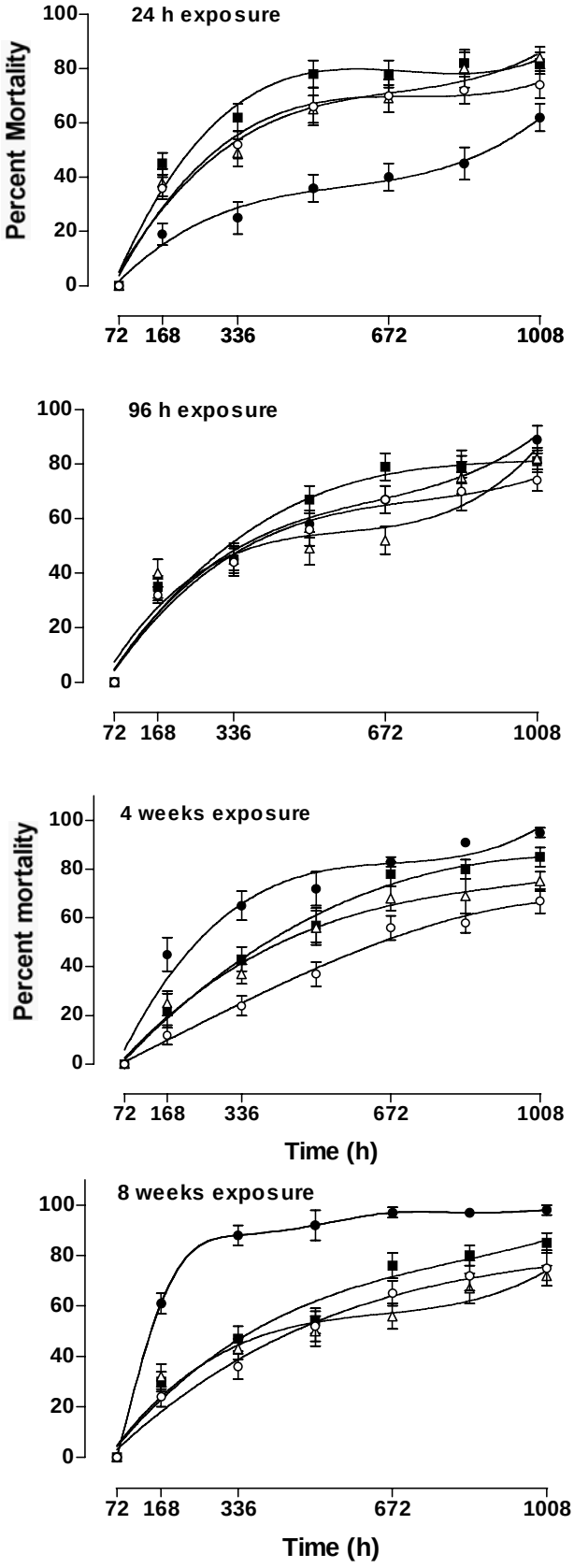

24 h exposure
Percent Mortality
100
80
60
40
20
0
72
168
336
672
1008
96 h exposure
100
80
60
40
20
0
72
168
336
672
1008
4 weeks exposure
Percent mortality
100
80
60
40
20
0
72
168
336
672
1008
Time (h)
8 weeks exposure
100
80
60
40
20
0
72
168
336
672
1008
Time (h)

Figure 5.

The Exxon Valdez Oil Spill Trustee Council administers all programs and activities free from discrimination based on race, color, national origin, age, sex, religion, marital status, pregnancy, parenthood, or disability. The Council administers all programs and activities in compliance with Title VI of the Civil Rights Act of 1964, Section 504 of the Rehabilitation Act of 1973, Title II of the Americans with Disabilities Action of 1990, the Age Discrimination Act of 1975, and Title IX of the Education Amendments of 1972. If you believe you have been discriminated against in any program, activity, or facility, or if you desire further information, please write to:

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