Exxon Valdez Oil Spill
Restoration Project Final Report

Responses of River Otters to Oil Contamination: A Controlled Study of Biological Stress Markers

Restoration Project 99348
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Study History: Project 99348 originated from the need to better understand the effects of contamination by crude oil on biomarkers in river otters (*Lontra canadensis*). Previous studies demonstrated elevated levels of biomarkers in river otters from oiled areas compared with those from non-oiled areas throughout Prince William Sound, Alaska, shortly following the *Exxon Valdez* oil spill (EVOS). Although the data collected to date strongly indicated a correlation between oil contamination and physiological stress in river otters, this evidence required verification through controlled experiments as identified by the EVOS Trustee Council review process (1997). This 2-year project was conducted at the Alaska SeaLife Center in Seward, Alaska, USA, between April 1998 and March 1999. Additional funding was provided by the Council for completion of additional 3 manuscripts in FY2000 for publication in a peer-reviewed journal.

Abstract: In this study, we experimentally determined the effects of oil contamination on river otters. Fifteen wild-caught male river otters were exposed to 2 levels of weathered crude oil (i.e., control, 5 ppm/day/ kg body mass, and 50 ppm/day/ kg body mass) under controlled conditions in captivity at the Alaska SeaLife Center in Seward, Alaska. Responses of captive river otters to oil ingestion provided mixed results in relation to biomarkers. Although hemoglobin, white blood cells, alkaline phosphatase, and possibly interleukin–6 immunoreactive responded in the expected manner, other parameters did not. Aspartate Aminotransferase Alanine Aminotransferase haptoglobin did not increase in response to oiling or decrease during rehabilitation. In addition, although expression of P450-1A increased in captive river otters during oiling, several inconsistencies in the data complicated data interpretation. Nonetheless, we were able to establish that reduction in hemoglobin led to increase in energetic costs of terrestrial locomotion, decrease in aerobic dive limit, and potential increase in foraging time due to a decrease in total length of submergence during each foraging bout. We offer a theoretical physiological model to describe interactions between the different biomarkers and advocate the exploration and development of other biomarkers that will be independent of the heme cycle.

Keywords: Aerobic dive limit, Alaska, captivity, CYP1A, crude oil, haptoglobin, hemoglobin, hydrocarbons, immuno-histochemistry, liver enzymes, *Lontra canadensis*, lymphocytes, oxygen consumption, quantitative RT-PCR.

Project Data: *Description of data* – data was collected from live animals held in captivity at ALSC. Blood and other tissues were sampled and processed in different laboratories. Additional samples are archived at the Institute of Arctic Biology, UAF. *Format* – All data were entered as Excel speardsheets. *Custodian* – contact Merav Ben-David, Institute of Arctic Biology, 311 Irving Bldg., University of Alaska Fairbanks, Fairbanks, AK 99775.

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TABLE OF CONTENTS

Biomarker responses in river otters experimentally exposed to oil contamination

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ABSTRACT: Investigations in Prince William Sound (Alaska, USA) following the Exxon Valdez oil spill (EVOS) revealed that river otters (Lontra canadensis) on oiled shores had lower body mass and elevated values of biomarkers, than did otters living on nonoiled shores. In addition, otters from oiled areas selected different habitats, had larger home ranges, and less diverse diets than animals living in nonoiled areas. These differences between river otters from oiled shores and those from nonoiled areas strongly suggested that oil contamination had an effect on physiological and behavioral responses of otters. In this study, we explored the effects of crude oil contamination on river otters experimentally. We hypothesized that exposure to oil would result in elevated values of biomarkers, indicating induced physiological stress. Fifteen wild-caught male river otters were exposed to two levels of weathered crude oil (i. e., control, 5 ppm/day/ kg body mass, and 50 ppm/day/ kg body mass) under controlled conditions in captivity at the Alaska Sealife Center in Seward, Alaska, USA. Responses of captive river otters to oil ingestion provided mixed results in relation to our hypotheses. Although hemoglobin (Hb, and associated red blood cells) and white blood cells, and possibly interleukin–6 immunoreactive responded in the expected manner, other parameters did not. Aspartate aminotransferase, alanine aminotransferase, and haptoglobin (Hp), did not increase in response to oiling or decreased during rehabilitation. Conversely, principle-component analysis identified values of alkaline phosphatase as responding to oil ingestion in river otters. Our results suggested that opposing processes were concurring in the oiled otters. Elevated production of Hp in response to tissue damage by hydrocarbons likely occurred at the same time with increased removal of Hp-Hb complex from the serum, producing an undetermined pattern in the secretion of Hp. Thus, the use of individual biomarkers as

indicators of exposure to pollutants may lead to erroneous conclusions because interactions in vivo can be complicated and act in opposite directions. Additionally, the biomarkers used in investigating effects of oiling on live animals usually are related to the heme molecule. Because of the opposing processes that may occur within an animal, data from a suite of heme-related biomarkers may produce results that are difficult to interpret. Therefore, we advocate the exploration and development of other biomarkers that will be independent from the heme cycle and provide additional information to the effect of oiling on live mammals.

Keywords: Captivity, crude oil, hemoglobin, haptoglobin, hydrocarbons, liver enzymes, Lontra canadensis, season.

INTRODUCTION

Investigations in Prince William Sound (PWS), Alaska, USA, following the Exxon Valdez oil spill (EVOS), revealed that coastal river otters (Lontra canadensis) on oiled shores had lower body mass and elevated levels of biomarkers than those living in nonoiled areas (Duffy et al. 1993; 1994a; 1994*b*; 1996; Blajeski et al., 1996; Bowyer et al., in review; Taylor et al., in press). In addition, otters from oiled areas selected different habitats, had larger home ranges, and less diverse diets than those living in nonoiled areas (Bowyer et al. 1994; 1995). These observed differences between river otters from oiled shores and those from nonoiled areas indicated that oil contamination had an effect on physiological and behavioral processes in river otters. Moreover, these effects had the potential to become chronic and may have impeded recovery of

populations of river otter if the exposure to hydrocarbons continued. Between 8-16% of the 39,000 metric tons of crude oil spilled by the super tanker Exxon Valdez remains buried in marine sediments (Wolfe et al., 1994; O'Clair et al., 1996). Moreover, microbial analyses indicated that oil in sediments along some shorelines was still several orders of magnitude more common than in nonoiled areas (Braddock et al., 1996). Oil buried in sediments, which may be re-suspended during storms and tidal action, is not subject to degradation by aerobic bacteria and therefore remains in a form that is toxic to many vertebrates (Braddock et al. 1996). Thus, oil may still be available for biological transport from benthic invertebrates through the food web.

Recent studies exploring effects of pollution on wild animals sought nonlethal methods to replace the traditional extraction of tissues from carcasses. Such traditional studies involve killing a sufficient number of animals to allow for proper statistical evaluations (for examples see Evens et al., 1998; Gutleb et al., 1998). This approach may lead to extirpation of subpopulations or cause genetic bottlenecks when populations are small (Bowyer et al., in review) and add to the damage already created by the pollution event. Thus, researchers embraced the use of biomarkers to assess effects of pollution, including exposure to and damage from hydrocarbons in living vertebrates (Miranda et al., 1987; Stegeman et al., 1992; Akins et al., 1993; Duffy et al., 1996; Zentano-Savin et al., 1997).

Several specific biomarkers as well as general blood panels have been used in studies related to hydrocarbon exposure (Bowyer et al., in review). Haptoglobin (Hp) and interleukin–6 immunoreactive (IL-6ir) were used to indicate increased liver activity. These acute-phase proteins are synthesized in response to trauma, toxicological damage, or infection (Duffy et al. 1993, 1994*a*, 1994*b*; Prichard et al., 1997). Additionally,

researchers assayed porphyrins extracted from fecal samples (Akins et al., 1993; Blajeski et al. 1996; Taylor et al, in press). Chemically induced changes in patterns of porphyrins have been observed in several avian species following exposure to aromatic hydrocarbons (Miranda et al. 1987). Cytochrome P450-1A was added to the suite of biomarkers used for assessing exposure to hydrocarbons. Cytochrome P450s are a group of enzymes that metabolize a wide variety of xenobiotic compounds. P450-1A is induced by planar aromatic or chlorinated hydrocarbons, and thus its presence serves as a bioindicator of hydrocarbon exposure (Woodin et al. 1997).

Studies initiated following EVOS indicated that several vertebrate predators displayed physiological stress related to oil toxicity, in addition to data collected on river otters (Collier et al., 1996; Laur and Haldorson, 1996; Loughlin et al., 1996; Piatt and Ford, 1996; Ballachey and Kloecker, 1997; Woodin et al. 1997). Oiled sea otters (Enhydra lutris), collected for rehabilitation, suffered from emphysema, ulcers, anemia, lesions and organ congestion (Williams et al. 1995). Similarly, free-ranging sea otters from oiled regions had greater antigenic stimulation than animals from nonoiled areas, and young sampled in those regions had lower hemoglobin (Hb) levels than young from nonoiled areas (Rebar et al., 1994). Pigeon guillemots (Cepphus columba) had elevated levels of Hp and blood proteins in specific locations and years, although dosing experiments in the field failed to demonstrate the connection between oiling and those parameters (Prichard et al., 1997; Seiser et al., 2000). Changes in plasma proteins and white blood cells (leukocytes), reduction in the number of red blood cells (erythrocytes), and electrolyte imbalance have also been observed in mink (Mustela vison) and polar bears (Ursus maritimus) following exposure to hydrocarbons (Øristsland et al., 1981; Mazet et al., in press).

Although previously collected data strongly indicated a relation between oil contamination and physiological stress in river otters and other vertebrates in Prince William Sound, interpretation of that evidence will be aided by controlled experiments. In this study, we investigated the effects of exposure to oil on physiological processes in captive river otters and hypothesized that exposure to oil would result in changes in levels of biomarkers, indicating induced physiological stress.

We adopted two approaches to data evaluation. First, we tested whether exposure to known doses of oil resulted in significant changes in levels of biomarkers for those markers that were previously used in the field studies on river otters (Bowyer et al., in review), or were reported for other species (Mohn and Nordstoga, 1975; Øristsland et al., 1981; Prichard et al., 1997; Zentano-Savin et al., 1997). Second, we used a data-exploration approach to detect other measures that may have responded to oil exposure in hopes to provide additional tools for future studies of biomarkers in vertebrates.

MATERIALS AND METHODS

Trapping and handling the experimental animals

River otters were live-captured in northwestern PWS (60° 40' N 147°50' W) from late April to late May 1998, with No. 11 Sleepy Creek® leg-hold traps (Blundell et al., 1999). Traps were placed on trails at latrine sites and monitored by trap transmitters (Telonics, Mesa, Arizona, USA) that signal when a trap is sprung (Blundell et al., 1999; Bowyer et al., in review). Processing of otters began within 1 - 2 h from capture. Otters were anesthetized with Telazol (9mg/kg; A. H. Robins, Richmond, Virginia, USA) administered using Telinject® darts and a blowgun. Data on morphometrics, collected

from anesthetized otters, included body mass (nearest 0.1 kg); body length, tail length, and total length (nearest 1 mm); total skull length and width of zygomatic arch (nearest 1 mm). Age of otters (juvenile, young adult, adult, and old adult) was estimated based on body size, tooth wear and staining. Blood and tissues were sampled from each individual otter at that time. Of the 51 individuals captured (Bowyer et al., in review), 15 young adult males (estimated age 1 – 5 yrs) were selected for the controlled experiments and transferred under sedation via air to the Alaska Sealife Center (ASLC) in Seward, Alaska, USA.

Wild-caught river otters were held in captivity at ASLC from May 1998 to March 1999. Animals were housed as one large group in an area of 90 m^2 surrounding six pools (one large salt-water pool - 4.5 m diameter by 3 m depth; four small salt-water pools - 2 by 1.5 by 1.5 m; and one small fresh-water tote - 1 by 1 by 1 m). Fences were covered with clear acrylic panels to prevent the otters from climbing, and an electric fence was mounted at a height of 1.6 m to further deter the otters from escaping the pen. The gate to the enclosure was locked at all times, and only trained personnel were allowed access to the pen. Thirteen plywood sleeping boxes (1.3 x 0.6 x 0.6m) were equipped with polyester fleece blankets and stationed throughout the enclosure. Otters were fed twice daily ad libitum on a diet of frozen fish of the following species: pink salmon (Oncorhynchus gorbuscha), capelin (Mallotus villosus), Pacific herring (Clupea pallasii), walleye pollock (Theragra chalcogramma), prawns (Pandalus platyceros), and eulachon (Thaleichthys pacificus). These fishes were purchased from commercial harvest and approved for human consumption. Two to three times per week, the diet was supplemented with live prey (pink salmon, kelp greenling (Hexagrammos decagrammus), and rockfish (Sebastes sp.)) captured in Resurrection Bay, Alaska (ADFG permit CF 98-

024; Ben-David et al., 2000). Initially, vitamins (HiVite™, EVSCO Pharmaceuticals, IGI, Inc., Buena, New Jersey) were provided with food, but because otters seemed reluctant to consume those fishes, we injected B complex vitamins (0.5 ml Phoenix Scientific, St. Louis, Missouri) intramuscularly during the blood-sampling sessions every 3 weeks. Minerals were provided as a mineral block and otters had continuous access to that resource.

Administration of crude oil

Experiments began in August after allowing the animals 2.5 months to acclimate to the enclosure, feeding regimes, and handling procedures. During that time, the average daily food intake of otters was monitored to quantify the amount of oil required for achieving each oiling level. Daily food intake per animal averaged 1,000 g/day over the acclimation period. At the end of that period, otters were randomly assigned to 3 experimental groups of 5 individuals each: a control group that received no oil; a low-dose group that received 5 ppm of oil/day/kg body mass (i.e., 0.1 g every other day or 100 ppm/kg of food every other day); and a high-dose group that received 50 ppm of oil/day/kg body mass (i.e., 1.0 g every other day or 1000 ppm/kg of food every other day). Individuals were assigned to treatment groups with a randomized complete-block design. Original assignment of otter identification numbers were based on body mass at capture to control for potential differences in age and size. For the complete-block design otter identification numbers were randomly permutated within blocks (performed by T. L. McDonald, West, Inc. Cheyenne, Wyoming).

Prudhoe Bay crude oil (obtained from Williams Inc. Fairbanks, Alaska, USA) was mixed in seawater and stirred continuously for 10 days at 25°C. Two batches of oil were weathered separately and a sample from each batch was sent for analysis at Auke Bay

Laboratory (J. Short, National Oceanographic and Atmospheric Administration, Juneau, Alaska). The two batches differed slightly in composition, but both were comparable to the oil profile of EVOS shortly after landfall in 1989 (Table 1; Short and Heintz, 1997). Weathered oil was separated from water and administered to otters in gel capsules (500 mg) hidden in fishes every other day. Quantity of oil was measured with a micro-pipette (Rainin Instruments Co., Emeryville, California) and weighed on an analytical micro-balance (nearest 0.01g). Occasionally the otters bit into the capsule when feeding and subsequently dropped the fish. On such occasions, oil was administered again during the following feeding to ensure that each otter ingested the amount of oil required for the experiment. Careful notes on feeding of oil were kept throughout the experiment. Feeding of oil lasted 100 days from 21 August to 28 November 1998. The first batch of oil was fed to the otters between August 21 and October 19, as well as between November 19 and November 28. The second batch was fed to the otters between October 20 and November 18 (Table 1). Data collection continued for additional 100 days of rehabilitation. Animals were then fitted with radiotransmitters and released at the site of their capture in PWS. Animals are currently being monitored to determine post-release survival.

Sampling of blood

Prior to exposure to oil, a series of blood and tissue samples were collected (29-30 June, and 15-16 August 1998) from each individual otter for analysis of biomarkers (Table 2). Blood and tissue sampling continued from 15 August 1998 until 12 January 1999 every three weeks. An additional sampling session occurred on 22-24 February 1999 in conjunction with implanting of radiotransmitters.

Otters were anesthetized with a combination of ketamine hydrochloride (100 mg/ml, Ketaset®, Aveco Co., Fort Dodge, Iowa) at a dosage of 10 mg/kg, and midazolam hydrochloride (5 mg/ml, Versed®, Hoffman-LaRoche, Nutley, New Jersey) at a dosage of 0.25 mg/kg (Spelman et al. 1993). The dosage was mixed in the same syringe and administered intramuscularly with Telinject® darts and a blowgun or hand injected while the otter was immobilized in a squeeze-box. Before darting the otter, each individual received one fish containing 0.5 cc Versed®. This dosage calmed the animals and reduced the stress associated with handling.

We drew blood from the jugular vein of each otter with care to keep samples sterile. A portion of the sample was preserved with EDTA (purple top Vacutainer®) for complete blood counts (CBC) and refrigerated until analysis (within 48 h; Table 2). The remaining blood (approximately 10 ml) was collected in a red top Vacutainer® and allowed to clot; serum was removed (within 8 h) following centrifugation at 3,000 rpm for 10 min, and refrigerated until analyses of serum chemistry (Table 2). Samples for haptoglobin (Hp) and IL-6ir analyses were frozen at –20°C. In addition, 4 blood smears were made for each river otter at the time the blood was drawn and samples were investigated later for cell abnormalities (Duncan et al., 1994).

Biomarker analysis

Serum-chemistry profiles were assayed with an Olympus 7000 analyzer (Olympus, Melville, New York) and complete blood counts were performed with a Stack-S whole blood analyzer (Coulter, Miami, Florida). Samples were analyzed at Quest Diagnostics Incorporated (Portland, Oregon)

Haptoglobins (Hp) are alpha$_2$ glycoproteins that stoichiometrically bind free hemoglobin (Hb) in a haptoglobin-hemoglobin (Hb-Hp) complex. Using the standard protocol described by Duffy et al. (1993; 1994*a*; 1994*b*) the Hp-Hb complex, was quantified by densitometry and results were expressed as mg Hb-bound/100 ml serum.

Values of IL-6ir were determined with an immunochemical assay. Replicates of each sample were added to a microtiter plate coated with a monoclonal antibody for IL-6 (Quantikine, R&D systems, Minneapolis, MN). After washing away any unbound proteins, an enzyme-linked polyclonal antibody for IL-6 was added to the wells and incubated to allow for binding. After a final wash, a substrate solution was added to the wells. Following color development, sample concentrations were determined from a standard curve (Duffy et al. 1994*a*, 1994*b*).

Statistical analyses

Of 165 samples collected through the experiment, 6 samples clotted thus providing no data for CBC. Missing values for those samples were replaced by a means of near points approach (Johnson and Wichern, 1992). To determine effects of oiling on values of biomarkers (dependent variable) in river otters, we used repeated measures ANOVA with oiling group (i.e., control, low dose, and high dose) and bleeding session as factors (Johnson and Wichern, 1992; GLM procedure, SPSS for Windows). The first bleeding session, that occurring at capture, and the 2 following sessions (June and August) provided information on the acclimation period. The oiling period included 5 sessions (three weeks interval; depicted as Sept I to Nov II), and rehabilitation period was represented by 3 sessions (Dec – Feb II). We opted to present data in this manner to better illustrate response curves for the different biomarkers. This approach, however, is

conservative as significant differences may be obscured by the slow changes over time in the values of biomarkers.

Analysis was conducted separately, as hypothesis tests, for each of the following variables: Aspartate aminotransferase and alanine aminotransferase (AST and ALT respectively; Duffy et al. 1994*a*) hemoglobin (Øristsland et al., 1981; Rebar et al., 1994; Williams et al., 1995), haptoglobin (Duffy et al., 1993; 1994*a*; 1994*b*; Prichard et al., 1997), IL-6ir (Duffy et al., 1994*a*), white cell count (Øristsland et al., 1981; Rebar et al., 1994). Analysis was followed by Tukey's multiple comparisons to establish where significant differences occurred for those models in which either group or session effects were significant. α level was set at 0.05.

For our data-exploration approach, we used principal components analysis (PCA, SPSS for Windows) to reduce the dimensionality of data while considering effects of all variables simultaneously for each bleeding session (Johnson and Wichern 1992; Bowyer et al., in review), because the number of blood variables that were analyzed was greater than the number of individual otters in this experiment. Under such conditions employing a series of univariate tests on the individual blood values would have been incorrect. We developed the PCA from a correlation matrix rather than the variance-covariance matrix to avoid the bias in results when original data vary markedly in scale (a common occurrence in blood values). A well-known difficulty with PCA is in determining the meaning of a particular axis (Johnson and Wichern 1992). Therefore, we followed the PCA with repeated measures ANOVA with oiling group (i.e., control, low dose, and high dose) and bleed session as factors, for those dependent variables that had the highest contribution to the variability in the data in at least 5 of the 11 bleeding sessions. Analysis was followed by Tukey's multiple comparisons to establish where significant

differences occurred for those significant models in which either group or session effects were significant (Johnson and Wichern, 1992: GLM procedure, SPSS for Windows). We also used correlation analysis (Peasrson's ρ; Zar, 1984, SPSS for Windows) to determine the relation between each two variables that were identified as important with PCA. A significant relation at the $\alpha = 0.05$ level was reported following a sequential Bonferroni correction when multiple comparisons were conducted (Rice, 1989).

RESULTS

Hypothesis tests

Values of AST significantly declined between capture and all other bleeding sessions (Fig. 1a; repeated measures 2-way ANOVA; $\underline{P} < 0.001$) for all 15 river otters. Although session effects were highly significant ($\underline{P} < 0.001$), group had no effect on AST values ($\underline{P} = 0.124$) suggesting that oil ingestion did not influence values of this variable. This observation is further supported by lack of increases in AST values in either the low-dose or the high-dose groups during the oiling period (21 August – 28 November; Fig. 1a). Values of AST for all otters were well below the values observed in the same individuals at capture (Fig. 1a). On 9 November (Nov I session; Fig.1a) mean values of AST were elevated in the high-dose group although that increase was not statistically significant (Tukey's multiple comparisons; $\underline{P} > 0.05$). These elevated values of AST resulted from an increase in one individual (908 U/L; EP06) that suffered from a gangrened toe (which was amputated immediately by a veterinarian). Data from that individual were excluded from subsequent analyses.

Values of ALT did not change significantly for any of the groups throughout the experiment (Fig. 1b; repeated measures 2-way ANOVA: $\underline{P} = 0.271$). For that analysis we excluded the one individual that suffered from a gangrened toe and that had exceedingly high values of this enzyme throughout the experimental period (up to 2,523 U/L; EP06). Group association dictated the values of ALT ($\underline{P} = 0.012$), with the high-dose animals having higher values than the low-dose or control animals (Fig. 1b) during the oiling period (Tukey's multiple comparisons; $\underline{P} < 0.05$); no difference was detected between the low-dose and the control groups ($\underline{P} > 0.7$). In addition, within each group, no significant difference in values of ALT was detected between the different sessions ($\underline{P} > 0.14$), suggesting that oil ingestion did not influence values of this variable. Values of AST and ALT were correlated for the entire data set ($\underline{r} = 0.52$; $\underline{P} < 0.001$; $\underline{n} = 165$).

Values of haptoglobin (Hp) significantly changed throughout the experimental period (Fig. 2a; repeated measures 2-way ANOVA; $\underline{P} = 0.002$). Although we were unable to detect a group effect ($\underline{P} = 0.48$), session seemed to influence the values of Hp in our otters ($\underline{P} < 0.001$). Values significantly increased between capture and the first bleeding session at the end of June for all otters (Tukey's multiple comparisons; $\underline{P} < 0.05$). Values then declined slowly for all otters until early November, after which values began to increase again (Fig. 2a). Values of Hp for both low-dose and high-dose animals were at the lowest values at the height of the oiling period (September to November), and in one session (October) significantly lower than those of the control group (Tukey's multiple comparisons; $P < 0.05$; Fig. 2a).

Values of IL-6ir did not change significantly throughout the experimental period (Fig. 3a; repeated measures 2-way ANOVA; $\underline{P} = 0.32$). No group or session effects were

detected ($\underline{P} = 0.12$ and $\underline{P} = 0.74$, respectively). In addition, no pattern related to the oiling period could be deciphered (Fig. 3a).

We observed a significant reduction in values of hemoglobin (Hb) from oiling in our experimental otters (Fig. 4a; repeated measures 2-way ANOVA; $\underline{P} < 0.01$; group effect $\underline{P} = 0.001$; session effect $\underline{P} < 0.001$), and values of Hb were highly correlated with values of red blood-cell counts for the entire data set (RBC; $\underline{r} = 0.875$; $\underline{P} < 0.001$). Hb values decreased in a similar fashion for all animals over the 3 months from capture to sampling in August. No significant differences ($\underline{P} > 0.8$) were detected between groups for that period (Fig. 4a). During the oiling period, values of Hb stabilized for the control group, while values continued to significantly decline for the oiled animals ($\underline{P} < 0.05$). No significant differences ($\underline{P} > 0.2$) were detected between the low and high dose during that period (Fig. 4a) and the lowest values of Hb were observed in the oiled otters in October. After administration of oil ended (28 Nov. 1998), Hb values increased and no significant differences were detected between oiled and control animals in the December sampling (Tukey's multiple comparisons; $\underline{P} > 0.05$). Similarly, no differences in Hb values were detected between the high dose and the control groups in January and February (Tukey's multiple comparisons; $\underline{P} > 0.05$), but the low-dose group experienced a significant decline in Hb values during that time (Fig. 4a; Tukey's multiple comparisons; $\underline{P} < 0.05$). We were able to determine that this decline was caused by an iron deficiency (62 ± 17 µg/L; mean ± SE; reported normal values 127.9 ± 40.5µg/L; Davis et al., 1992). We reversed the deficiency with weekly iron injections before the animals were released. Scanning of blood smears for cell abnormalities revealed nucleated erythrocytes in the anemic animals but no Heinz bodies were apparent.

Hb and Hp were slightly negatively correlated (r = -0.18; P = 0.02) as were Hb and IL-6ir (r = -0.22; P = 0.005). In addition, AST was positively correlated with Hb (r = 0.4; $P < 0.001$). A repeated measures ANOVA model with Hp as the dependent variable, cumulative dose of oil and session as the factors, and Hb as a covariate (P = 0.007) indicated that values of Hb had a significant influence on values of Hp (P = 0.013).

Individual variation in reduction in Hb was high among the oiled animals. Although several individuals lost only 15 – 20% of their Hb during the experiment, others lost up to 32%. This is further supported by the large influence of animals ID on the result of the ANOVA model ($P < 0.001$). We were unable to correlate the reduction in Hb with the cumulative amount of oil ingested by each animal (r = 0.08; P = 0.26).

White blood-cell count (WBC) differed significantly between groups and sessions (Fig. 4b; repeated measures 2-way ANOVA; $P < 0.001$; group effect $P < 0.001$; session effect $P < 0.001$). Large variations in WBC occurred throughout the experiment for all groups but a trend of continuous decline was more evident for the oiled groups (Fig. 4b; Tukey's multiple comparisons; $P < 0.05$). The pattern of recovery we observed in Hb at the end of the oiling period was not evident in WBC (Fig. 4b; Tukey's multiple comparisons; $P > 0.05$). Nonetheless, WBC exhibited a weak but significant correlation with Hb (r = 0.185, P = 0.017) and a weak negative correlation with IL-6ir (r = -0.177, P = 0.023).

Data exploration

The first 3 principle components in the PCA analyses identified 8 variables as explaining most to the variation in these data and accounted for 50.8 – 80.6% of the variation in each session. Those variables included albumin (ALB; Fig. 2b), alkaline phosphatase (ALK PHOS; Fig. 4c), ALT (Fig. 1b), globulin (GLOB; Fig. 2c), Hb (Fig.

4a), lactate dehydrogenase (LDH; Fig. 1c), platelet counts (PLAT; Fig. 3b), and triglycerides (TRIG; Fig. 1f). All those variables, excluding LDH, differed significantly between groups and sessions.

Values of ALK PHOS differed significantly between groups and sessions (repeated measures 2-way ANOVA; $\underline{P} = 0.001$; group effects $\underline{P} = 0.013$, session effect $\underline{P} < 0.001$). Values of ALK PHOS significantly declined for the control group through the experiment (Tukey's multiple comparisons; $\underline{P} < 0.05$), but did not for the low and high dose animals (Tukey's multiple comparisons; $\underline{P} > 0.05$). Significant differences between controls and treatment animals were only evident during October to November II sessions (Fig. 4c), as well as the January session. That pattern may indicate effects of oiling although the process is difficult to interpret. ALK PHOS was not correlated with either Hb or ALT ($\underline{r} = 0.115$ and $\underline{r} = 0.142$ respectively), and only slightly correlated with WBC ($\underline{r} = 0.152$, $\underline{P} < 0.05$). In contrast, ALK PHOS was negatively correlated with Hp ($\underline{r} = -0.21$, $\underline{P} < 0.05$) and positively with ALB and AST ($\underline{r} = 0.191$ and $\underline{r} = 0.27$ respectively, $\underline{P} < 0.05$).

ALB (Fig. 2b) and GLOB (Fig. 2c) exhibited opposite patterns to each other, although both had a significant group and session effects (ALB: repeated measures 2-way ANOVA; $\underline{P} < 0.001$; group effects $\underline{P} < 0.001$, session effect $\underline{P} < 0.001$; GLOB: repeated measures 2-way ANOVA; $\underline{P} < 0.001$; group effects $\underline{P} = 0.001$, session effect $\underline{P} < 0.001$). Although a significant group effect occurred for both variables, no relation to oiling could be detected because the control group had values in between those of the high-dose and the low-dose animals (Fig. 2b and Fig. 2c). Both ALB and GLOB were significantly correlated with Hp (ALB: $\underline{r} = -0.36$, $\underline{P} < 0.001$; GLOB: $\underline{r} = 0.29$, $\underline{P} < 0.001$), but only

ALB was correlated with Hb ($r = 0.36$, $P < 0.001$). ALB and GLOB were also negatively correlated ($r = -0.4$, $P < 0.001$).

LDH significantly decreased from the capture session, but no difference was detected between the treatment groups (Fig. 1c; repeated measures 2-way ANOVA; $P < 0.001$; group effects $P = 0.08$, session effect $P < 0.001$). The pattern in values of LDH was similar to that of AST (Fig. 1a), and indeed those 2 variables were significantly correlated ($r = 0.73$, $P < 0.001$). In addition, a similar pattern was observed in values of glucose and BUN (Fig. 1d and 1e), which were positively correlated with LDH ($r = 0.263$ and $r = 0.39$ respectively, $P < 0.05$).

Platelet counts significantly differed between groups and sessions (Fig. 3b; repeated measures 2-way ANOVA; $P = 0.002$; group effects $P = 0.009$, session effect $P = 0.038$), but no pattern could be discerned from these data (Fig. 3b). Similarly, although identified by the PCA as explaining part of high variability in these data, triglycerides exhibited a trend of increase in the captive otters (Fig. 1f; repeated measures 2-way ANOVA; $P = 0.113$; group effects $P = 0.002$, session effect $P = 0.05$), but no pattern related to oiling could be deciphered. Otter ID had significant effect on values of TRIG ($P = 0.009$). None of the other variables measured in this study exhibited any noticeable patterns related to oil ingestion, captivity, or season (Table 2).

DISCUSSION

Responses of the captive river otters to oil ingestion provided mixed results in relation to our hypotheses. Although Hb (and associated RBC), WBC and possibly IL-6ir responded in the expected manner, other parameters did not. AST, ALT, and Hp did not

increase in response to oiling or decreased during rehabilitation. In addition, of those variables identified in the PCA, only ALK PHOS responded to oil ingestion.

Responses to captivity

Although AST and ALT were strongly correlated with each other, they exhibited different patterns in relation to group association and bleeding session. That outcome probably occurred because values of ALT did not decrease significantly between capture and captivity. Values of AST during the acclimation, oiling, and rehabilitation periods were significantly lower than those recorded at capture, and comparable to values recorded for other captive otters (Table 2). Thus, it seems that the responses of AST to conditions in captivity may have obscured any possible responses associated with ingestion of oil.

The increase in Hp between captures and the first bleeding session (June) may be related to stress associated with captivity. Although other studies documented similar increases associated with physical, environmental and psychological stress (Aikawa et al., 1990; Kalmovarin et al., 1991; Boosalis et al., 1992; Zentano-Savin et al., 1997), we are unable to determine whether the increases we observed were related to such factors. Nonetheless, the decrease in levels of Hp by the end of the acclimation period (August) without a change in either diet, or levels of activity may support the possibility of psychological stress. In this experiment we introduced 15 adult male river otters, captured at 4 geographical locations in PWS, to a relatively small enclosure. These animals probably have never been in close contact with humans before and also had to adjust to new members of their own species.

A group of variables exhibited a similar pattern of a significant decrease between capture and the June sampling session, followed by stable values throughout the

experiment. Values of AST, GLU, BUN, and LDH during the experiment were below the mean value recorded for 24 animals live-captured in nonoiled areas in 1998 (Table 2; Bowyer et al., in review), but in agreement with those reported for other captive river otters (Table 2; Reed-Smith, 1995), suggesting that these variables are associated with trapping stress. Several studies identified these parameters as indicators of capture stress in a variety of wild animals (Seal and Hoskinson, 1978; Williams et al., 1992; Boonstra et al., 1998; Keech et al., 1998; Hartup et al., 1999). Unfortunately, this result may seem to contradict our interpretation of the initial increase in Hp in captivity. Values of Hp in Alaskan pinnipeds, however, were similar between anesthetized and non-anesthetized animals, and no increases were documented with increases in handling time suggesting that elevation in Hp is not directly related to capture (Zentano-Savin et al., 1997). Therefore, the initial increase in Hp was likely a result of stressors not directly related to handling the animals for drawing blood. That these stress-related variables were low throughout our experiment indicates that the otters acclimated well to the enclosure and our handling. Thus, we believe that interpreting the results from other variables can be accomplished without accounting for handling stress.

In contrast, the high variability within and between groups in values of triglycerides was probably a result of diet preference of individuals in captivity. Although some individuals preferred capelin, others consumed more salmon and yet others showed preference to eulachons. This outcome is particularly evident when the effect of otter ID on levels of TRIG is considered. Values of triglycerides were higher than those observed for 24 river otters live-captured in nonoiled areas in Prince William Sound (Table 2; Bowyer et al., in review). These values probably reflect the higher lipid contents of the food we offered to the captive otters (herring, salmon and, capelin) in comparison with

the intertidal fishes otters usually consume in the wild (Bowyer et al., 1994; in review). This conclusion is supported by the high values of cholesterol, and high and low density lipids observed in the captive otters compared to the ones caught in PWS (Table 2).

Responses to seasonal factors

Hp, ALB, and GLOB did not exhibit a pattern related to oiling, indicating that additional processes may have been operating in our animals. Values of ALB were lower than those reported for 24 river otters live-captured in nonoiled areas in Prince William Sound (Table 2; Bowyer et al., in review) as well as for other captive otters (Table 2; Reed-Smith, 1995), except for the high-dose group. Similarly, levels of GLOB for the low-dose group were higher than those reported for 24 river otters live-captured in nonoiled areas in Prince William Sound (Table 2; Bowyer et al., in review) as well as for other captive otters (Table 2; Reed-Smith, 1995) at least during the oiling period. We recorded lower values of Hp and other globulins from September to December followed by an increase in January and February. During this later period, we noted changes in testicular size as well as increased aggression among our otters that suggest the onset of the breeding season. Investigating the relations between levels of Hp, ALB, and GLOB and those of testosterone in male river otters will enhance our understanding of the potential effects of season on these parameters.

Responses to ingestion of oil

Our expectation that Hp levels would be elevated in the oiled animals was based on observations in the field immediately following EVOS (Duffy et al. 1993; 1994*a*; 1994*b*). Nonetheless, previous studies demonstrated that serum concentrations of Hp are significantly reduced during hemolytic episodes because of increased removal rate of the Hp-Hb complex (Laurell and Gronwall, 1962). Indeed, Hp and Hb were negatively

correlated in our experiment and levels of Hb had a significant influence on levels of Hp, supporting this interpretation. We hypothesize that two opposing processes were occurring in our oiled otters: the first, elevated production in Hp in response to tissue damage by hydrocarbons, and the second increased removal of Hp-Hb complex from the serum (Table 3). That the Hp and Hb were only slightly correlated, suggests that additional factors were interacting in these animals. Hp is known to block the exchange of heme between methemoglobin and albumin (Koj, 1974). Albumin and hemoglobin were significantly positively correlated in our animals and both were negatively correlated with Hp. The complex interactions between those parameters may mask any clear pattern originating from exposure to hydrocarbons in live animals, which may explain the inconclusive results obtained in a study of pigeon guillemots (Prichard et al., 1997). Figure 5 depicts the potential relations between WBC, IL-6, Hp, Hb, ALB and GLOB (Marks, 1985; Heinrich et al., 1990). IL-6 secreted from WBC (mostly macrophages and monocytes; Heinrich et al., 1990) induces synthesis of Hp and other α- and β-globulins in the liver. The increased levels of Hp are associated with a decrease in ALB (Duncan et al., 1994). In instances where hemolytic processes are concurring, Hp binds with free Hb resulting in an overall decrease in levels of Hp (Laurell and Gronwall, 1962). The directions of the correlation between those variables in our experiment support the idea that the complex interactions between them are responsible for the observed pattern in Hp, ALB, and GLOB. Duffy et al. (1993) suspected that river otters in oiled areas in PWS in 1990 experienced anemia, although they were limited in their analysis to examination of blood smears. If this indeed occurred, the anemia experienced by those animals likely was not severe. If the removal of Hp – Hb complex from blood can result in a significant reduction of Hp levels as was observed by Laurell and

Gronwall (1962), and as indicated by our results, the high levels of Hp recorded by Duffy et al. (1993) will indicate low levels of anemia in otters exposed to hydrocarbons (Table 3). Indeed, values of 16.3 ± 0.6 g/dL of Hb were recorded in river otters captured in oiled areas in PWS in 1991 (n = 11; Duffy et al., 1994*a*), whereas levels of Hp in these same individuals were 156.9 ± 27.9 (mg Hb binding/dL), and IL-6ir 48.3 ± 13.8 (pg/ml). Values for both these variables are higher than those observed in our study.

IL-6ir did not exhibit the expected pattern and was not correlated with either globulins or Hp. The pattern we observed in IL-6ir suggests pulsating secretion as is common in several hormones such as testosterone (Wong et al., 1983). Therefore, our sampling interval may have been insufficient to detect such secretions. Therefore, the individual results should be interpreted with caution. Nonetheless, overall mean values for the control group were comparable to those of 24 river otters live-captured in nonoiled areas in Prince William Sound (Table 2; Bowyer et al., in review). In comparison, those overall mean values were double for the high-dose animals (1.47 ± 0.5) and triple for the low-dose animals (2.2 ± 0.5). That the overall values were higher in the oiled animals compared with the controls indicates that oil ingestion may have influenced the secretion of this factor. Similarly, the high variability in WBC complicates the interpretation of our results, although the pattern of reduction in WBC was more evident in the oiled animals. Production and elimination of WBC is an intricate process and is affected by conditions in the bone marrow, exposure to antigens, nutrition, and immuno-suppression (Duncan et al., 1994). Nonetheless, the values of WBC we recorded in our experimental otters were lower than those reported for 24 river otters live-captured in nonoiled areas in Prince William Sound (Table 2; Bowyer et al., in review) as well as for other captive otters (Table 2; Reed-Smith, 1995). That WBC and IL-6ir were slightly

negatively correlated supports our interpretation that oil ingestion may have influenced the secretion of IL-6.

The reduction in WBC, and the reduction in RBC and Hb we documented in the river otters agree with findings of other studies (Fry and Lowenstine, 1985; Leighton et al., 1983; Øristland et al, 1981; Rebar et al., 1994; Williams et al., 1995). These observations point to potential damage to bone marrow tissues. Examinations of smears made from bone marrow of oiled and nonoiled animals should provide insights to the effects of hydrocarbons on production of blood cells by that tissue. The reduction in WBC potentially points to suppression of the immune system and may have profound effects for free-ranging animals challenged with other antigens.

That the reduction in Hb and RBC did not differ between the low and the high dose groups indicates that even low doses of hydrocarbons can cause significant responses in these variables, and that the response probably is threshold activated. Alternatively, ingestion of higher doses of oil may reduce the levels of hydrocarbons assimilated in the animal because the oil acts as a lubricant in the gut. Our investigation of the effect of oiling dose on passage rate and assimilation efficiency in these captive otters suggested that consumption of oil increased passage rate of food in the gut and reduced assimilation of both food and oil (Ormseth and Ben-David, 2000). This outcome may explain results for other biomarkers in our study such as those of IL-6ir (high dose animals had values between those of low dose and control animals).

Values of Hb in our experiment were lower than those reported for 24 river otters live-captured in nonoiled areas in Prince William Sound (Table 2; Bowyer et al., in review) as well as for other captive otters (Table 2: Reed-Smith, 1995) suggesting clinical anemia. The occurrence of nucleated erythrocytes in blood smears is indicative of

regenerative anemia and abnormal release from the bone marrow (Duncan et al., 1994), and supports our interpretation of damage from hydrocarbons to bone marrow tissues. That Hb and RBC significantly declined in response to oiling could have major implications to maintenance of body condition, survival, and reproduction of coastal river otters as well as other diving mammals and birds. Ben-David et al. (2000) documented an increase in energetic costs of terrestrial locomotion, decrease in aerobic dive limit, and a potential increase in foraging time in a companion study on these same captive river otters.

The values of ALT for the high-dose animals were elevated compared with levels reported for captive otters (Table 2; Reed-Smith, 1995), but significantly lower than the levels reported for 24 free-ranging river otters captured in Prince William Sound (Table 2; Bowyer et al., in review). In many cases, increases in both enzymes are indicative of liver or muscle necrosis, as was evident in the otter with the gangrenous toe. Nonetheless, in acute liver disease a 50% decrease or more in circulating ALT levels are common (Duncan et al., 1994). That we observed no significant differences in values of ALT between groups during the oiling period indicates that our animals did not experience an acute liver damage, although some necrosis may have occurred as can be inferred from the increased values of ALK PHOS. The values of ALK PHOS for the oiled otters were higher than those reported for 24 river otters live-captured in nonoiled areas in Prince William Sound (Table 2; Bowyer et al., in review) as well as from those of other captive otters (Table 2; Reed-Smith, 1995). Nonetheless, ALK PHOS can increase in response to several physiological processes such as bone growth, intestinal lesions and hepatic function and can not be considered a specific marker (Duncan et al., 1994). Because AST occurs in erythrocytes, hemolysis may increase serum concentrations of this variable

(Duncan et al., 1994). Values of AST in our experimental animals were positively correlated with levels of hemoglobin (Hb).

Not all individuals responded to oiling in the same fashion. The reduction in Hb was doubled in several individuals compared with their counterparts. This result may point to genetic variability in the ability of animals to acclimate and compensate for chronic exposure to toxins, and may have far reaching implications from an evolutionary perspective. Zentano-Savin et al. (1997) suggested that the observed differences in Hp levels in Alaskan pinnipeds could be associated with different genetic stocks. In this study we investigated responses to oil ingestion in young adult male river otters. Therefore, inferences from our results to other age and gender categories should be made with caution. Bowyer et al. (in review) explored differences between gender and age classes in wild river otters captured in oiled and nonoiled sites in PWS from 1996-1998. Only diet related parameters (i.e., cholesterol and lipid values) differed between the genders in their analysis, suggesting that age and gender classes would have little effect on individual responses to ingestion of hydrocarbons.

Conclusion

Two main conclusions can be drawn from our study. First, the use of individual biomarkers as indicators of exposure to pollutants can lead to erroneous conclusions because interactions in vivo can be complicated and act in opposite directions (Fig. 5, Table 3). Additionally, biomarkers used in investigating effects of oiling on live animals are usually related to the heme cycle (Fig. 5). Because of the opposing processes that may occur within an animal, data from the suite of heme-related biomarkers may produce results that are difficult to interpret. Therefore, we advocate the exploration and

development of other biomarkers that will be independent from the heme cycle and will provide additional verification to the effect of oiling on live animals.

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Table 1 – Concentrations of hydrocarbons in the two batches of weathered Prudhoe Bay Crude oil fed to river otters at the Alaska Sealife Center in Seward Alaska from 21 August to 28 November 1998. The first batch of oil was fed to the otters between August 21 and October 19, as well as between November 19 and November 28. The second batch was fed to the otters between October 20 and November 18.

	Analyte concentrations: (μg/g)	
Compound	Batch 1	Batch 2
naphthalene	283.14	60.16
2-methylnaphthalene	904.43	616.81
1-methylnaphthalene	753.80	531.44
2,6-dimethylnaphthalene	641.37	577.26
C-2 naphthalenes	2601.33	2359.91
2,3,5-trimethylnaphthalene	268.37	278.51
C-3 naphthalenes	2284.48	2305.84
C-4 naphthalenes	609.96	593.84
biphenyl	155.55	120.85
acenaphthylene	0.00	0.00
acenaphthene	13.42	12.43
fluorene	97.31	100.48
C-1 fluorenes	228.37	247.95
C-2 fluorenes	273.45	309.82
C-3 fluorenes	169.23	183.75
dibenzothiophene	177.90	188.40
C-1 dibenzothiophenes	359.69	378.64
C-2 dibenzothiophenes	473.14	511.84
C-3 dibenzothiophenes	371.25	387.41
phenanthrene	244.29	263.38
1-methylphenanthrene	182.57	196.85
C-1 phenanthrenes/anthracenes	759.29	820.86
C-2 phenanthrenes/anthracenes	874.31	940.14
C-3 phenanthrenes/anthracenes	485.21	540.15

C-4 phenanthrenes/anthracenes	80.23	85.26
anthracene	7.72	3.41
fluoranthene	4.34	4.46
pyrene	11.70	12.34
C-1 fluoranthenes/pyrenes	66.05	644.75
benz-a-anthracene	6.64	4.28
chrysene	44.48	45.27
C-1 chrysenes	66.13	67.56
C-2 chrysenes	66.78	71.22
C-3 chrysenes	15.75	33.08
C-4 chrysenes	2.76	3.25
benzo-b-fluoranthene	14.73	15.66
benzo-k-fluoranthene	0.00	0.00
benzo-e-pyrene	9.89	11.76
benzo-a-pyrene	2.90	2.21
perylene	1.03	11.09
indeno-123-cd-pyrene	1.03	0.58
dibenzo-a,h-anthracene	1.31	1.07
benzo-g,h,i-perylene	2.50	2.97

Table 2 – List of blood variables, abbreviations, units of measurement, and values for 15 young adult male river otters live captured in PWS and held in captivity at the Alaska Sealife Center in Seward, Alaska, USA, from May 1998 to March 1999. For comparison, published values for these variables in river otters live-trapped in nonoiled areas in PWS 1998 are presented (adapted from Bowyer et al., in review), as well as values for zoo animals (adapted from Reed-Smith, 1995).

Variable Name	Abbreviation	Values for river otters in this study			Values for river otters from nonoiled areas in PWS			Values for zoo otters (from Literature)		
		n	$\bar{x}$	SE	n	$\bar{x}$	SE	n	$\bar{x}$	SE
Alanine Aminotransferase (U/L)	ALT	15	*		24	418.92	74.21	31	91.1	6.3
Albumin (g/dL)	ALB	15	*		24	3.09	0.05	37	3.0	0.05
Alkaline Phosphatase (U/L)	ALK PHOS	15	*		24	139.79	9.21	40	95.7	6.15
Aspartate Aminotransferase (U/L)	AST	15	*		24	176.88	13.68	39	89.9	6.5
Blood Urea Nitrogen (mg/dL)	BUN	15	*		24	43.50	3.26	40	27.6	1.2
Calcium (mg/dL)	Ca	15	8.86	0.02	24	8.86	0.08	40	9.1	0.1

Chloride (mEq/L)	Cl	15	110.13	5.03	24	112.25	0.76	36	113	4.0
Cholesterol (mg/dL)	CHOL	15	276.33	0.2	24	166.04	10.91	35	254	12.0
Cholesterol/High Density Lipid Ratio	CHOL/HDL	15	2.43	0.04	24	1.83	0.07			
Direct Bilirubin (mg/dL)	Dir Bili	15	0.03	0.05	24	0.08	0.01			
Gamma Glutamyl Transpeptidase (U/L)	GGT	15	17.33	0.74	24	27.79	5.55			
Globulin (g/dL)	GLOB	15	*		24	3.83	0.10	23	3.2**	0.1
Glucose (mg/dL)	GLU	15	*		24	136.79	10.14	40	88.2	3.5
Haptoglobin mg hb-bound/100ml	Hp	15			24	22.38	8.28			
Hemoglobin (g/dL)	Hb	15	*		21	15.72	0.24	51	14.2	0.3
High Density Lipids mg/dL	HDL	15	120.18	6.33	24	89.13	3.78			
Interleukin-6 immunoreactive (pg/ml)	IL-6ir	15	*		24	0.75	0.31			
Lactate Dehydrogenase (U/L)	LDH	15	*		24	221.33	27.30	34	324	76.9
Low Density Lipids (mg/dL)	LDL	15	146.85	4.04	24	65.92	6.74			

Phosphorous (mg/dL)	P	15	5.21	0.06	24	4.62	0.27	41	5.9	0.3
Platelet Count (Th/cmm)	PLAT	15	*		21	357.24	11.06			
Potassium (mEq/L)	K	15	3.97	0.02	24	3.95	0.07	39	4.3	0.06
Red Blood Cell Count m/cmm (million)	RBC	15	*		21	9.14	0.15	26	10.2	0.5
Serum Creatinine (mg/dL	SCREAT	15	0.3	0.01	24	0.26	0.01	39	0.6	0.02
Sodium (mEq/L)	Na	15	148.93	0.15	24	149.50	0.63	39	150	0.7
Total Bilirubin (mg/dL)	T. Bili	15	0.22	0.02	24	0.33	0.01	40	0.2	0.02
Total Protein (g/dL)	TP	15	6.75	0.02	24	6.92	0.09	40	6.5	0.1
Triglycerides (mg/dL)	TRIG	15	*		24	55.29	10.50			
Uric Acid (mg/dL)	UA	15	1.55	0.06	24	1.83	0.20	33	2.1	0.4
Very Low Density Lipids (mg/dL)	VLDL	15	15.53	0.75	24	11.00	2.00			
White Blood Cells Th/cmm (thousand)	WBC	15	*		21	10.33	1.11	55	8.8	0.4

*differences due to oil ingestion or conditions of captivity (see Figures 1 – 4)
**Gamma globulins only

Table 3 – Theoretical predictions for the direction of response (plus = high, minus = low) in several biomarkers under differing levels of exposure to hydrocarbons. Pathways are described in Fig. 5. Levels of P450 are expected to be low in high-exposure with anemia because of competition for the heme molecule. Copro III is expected to be low because of increase in demand for heme.

Level of exposure	IL-6	Hp	Hb	ALB	P450	Copro III
None (without anemia)	-	-	+	+	-	-
Low (without anemia)	+	+	+	-	+	+
High (with anemia)	+	-	-	-	-	-

Figure legends.

Figure 1 – Values (mean ± SE) of AST (a), ALT (b), LDH (c), glucose (d) BUN (e) and triglycerides (f), for control, low-dose and high-dose groups (n = 15; 5 in each group) of river otters held in captivity at the Alaska Sealife Center in Seward, Alaska, USA. The period of oil administration occurred between 21 August and 28 November 1998 as indicated by arrow. For description of differences see text. This group of variables exhibited a response to captivity.

Figure 2 - Values (mean ± SE) of haptoglobin (Hp, a), albumin (b), and globulins (c), for control, low-dose, and high-dose groups (n = 15; 5 in each group) of river otters held in captivity at the Alaska Sealife Center in Seward, Alaska, USA. The period of oil administration occurred between 21 August and 28 November 1998 as indicated by arrow. For description of differences see text. This group of variables exhibited a response to seasonal factors.

Figure 3 - Values (mean ± SE) of IL-6ir (a), and platelet counts (b) for control, low-dose and high-dose groups (n = 15; 5 in each group) of river otters held in captivity at the Alaska Sealife Center in Seward, Alaska, USA. The period of oil administration occurred between 21 August and 28 November 1998 as indicated by arrow. For description of differences see text. Both variables were identified as important in explaining the high variability in the data by PCA analysis. While IL-6ir exhibited a potential response to oil

ingestion, no relation to oil ingestion, season or captivity could be determined for platelet counts.

Figure 4 - Values (mean ± SE) of hemoglobin (Hb, a), white blood cells (WBC, b), and alkalaine phosphatase (ALK PHOS, c) for control, low-dose and high-dose groups (n = 15; 5 in each group) of river otters held in captivity at the Alaska Sealife Center in Seward, Alaska, USA. The period of oil administration occurred between 21 August and 28 November 1998 as indicated by arrow. For description of differences see text. This group of variables exhibited a response to oil ingestion.

Figure 5 – Schematic description of the potential interactions between different biomarkers related to the heme cycle (based on information from Heinrich et al., 1990; Koj, 1974; Laurell and Gronwall, 1962; Marks, 1985). Effects of PAH on heme synthesis depicted here in the liver are assumed to be similar in other tissues.

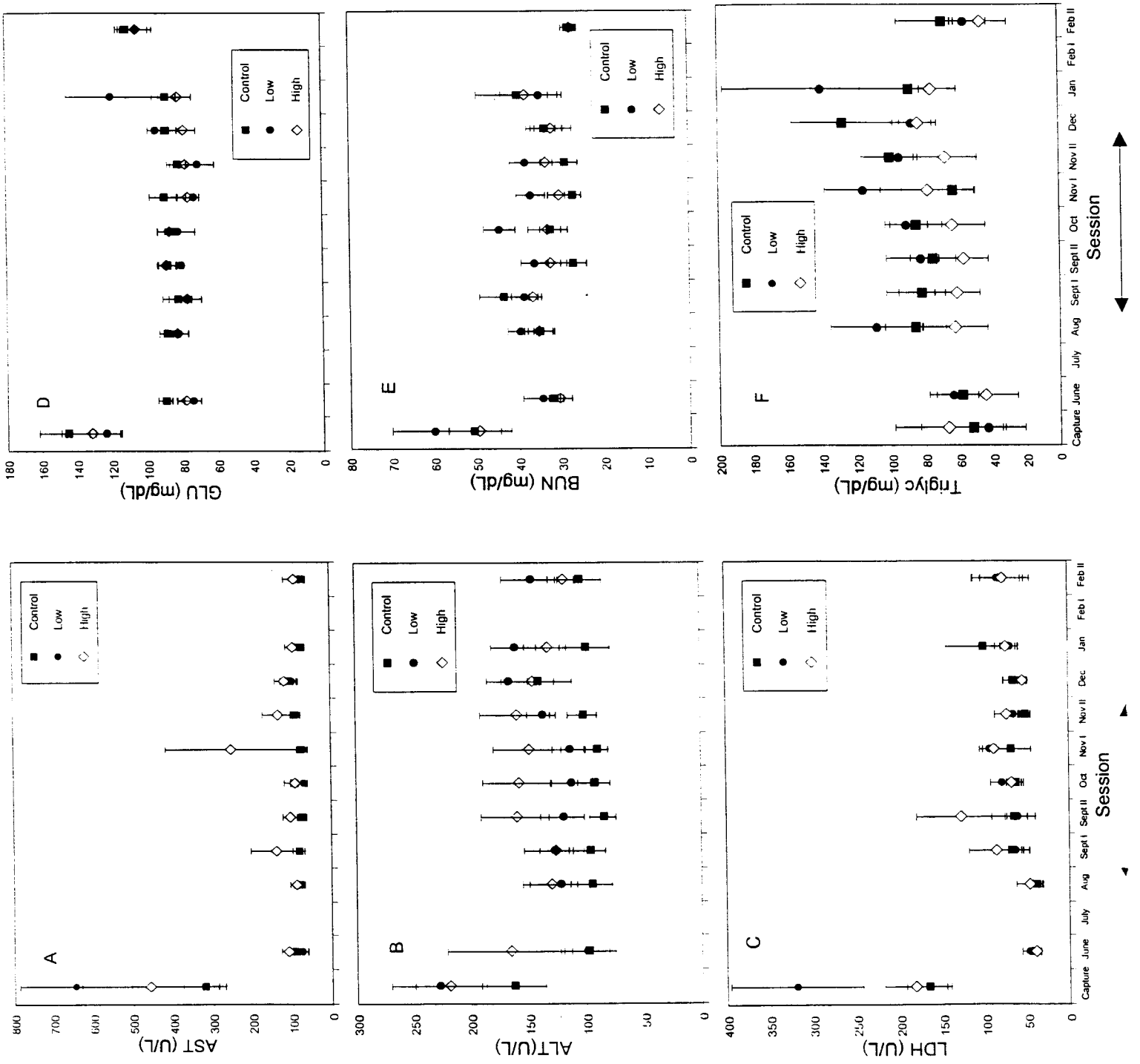
A
AST (U/L)
B
ALT(U/L)
C
LDH (U/L)
D
GLU (mg/dL)
E
BUN (mg/dL)
F
Triglyc (mg/dL)
Control
Low
High
Capture
June
July
Aug
Sept I
Sept II
Oct
Nov I
Nov II
Dec
Jan
Feb I
Feb II
Session

Figure 1.

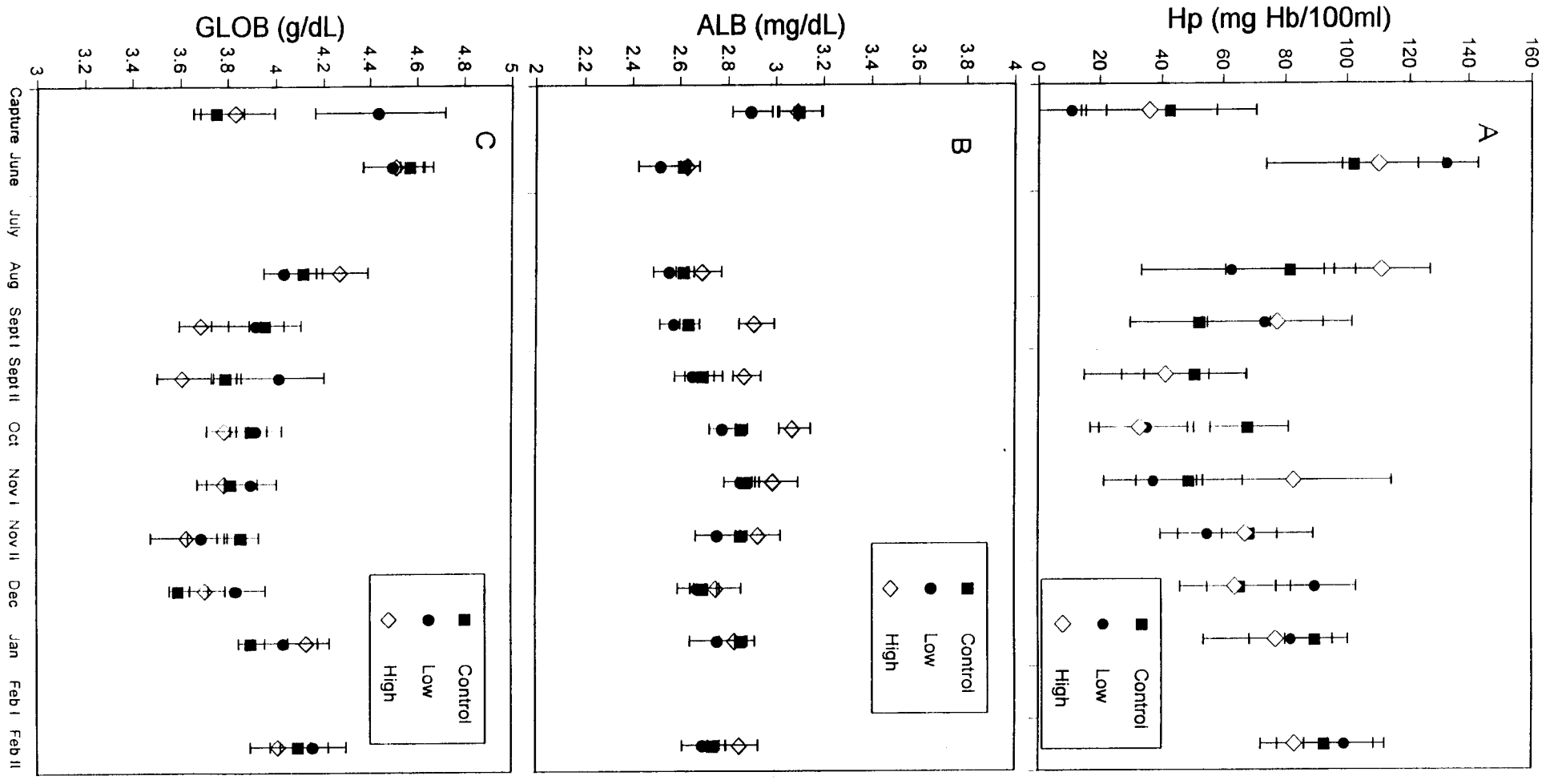
Hp (mg Hb/100ml)
A
Control
Low
High
ALB (mg/dL)
B
Control
Low
High
GLOB (g/dL)
C
Control
Low
High
Capture June
July
Aug
Sept I
Sept II
Oct
Nov I
Nov II
Dec
Jan
Feb I
Feb II
Session

Figure 2.

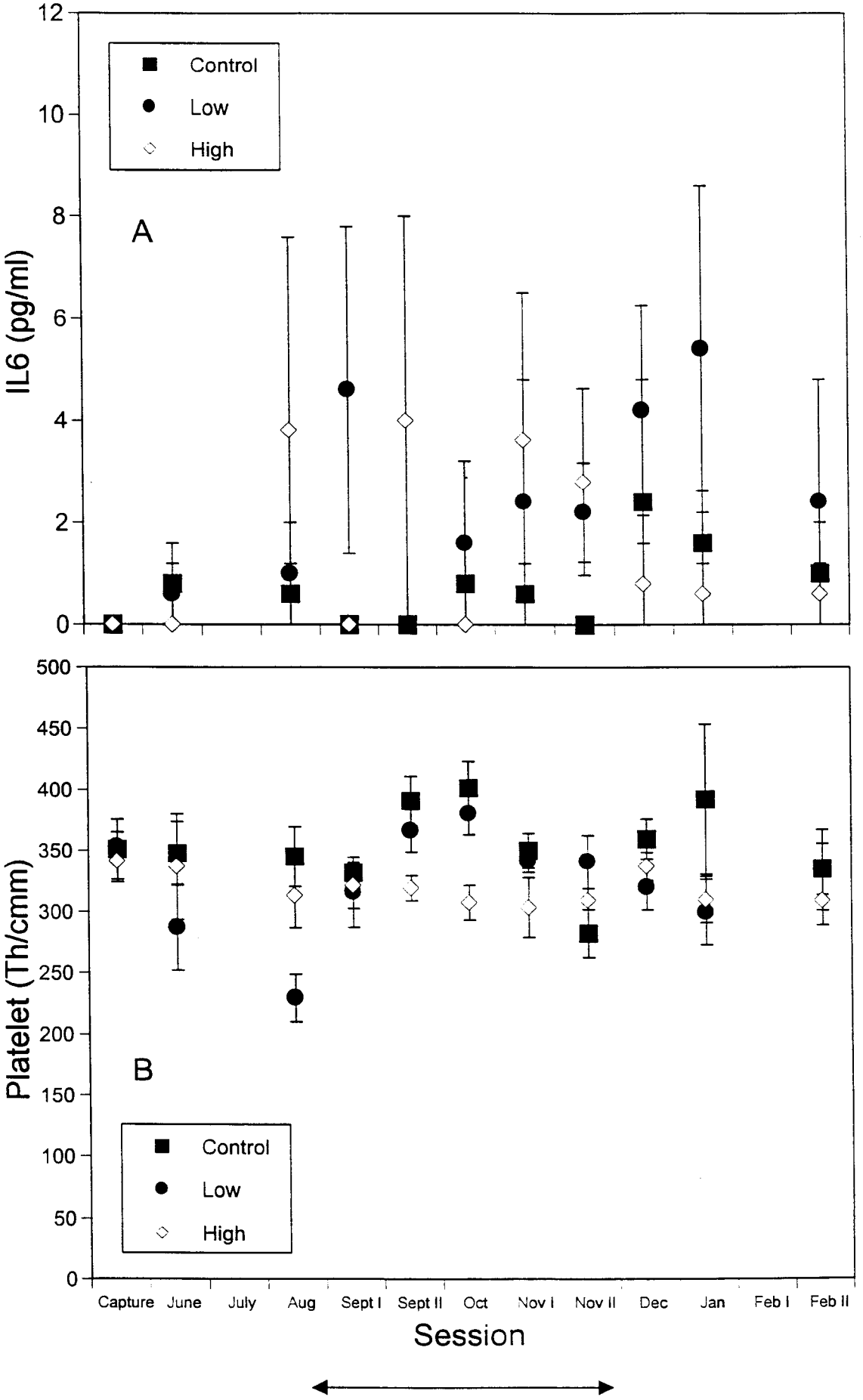
12
10
8
6
4
2
0
IL6 (pg/ml)
Control
Low
High
A
500
450
400
350
300
250
200
150
100
50
0
Platelet (Th/cmm)
B
Control
Low
High
Capture
June
July
Aug
Sept I
Sept II
Oct
Nov I
Nov II
Dec
Jan
Feb I
Feb II
Session

Figure 3.

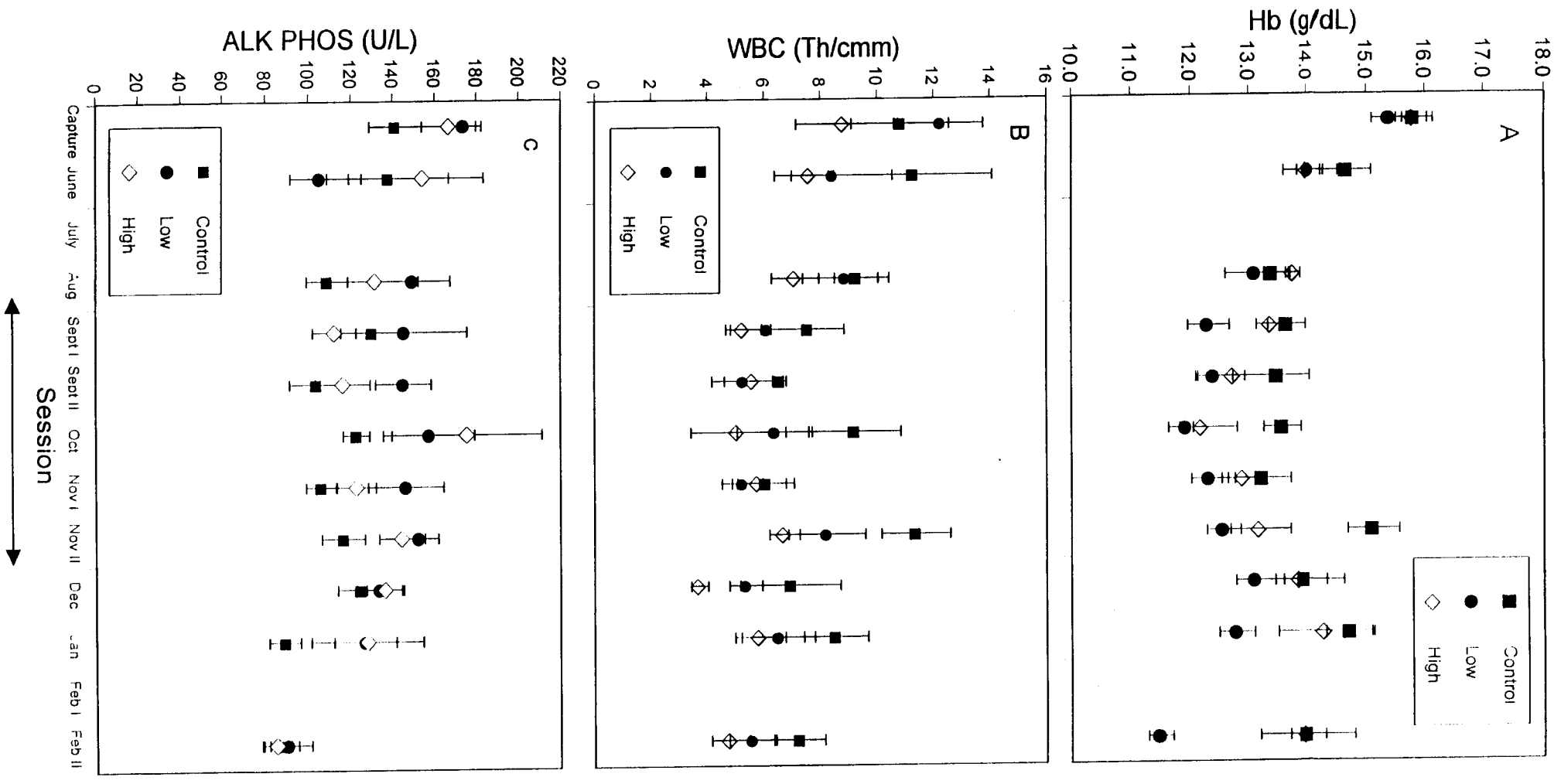
A
Hb (g/dL)
10.0
11.0
12.0
13.0
14.0
15.0
16.0
17.0
18.0
Control
Low
High
B
WBC (Th/cmm)
0
2
4
6
8
10
12
14
16
Control
Low
High
C
ALK PHOS (U/L)
0
20
40
60
80
100
120
140
160
180
200
220
Control
Low
High
Capture June
July
Aug
Sept I
Sept II
Oct
Nov I
Nov II
Dec
Jan
Feb I
Feb II
Session

Figure 4.

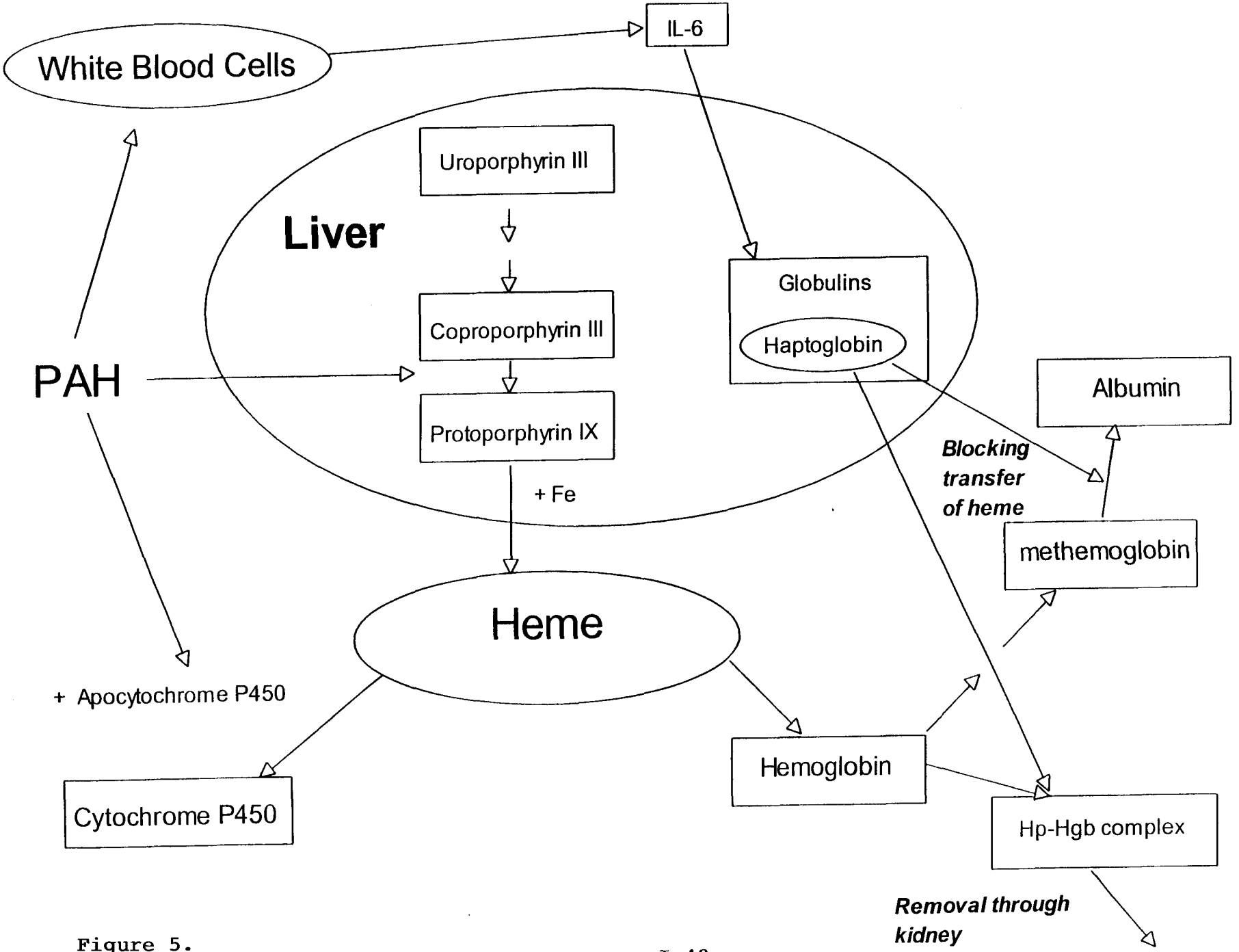
White Blood Cells
IL-6
Uroporphyrin III
Liver
Coproporphyrin III
Protoporphyrin IX
+ Fe
Globulins
Haptoglobin
PAH
Albumin
Blocking transfer of heme
methemoglobin
Heme
+ Apocytochrome P450
Cytochrome P450
Hemoglobin
Hp-Hgb complex
Removal through kidney

Figure 5.

Induction of cytochrome P450 1A1 expression in captive river otters fed Prudhoe Bay crude oil: evaluation by immunohistochemistry and quantitative RT-PCR

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Running Head: Induction of cytochrome P450 1A1 in captive river otters

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Numerous studies have explored relations between exposure to a variety of environmental contaminants, such as polycyclic aromatic hydrocarbons, and induction of cytochrome P450 1A (CYP1A) in different vertebrates. Few controlled studies, however, simulated chronic long-term exposure with repeated non-lethal sampling of the same individuals, which should better represent repeated exposure incidents in animals inhabiting polluted areas. In this study, we investigated the effects of chronic exposure to crude oil on levels of CYP1A1 in endothelial cells of skin biopsies and peripheral mononuclear blood cells in captive river otters (*Lontra canadensis*) using repeated sampling of the same individuals. We hypothesized that ingestion of oil would result in an increase in levels of CYP1A1 in both targets, and predicted that the relation between prolonged exposure and expression of CYP1A1 would reach a plateau indicative of continuous detoxification of hydrocarbons. Fifteen wild-caught male otters were acclimated to captivity, and then fed diets containing no oil (control) or diets containing weathered crude oil at 5mg/day/kg body weight (low-dose) and 50mg/day/kg body weight (high-dose), at the Alaska Sealife Center in Seward, Alaska, USA. Expression of CYP1A1 was assessed with immunohistochemical analysis of CYP1A1 protein in skin biopsies and by quantitative RT-PCR analysis of CYP1A1 mRNA in mononuclear blood cells. Both assays revealed a decrease between capture and the transfer to captivity, indicating that the enclosure at the Alaska Sealife Center, and the food we offered to the otters were free of potential inducers of CYP1A1. During the exposure period, increases in CYP1A1 expression were registered by both techniques, followed by a decline in CYP1A1 after oil administration ended. Levels of endothelial CYP1A1 in the high-dose group were comparable to those recorded for wild river otters in PWS in 1996 and 1997.

Levels of CPY1A1 mRNA in mononuclear blood cells, however, were well below levels recorded for river otters in Prince William Sound, and no correlation was detected between values obtained from the two methods. Thus, our results from this longitudinal study with repeated sampling of the same individuals provide support for the use of cytochrome P450 1A1 as a biomarker for hydrocarbon exposure. Nonetheless, our results also suggest that the induction process of CYP1A1 may be complicated and interacting with other processes in vivo. Such interactions may obscure our ability to describe specific, quantitative, predictable, dose-response relations between exposure to hydrocarbons and induction of CYP1A1, which are required of reliable biomarkers. Evaluations of such interactions based on theoretical physiological models in live-animals merit further investigation.

Keywords: *Alaska, biomarkers, chronic exposure, CYP1A, dose response, hydrocarbons,* Lontra canadensis, *mononuclear blood cells, mono-oxygenases, skin samples.*

Introduction

Many studies assessing the risk of pollutant exposure in wildlife populations involve the measurement of chemical residues in sediments, water, or soil. Because chemical residue analysis can be expensive, laborious, and often inadequately measure bioavailability, biomarker analyses have become widely used in ecotoxicological studies (Vanden Heuvel and Davis 1999). Members of the Cytochrome P450 (CYP) family of hemoproteins that catalyze oxidative transformation of organic chemicals are important in the detoxification of environmental contaminants (Marks 1985, Stegeman and Lech 1989, Buchelli and Fent 1995). Evaluations of the mRNA, protein or catalytic activity of CYP1A have been used as a biomarker for exposure to a variety of contaminants such as polycyclic aromatic hydrocarbons (PAH), naphthoflavones, dioxins, planar polyhalogenated biphenyls (i.e., 3-methylcholanthere (MC) type agents), which induce CYP1A via the Aryl hydrocarbon receptor (AhR; Miranda *et al.* 1987, Stegeman *et al.* 1988, Kloepper-Sams and Stegeman 1989, Juchau 1990, Goksyør and Husoy 1992, Rattner *et al.* 1993, Stegeman *et al.* 1992, Willet *et al.* 1997, Woodin *et al.* 1997). CYP1A1 is broadly distributed in different species and tissues, and is not readily induced by steroids and other lipids (Juchau 1990, Vanden Heuvel and Davis 1999). These characteristics make CYP1A1 a good indicator of exposure to MC-type agents in vertebrates (Vanden Heuvel and Davis 1999).

Petroleum, including that spilled by the *Exxon Valdez* oil spill (*EVOS*), contains numerous PAH compounds (Short *et al.* 1996), that potentially induce the expression of CYP1A1 in vertebrates. Early investigations in Prince William Sound (PWS), following *EVOS*, revealed that coastal river otters (*Lontra canadensis*) on oiled shores had lower

body mass and elevated levels of liver enzymes (ALT, AST), haptoglobin, interleukin-6, and fecal porphyrins than animals living in nonoiled areas (Duffy *et al.* 1993, 1994*a*, 1994*b*, 1996, Blajeski *et al.* 1996, Bowyer *et al. in review*). In addition, otters from oiled areas selected different habitats, had larger home ranges, and less diverse diets than those in nonoiled areas (Bowyer *et al.* 1994, 1995). These differences between river otters from oiled shores and those from nonoiled areas suggest that oil contamination had an effect on physiological and behavioral processes in otters. Continued exposure could have the potential to impede recovery of populations of river otter inhabiting areas affected by *EVOS*. Later studies of river otters in PWS between 1996 –1998 employed two different techniques for evaluating expression of CYP1A1 in an attempt to determine the occurrence and level of exposure to hydrocarbons in these animals. The first technique involved evaluation of immunohistochemical (IHC) staining of CYP1A1 hemoprotein in endothelial cells from skin tissue. The second employed quantitative reverse transcriptase - polymerase chain reaction (RT-PCR) to measure CYP1A1 mRNA content in peripheral blood lymphocytes and monocytes (i.e., mononuclear blood cells; Ballachy *et al. in review*). Endothelial CYP1A1 content was significantly higher in river otters captured in oiled compared with nonoiled areas in 1996, but values of CYP1A1 levels in samples collected in 1997 and 1998, were nearly identical for otters living in oiled versus nonoiled areas (Bowyer *et al. in review*). Similarly, expression of CYP1A1 mRNA in lymphocytes, measured by quantitative RT-PCR, revealed no differences in river otters from oiled and nonoiled areas in 1998 (Ballachy *et al. in review*). To adequately interpret these findings it became necessary to establish the relation between exposure to

hydrocarbons, dose levels, and expression of CYP1A1 in these mustelids under controlled conditions.

Although numerous studies explored the relation between hydrocarbon exposure and expression of CYP1A1 in different vertebrates (Payne *et al.* 1987, Stegeman *et al.* 1988, Juchau 1990, Buchelli and Fent 1995), few controlled studies simulated chronic long-term exposure with repeated non-lethal sampling of the same individuals. Such controlled chronic-exposure studies would likely provide better representation of conditions experienced by wild animals inhabiting polluted areas and a better frame for interpreting data collected from free-ranging exposed-animals. In this study, we investigated the effects of chronic exposure to crude oil on levels of CYP1A1 in endothelial cells of skin biopsies and lymphocytes in captive river otters using repeated sampling of the same individuals. We hypothesized that ingestion of oil would result in an increase in levels of CYP1A1 in both targets, as both mononuclear blood cells and endothelial cells would be in immediate contact with the assimilated hydrocarbons transported in the blood. We predicted that the relation between prolonged exposure (i.e., cumulative dose) and expression of CYP1A1 would reach a plateau suggesting continuous detoxification of the ingested hydrocarbons. In addition, we expected a correlated response in the two target cells indicative of a systemic response to exposure.

MATERIALS AND METHODS

General

Fifteen wild male river otters were captured in northwestern PWS using No. 11 Sleepy Creek® leg-hold traps (Blundell *et al.* 1999). Traps were placed on trails at latrine

sites and monitored by trap transmitters (Telonics, Mesa, Arizona, USA) that signal when a trap is sprung (Bowyer *et al. in review*). Processing of otters began within 1 - 2 hours from capture and lasted between 0.5 (regular processing) to 5 hours (processing involved implanting the individual with radio transmitter). Otters were anesthetized with Telazol (9mg/kg; A. H. Robins, Richmond, Virginia, USA) administered using Telinject® darts and a blowgun. Blood and tissues were sampled from each individual otter within 15 minutes of sedation.

The river otters were then transferred under sedation via air to the Alaska Sealife Center in Seward, Alaska (ASLC). The otters were held in captivity at ASLC from May 1998 to March 1999. The animals were housed as one large group in an area of 90 m^2 surrounding 6 pools (1 large salt-water pool - 4.5 m diameter by 3 m depth; 4 small salt-water pools - 2 by 1.5 by 1.5 m; and 1 small fresh-water tote - 1 by 1 by 1m). Otters were fed twice daily *ad libitum* on a diet of frozen fish of the following species: pink salmon (*Oncorhynchus gorbuscha*), capelin (*Mallotus villosus*), Pacific herring (*Clupea pallasii*), walleye pollock (*Theragra chalcogramma*), prawns (*Pandalus platyceros*), and eulachon (*Thaleichthys pacificus*). These fishes were purchased from commercial harvest and approved for human consumption. Two to three times per week, the diet was supplemented with live prey (pink salmon, kelp greenling (*Hexagrammos decagrammus*), and rockfish (*Sebastes* sp.)) captured in Resurrection Bay, Alaska (ADFG permit CF 98-024). Initially, vitamins (HiVite™, EVSCO Pharmaceuticals, IGI, Inc., Buena, NJ) were provided with food, but because otters seemed reluctant to consume those fishes, we injected Butler B-complex vitamins (0.5 ml; Phoenix Scientific, St. Louis, MO)

intramuscularly during the blood-sampling sessions every 3 weeks. Minerals were provided as a standard cattle mineral block to which otters had continuous access.

Experiments began in August after 2.5 months of acclimation to the enclosure, feeding regimes, and handling. During that time, the average daily food intake of the otters was monitored to quantify the amount of oil required for achieving each oiling level. Daily food intake per animal averaged at 1000 g/day over the acclimation period. Average body weight of the otters at the end of the acclimation period was 10.7 kg (± 0.3, SE). At the end of that period, otters were randomly assigned to 3 experimental groups of 5 individuals each. Group assignment was accomplished with a randomized complete block design. Original assignment of otter identification numbers were done based on body mass at capture to control for potential differences in age and size. For the complete block design otter identification numbers were randomly permutated within blocks (T. L. McDonald, West, Inc. Cheyenne, WY). The three treatment groups were: a control group that received no oil; a low dose group that received 100mg of oil every other day (i.e., 5mg/day/ kg body weight); and a high dose group which received 1000mg every other day (i.e., 50mg/day/ kg body weight). The exposure level for the low-dose group was determined based on levels of Prudhoe Bay crude oil (PBCO) found in mussel beds in PWS in 1995 (Short *et al.* 1996), in an attempt to simulate conditions of chronic exposure in the wild. The high-dose level was selected to simulated conditions in PWS immediately following *EVOS*.

Prudhoe Bay crude oil (PBCO; obtained from Williams Inc. Fairbanks, Alaska, USA) was mixed in seawater and stirred continuously for 10 days at 25°C. Two batches of oil were weathered separately and a sample from each batch was sent for analysis at

Auke Bay Laboratory (J. Short, NOAA, Juneau, Alaska). The two batches differed slightly in composition, but both were comparable to the oil profile of *EVOS* shortly after landfall in 1989 (table 1; Short *et al.* 1996). Weathered oil was separated from water and administered to otters in gel capsules hidden in fishes every other day. The quantity of oil placed in the gel was measured with a micro-pipette (Rainin Instruments Co., Emeryville, CA) and weighed on a micro-balance (nearest 0.01g). Occasionally the otters bit into the capsule when feeding and subsequently dropped the fish. On such occasions, oil was administered again during the following feeding.to ensure that each otter ingested the amount of oil required for the experiment. Careful notes on oil feeding were kept throughout the experiment. Oil feeding lasted 100 days, from 21 August to 28 November 1998. The first batch of oil was fed to the otters between August 21 and October 19, as well as between November 19 and November 28. The second batch was fed to the otters between October 20 and November 18 (table 1). Data collection continued for additional 100 days of rehabilitation. Cumulative dose of oil provided in food throughout the period of exposure was determined for each individual otter and used in later analyses. Analysis of passage rate and assimilation efficiency indicated that some of the ingested oil was occasionally deposited in feces (Ormseth and Ben-David 2000). At the end of the rehabilitation period, animals were fitted with radio transmitters and released at the site of their capture in PWS. Animals are currently being monitored to determine post-release survival (Ben-David unpublished data).

[Insert table 1 around here]

Collection of blood and tissues

Prior to the exposure to oil (29-30 June and 15-16 August 1998), blood and tissues were sampled from each individual otter. Blood and tissue sampling continued every three weeks from 15 August 1998 until 12 January 1999. An additional sampling session occurred on 22-24 February 1999 in conjunction with implanting of radio transmitters.

Otters were anesthetized with a combination of ketamine hydrochloride (100 mg/ml, Ketaset®, Aveco Co., Fort Dodge, Ia., USA) at a dosage of 10 mg/kg, and midazolam hydrochloride (5 mg/ml, Versed®, Hoffman-LaRoche, Nutley, N.J., USA) at a dosage of 0.25 mg/kg (Spelman *et al.* 1993). The dosage was mixed in the same syringe and administered intramuscularly with Telinject® darts and a blowgun or hand injected while the otter was immobilized in a squeeze-box. Before beginning to dart the otter, each individual received one fish containing 0.5 ml Versed®. This calmed the animals and reduced the stress associated with handling.

A 3-mm disposable skin-biopsy punch was used to obtain a tissue sample from each river otter for analysis of endothelial cytochrome P450 1A1. This amount of tissue was a maximum approved by the ASLC veterinarian and the Independent Animal Care and Use Committee (IACUC) given the repeated nature of tissue sampling. Prior to collecting the sample, we clipped hair on the medial surface of the triceps on the left front limb, and a surgical scrub was performed. The tissue specimen was preserved in 10% neutral-buffered formalin immediately after collection, and sent to Woods Hole Oceanographic Institution for analysis. Samples were coded and analysis conducted in a blind study (i.e., analyst had no knowledge of group assignment of samples).

A total of 10 ml of heparinized (preservative free heparin, Sigma Chemical Co., St Louis, MO) blood was collected by vena puncture from each animal for the isolation of peripheral blood mononuclear cells. Additional 12 ml of blood was collected for other analyses (Ben-David *et al. in press*). Blood was centrifuged at 2000 rpm for 10 minutes and plasma was siphoned off and frozen. Cells were re-suspended with RPMI 1640 (Cellgro™, Mediatech; Fischer MT-10-041-LV) medium supplemented with 10% CPSR-3 (Sigma C-9030) to produce 10 ml of solution. Solution was combined with 10 ml of Hank's balanced salt solution (HBSS; Cellgro™, Mediatech; Fischer MT-21-021-LV; with 1% antibiotic/antimycotic solution added (Sigma A-5955) and carefully layered on 4 ml of histopaque (Sigma). Samples were centrifuged at 1300 rpm for 25 minutes after which HBSS was removed and leukocytes were re-suspended and washed with 10 ml HBSS. After centrifugation at 1500 rpm for 10 minutes, HBSS was removed and cells were re-suspended in 3 ml freezing medium (i.e., a solution of 1:1 RPMI and CPSR-3 and 0.3 ml of 10% DMSO). Samples were aliquoted into two 1.5 ml nalgene cryovials and placed in a cryo-freezing container (Nalgene) with 70% alcohol. Samples were frozen and stored in -70 °C, and shipped to Purdue University in liquid nitrogen.

Laboratory procedures for CYP1A assays

Immunohistochemistry

For estimation of CYP1A1 in skin biopsies, preserved biopsy samples were embedded in paraffin tissue and were sectioned and examined with procedures modified from Smolowitz *et al.* (1991). Prior to immunochemical staining, standard 5 μm sections were deparaffinated and hydrated in 1% bovine serum albumin/ phosphate buffered

saline (BSA/PBS). During the hydration process, sections were incubated in 0.5% H_2O_2 in methanol for 45 minutes to block endogenous peroxidase. Hydrated sections were immunochemically stained using an indirect peroxidase stain (Universal Immunoperoxidase Staining Kit (Murine), Signet Laboratories, Inc., Dedham, MA) with the monoclonal antibody MAb 1-12-3 (Park *et al.* 1986) and peroxidase labeled goat anti-mouse IgG secondary antibody. MAb 1-12-3 is highly specific for cytochrome P450 1A proteins in vertebrates (Drahushuk *et al.* 1998). The specificity of monoclonal antibody CYP1A1 in mammals has been determined by evaluating cross-reactivity with proteins in liver microsomes and with heterologously expressed CYP1A1 and CYP1A2 from mice, rabbits and humans. The antibody recognizes only CYP1A1 (e.g., Drahushuk *et al.* 1998). Moreover, the epitope recognized by MAb 1-12-3 has been identified and it is a sequence that is present only in mammalian CYP1A1, not in CYP1A2 nor in any other mammalian P450 (J. J. Stegeman, unpublished observations). Specific staining by MAb 1-12-3 was evaluated by light microscopic examination of the stained sections (figure 1). Cell types that stain and their associated occurrence and staining intensity were recorded for each tissue section examined. At least two immunochemically stained sections were examined from each sample. Staining of CYP1A1 was scored for intensity of staining (0 = no stain, 1 = mild stain, 2 = moderate stain, 3 = strong stain, 4 = very strong stain) and for occurrence of staining (0 = no cells, 1 = rare cells, 2 = some cells, 3 = many cells, 4 = all cells staining). A stain index was computed by multiplying the intensity and occurrence for scale from 0 – 16. In other studies, this stain index compared well with CYP1A content measured by quantitative immunoblot (Woodin *et al.* 1997).

[insert figure 1 around here]

Quantative RT-PCR

For analysis of CYP1A1 mRNA expression in peripheral blood mononuclear cells, frozen cells were thawed rapidly in a 37°C water bath and immediately placed on ice. Samples were then transferred to a 15ml centrifuge tube and diluted to 10 ml with HBSS, centrifuged and pelleted. Cell viability was determined by trypan blue exclusion. The RT-PCR assay was performed on total RNA isolated from peripheral blood mononuclear cells (PBMC). The messenger RNA (mRNA) that codes for the CYP1A1 protein was quantified, using specific cDNA primers and a quantitative reverse transcriptase polymerase chain reaction (Vanden Heuvel *et al.* 1993, 1994). Initially, the RT-PCR assays required the isolation, cloning and sequencing of the PCR product, and the development of river otter specific primers for CYP1A (Ballachy *et al. in review*). Since mustelid CYP1A1 had not been previously described, we designed primers based on a comparison of several known CYP 1A1 cDNA sequences: sheep, human, mouse, guinea pig, hamster and rat. We aligned these sequences using clustal MegAlign method and chose primers from highly conserved areas. The primers were then utilized to amplify mustelid CYP1A from liver RNA isolated from sea otters (*Enhydra lutris*) exposed to high concentrations of oil (Ballachy *et al. in review*). The PCR product was cloned, into T7Blue T-vector, and sequenced. Using Primer Select (DNA Star, Madison, WI) and Prettyplot methods we obtained more efficient primers for mustelid CYP 1A1 cDNA. Because these primers are very short it is difficult to run them in BLAST even for epitope identification. Nonetheless, we have examined the PCR product sequence in BLAST and the sequence homology is highest with other known CYP1A1 sequences.

The primers were optimized for Mg, annealing temperature, pH, and number of cycles. The optimized conditions were: 4mM Mg, 54° C annealing temperature, pH 8.8, and 30 cycles. The PCR products were cloned into T7Blue T-vector according to the manufacture's instructions (Novagen, Madison, WI). Following isolation of plasmid DNA, fluorescence dideoxynucleotide sequencing was performed at the Purdue University DNA Facility. The sequence information was used to obtain more efficient primers specific for river otter cDNA using PrimerSelect (DNAStar, Madison, WI).

River otter 1A forward primer, 5'-TGGTCAATTTTCTGTTCCTAG-3'
River otter 1A reverse primer, 5'-AGGTCAGCTCAACCTTGAGA-3'

The use of an internal standard that contains target (i.e. river otter CYP1A1) primer sequences negates tube-to-tube variability in PCR amplification and is essential to quantifying mRNA expression by RT-PCR. We generated recombinant RNA (rcRNA) internal standards as described by Vanden Heuvel *et al.* (1993). Using this method, a rcRNA was generated that upon amplification with river otter CYP1A1 primers results in a product that is easily resolved from target product following agarose gel electrophoresis.

Competitive PCR was performed as described by Gilliland *et al.* (1990*a*; 1990*b*) and modified by Vanden Heuvel *et al.* (1994). For each sample 8-10 aliquots of total RNA (0.1 mg) were prepared, and a dilution series of the rcRNA internal standard was spiked into these aliquots. Reverse transcription of RNA was performed in a final volume of 20 µl containing 25 mM Tris-HCl (pH 8.3 at 25°C), 50 mM $(NH_4)_2SO_4$, 1MM

DTT, 0.1 mg/ml bovine serum albumin, 5 mM $MgCl_2$, 1 mM of each deoxynucleotide triphosphate, 1 unit RNase inhibitor, 2.5 units M-MLV Reverse Transcriptase (Life Technologies, Inc.), 2.5 mM oligo$(dT)_{16}$, 0.1 µg total RNA, and varying amounts of rcRNA internal standard. The samples were incubated at 42°C for 15 min., and reverse transcriptase was inactivated by heating to 99°C for 5 min. PCR reaction mixture contained 3 mM $MgCl_2$, 2.5 units Taq polymerase, and 6 pmol of forward and reverse primers. The reactions were heated to 94°C for 3 min. and cycled 30 times through 30-s denaturing step at 94°C, a 30-s annealing step at 54°C, and a 30-s elongation step at 72°C. Following the final cycle, a 5-min. elongation step at 72°C was included.

Aliquots of the PCR reaction were electrophoresed on 2.5% NuSieve® 3:1 agarose (FMC Bio Products, Rockland, ME) gels, and PCR fragments were visualized with ethidium bromide staining (figure 2). A photographic negative was prepared and densitometry was performed using a LKB Gel Scan II laser densitometer (LKB, Piscataway, NJ). Quantification of the amount of target mRNA present was determined as described by Gilliland *et al.* (1990*a*). The actual number of molecules of CYP1A1 were determined by comparing the ratio of the volume of the internal standard to CYP1A1 mRNA PCR products were plotted against the amount of internal standard added to individual tubes as previously described by Gilliland *et al.* (1990*a*). Linear regression analysis was used to define the equation for the line through the data points. The amount of CYP1A1 mRNA present for individual animals was defined as the amount of rcRNA present where the volume ratio was equal to 1.

[Insert figure 2 around here]

Statistical Analyses

Of 150 samples collected through the experiment for the quantitative RT-PCR, 3 were contaminated during processing. Missing values for those samples were replaced by means of near points approach (Johnson and Wichern 1992). To determine the effects of oil ingestion on levels of CYP1A1 we used repeated measures ANOVA with oiling group (i.e., control, low dose, and high dose) and session as factors (Johnson and Wichern 1992; SPSS for Windows). Analysis was followed by Tukey's multiple comparisons to establish where significant differences occurred for those models in which either group or session effects were significant. We used non-linear regression curve estimation to describe the relations between staining index of endothelial CYP1A1 as the dependent variable and cumulative dose levels as the independent one to determine the effects of cumulative effects of chronic exposure on this parameter (Zar 1984). In addition, we used correlation analysis (Pearson's ρ; Zar 1984) to determine the relation between the endothelial and mononuclear blood cells CYP1A1.

Results

Expression of CYP1A1 in endothelial cells in skin biopsies as determined by IHC was similar for all 15 otters at capture (figure 3; repeated measures ANOVA, $P > 0.05$). Significant changes occurred in levels of the index throughout the experimental period (figure 3; repeated measures ANOVA, $P < 0.001$; group effect $P = 0.282$; session effect $P = 0.001$). The index value declined significantly between capture and the first sampling in captivity in June (prior to oil administration) for otters in the control and low-dose groups ($P < 0.05$). A similar non-significant trend was observed for the high-dose group (figure 4;

$P = 0.09$).

[Insert figure 3 around here]

A significant increase in levels of the index occurred in all groups between the June sampling session and the first exposure sampling session 16 days after the beginning of oil administration (Sept I; figure 3). The levels in the animals receiving the high dose were significantly higher than those in the other 2 groups (figure 3; Tukey's multiple comparisons, $P < 0.05$). The levels in this group declined somewhat by the October and November II sampling but remained high until the end of the oil administration. The CYP1A1 staining signal declined significantly in the high-dose group by the middle of the rehabilitation period (January; figure 3; Tukey's multiple comparisons. $P < 0.05$).

In the low-dose group the levels of endothelial CYP1A1 at the first sampling session were between those detected in the control and high-dose group. A significant decline in levels of the index occurred during the October sampling (figure 3). During rehabilitation, the levels in both the low and high-dose groups were similar to those recorded during capture (figure 3; $P > 0.05$), but higher than those recorded before the start of the experiment (figure 3; $P < 0.05$).

Non-linear curve estimation detected no relation between cumulative dose of ingested PBCO for the low dose group (figure 4a; non-linear curve estimation; $R^2 = 0.06$, $P = 0.311$). A significant near asymptotic relation was observed for the high dose group (figure 4b; non-linear curve estimation; $R^2 = 0.36$, $P = 0.005$; $y = 1.53*(x^{0.32})$).

[Insert figure 4 around here]

QRT-PCR results showed that the CYP1A1 mRNA concentration in peripheral blood mononuclear cells (expressed as molecules of CYP1A1/100ng of total RNA) was

similar for all 15 otters at capture (figure 5; repeated measures ANOVA, $P > 0.05$). A significant decline in those levels occurred between capture and the experimental period (figure 5; repeated measures ANOVA, $P < 0.001$; group effect $P = 0.658$; session effect $P = 0.001$). As with the endothelial CYP1A1, there was a significant increase in CYP1A1 mRNA expression in all groups between the August sampling period and the second sampling after the beginning of oil administration (Sept II; figure 5). Concentrations fluctuated through the period of oil administration and then significantly declined during rehabilitation (figure 5; Tukey's multiple comparisons, $P < 0.05$). Concentrations during the oil administration period were significantly lower than those recorded during capture for all except two of the control animals (UI02: capture level = 0.64, highest during oiling = 3.43; UI03: capture level = 1.72, highest during oiling = 4.01 molecules of CYP1A1/100ng of total RNA).

[Insert figure 5 around here]

No correlation was detected between values of the CYP1A1 staining index in endothelium and the concentration of CYP1A1 mRNA in lymphocytes, for the entire data set ($r = 0.006$, $P = 0.96$, $n = 75$).

Discussion

The development of effective biomarkers for environmental agents depends on the ability to describe quantitative, predictable, dose-response relations between exposure and the biological indicator (Vanden Heuvel and Davis 1999). In addition, the biomarker examined should be specific to a particular chemical or class of compounds, and relatively non-invasive (Vanden Heuvel and Davis 1999). Evaluations of the mRNA,

protein or catalytic activity of CYP1A in a variety of vertebrates, have been widely used as a biomarker for exposure to MC type environmental contaminants (Miranda *et al.* 1987, Payne *et al.* 1987, Stegeman *et al.* 1988, Kloepper-Sams and Stegeman 1989, Stegeman and Lech 1989, Juchau 1990, Goksøyr and Husoy 1992, Stegeman *et al.* 1992, Rattner *et al.* 1993, Buchelli and Fent 1995, Willet *et al.* 1997, Woodin *et al.* 1997). A prominent induction of CYP1A in endothelium of mammals and fish was identified in the 1980s (Stegeman *et al.* 1989). Those observations prompted studies to evaluate CYP1A in biopsy samples collected from marine mammals, as a marker of exposure to CYP1A inducers in these protected animals (Fossi *et al.* 1992, Goksøyr, 1995). Similarly, CYP1A1 mRNA has been successfully measured in peripheral mononuclear blood cells of humans, sea otters, and river otters (Ballachy *et al. in review*; Vanden Heuvel and Davis 1999). Thus, given the specificity of CYP1A1 to MC type inducers (as reviewed by Juchau 1990), the evidence of dose response in CYP1A1 expression (as reviewed by Vanden Heuvel and Davis 1999), and the ability to conduct non-destructive sampling for measurement of CYP1A1 expression in skin biopsies and peripheral blood cells (Fossi *et al.* 1992, Goksøyr 1995, Vanden Heuvel and Davis. 1999, Ballachy *et al. in review*), CYP1A1 should be considered a reliable biomarker.

Wild animals, inhabiting polluted areas, likely experience repeated exposures to CYP1A inducers. Nonetheless, chronic long-term exposure with repeated non-lethal sampling of the same individuals was rarely adopted in controlled studies designed to obtain a dose response in CYP1A expression. In this study, we simulated such chronic exposure in captive river otters fed weathered Prudoe Bay crude oil and measured levels of CYP1A1 in endothelial cells of skin biopsies and peripheral blood mononuclear cells

using repeated sampling of the same individuals. Despite the lack of correlation between those assays, both the IHC analysis and QRT-PCR exhibited a similar overall pattern in relation to exposure to hydrocarbons. Our results indicated the occurrence of induction of CYP1A1 expression in both the peripheral vascular endothelium and in peripheral blood mononuclear cells. In addition, this induction was measurable throughout the exposure period and declined shortly following the cessation of oil administration (figures 3 and 5). That the values of IHC for the low-dose group were on average between those of the control group and the high-dose group shortly following the initiation of dosing indicates a dose-response expression of CYP1A1 in the skin endothelium.

Our expectation that a systemic response to exposure would result in a correlated response between the two target tissues was not met. This could be a result of differences in turnover rate of endothelial and blood mononuclear cells or different responses to circulating xenobiotics by the two tissues. Other studies on fish and mammals documented differences in temporal accumulation of MC type agents and differential induction of CYP1A1 in different tissues (Brunström 1992, Woodin *et al.* 1997). Because of these potential differences, it would have been beneficial to conduct an IHC examination on peripheral blood mononuclear cells and conduct RT-PCR on endothelial cells. Unfortunately, the repeated sampling design of this experiment, and the need to obtain relatively large quantities of blood for other assays (Ben-David *et al. in press*) precluded the collection of additional samples for such a cross-check study. Such study, potentially conducted on larger animals, will enhance our ability to explore tissue specific responses to hydrocarbons as well as the applicability of both analytical methods.

Alternatively, the lack of correlation between endothelial and peripheral blood mononuclear cells CYP1A1 expression could be attributed to the occurrence of clinical anemia in the oiled captive otters (Ben-David *et al. in press*). In instances where toxins affect production of erythrocytes (anemia), and cause a regenerative response with release of nucleated erythrocytes into circulation from the bone marrow, the RNA of the nucleated erythrocytes would dilute the quantity of total RNA that is being evaluated from lymphocytes and monocytes. The anemia observed in our study animals was accompanied by the occurrence of nucleated erythrocytes suggesting a regenerative response (Ben-David *et al. in press*). Therefore, it is possible that we harvested nucleated erythrocytes with the other peripheral blood mononuclear cells thus reducing the overall measure of mRNA of CYP1A1. Furthermore, the observed reduction in white blood cells in the oiled animals (Ben-David *et al. in press*) may be indicative of suppression of the bone marrow and abnormal cell release from that tissue. Under conditions of long-term chronic exposure, new mononuclear blood cells could potentially be produced from an abnormal bone marrow tissue. The ability of cells, produced by such tissue, to respond to MC-type agents, in circulation, is unknown and merits further investigation. Analysis of CYP1A1 mRNA in mononuclear blood cells may produce quantitative dose-response results in cases where exposure is low enough so suppression of bone marrow does not occur. Regardless, the lack of quantitative, dose-response expression of CYP1A1 in mononuclear blood cells under conditions of clinical anemia in this study suggests that the use of this measure to describe exposure in free-ranging animals should be done with caution and determination of anemia should be done concurrently with evaluation of CYP1A1. Many controlled studies as well as field studies of wildlife exposed to

hydrocarbons frequently documented anemia in their subjects (Øristland *et al.* 1981, Leighton *et al.* 1983, Fry and Lowenstine 1985, Rebar *et al.* 1994, Williams *et al.* 1995).

As expected a significant near asymptotic relation between cumulative dose of ingested PBCO and values of IHC was observed for the high-dose group, indicative of induction of CYP1A1 and probable continuous oxidation of the hydrocarbons. It is important to note that the cumulative dose of ingested PCBO can not be linearly translated into accumulation of hydrocarbons in the target tissue (i.e., skin endothelium). Cumulative dose in this study should be regarded as a surrogate for continuous chronic exposure rather than accumulation of hydrocarbons. The increased variability in values of IHC with time (i.e., cumulative dose; figure 4) suggests that long-term chronic exposure may elicit different responses in individuals experiencing similar conditions. In addition, the lack of similar asymptotic relation in the low-dose group indicates that under chronic low exposure levels, induction of CYP1A1 may be unpredictable.

The reduction in levels of CYP1A1 mRNA in mononuclear blood cells, in our captive animals during rehabilitation, was more pronounced than that of CYP1A1 protein in endothelial cells. This is not an unexpected result because the half-life of the CYP1A1 protein is likely longer than that of mRNA (see review by Vanden Heuvel and Davis 1999). Thus, studies employing IHC techniques on skin endothelium may be able to record the occurrence of exposure to MC-type agents for longer periods of time than those using QRT-PCR. In contrast, the persistent and diminished signal in IHC may complicate interpretations of field data. Without additional information, a lingering, lower signal, resulting from elapsing time between exposure and sampling may be interpreted mistakenly as a recent exposure to a lower dose.

During the oiling period induction of CYP1A1 was detected in animals from the control group. Although this induction mainly occurred in mononuclear blood cells, by the end of the oiling period CYP1A1 values measured with IHC in endothelial cells for the control group were similar to the diminishing values of the oiled animals. The high levels of CYP1A1 in peripheral mononuclear blood cells recorded in the control animals may represent the fact that these individuals did not suffer from clinical anemia (Ben-David *et al. in press*), thus the levels of CYP1A1 in these animals were not diluted by nucleated erythrocytes. It is also possible that animals in our experiment were unintentionally exposed to MC-type agents through the fish they consumed. Although we did not test the foods we offered the otters for hydrocarbons, PCBs or other xenobiotics, otters were fed the same diet throughout the experiment (i.e., the frozen fish offered to the otters came from the same shipment and the live fish were caught in the same location throughout the experimental period). That both analytical approaches exhibited an initial decline in expression of CYP1A1, between capture and the experimental period (figures 3 and 5) indicated that the enclosure at the Alaska Sealife Center, and the food we offered to the captive otters were free of potential inducers of CYP1A1.

Alternatively, it is possible that the control animals were indirectly exposed to hydrocarbons because all the otters in our study were housed in one enclosure. Oiled animals occasionally dropped a partially consumed fish that contained oil. Although we cleaned the contaminated area with paper towels it is likely that we were unable to remove the more volatile compounds from the enclosure (table 1). In addition, an experiment designed to investigate the effects of oil ingestion on passage rate in the same individuals documented the excretion of hydrocarbons in feces of the oiled animals

(Ormseth and Ben-David, 2000). River otters use communal latrine site in the wild (Bowyer *et al.* 1995). Similarly, the experimental animals established communal latrine sites in the enclosure. Thus, although river otters do not consume feces, animals from the control group could have been exposed to hydrocarbons in feces of oiled animals while exploring the communal latrines. A study on captive Koalas demonstrated that animals kept in separate cages experienced induction of cytochrome P450 in response to the odor of leaves from a certain eucalyptus tree without actually consuming the foliage (Cork and Foley 1997). Whether similar processes occur in river otters is unknown. That a measurable induction of CYP1A1 occurred in the control animals which may have experienced a low and indirect exposure, illuminates the sensitivity of this biomarker.

The levels of endothelial CYP1A1 in the high-dose group during the oiling period were within the range of values reported for wild river otters in Herring bay, Knight Island, PWS (oiled area) in 1996 and 1997 (3.2 ± 0.5, mean ± SE and 4.7 ± 0.6, respectively; Bowyer *et al. in review*). In contrast, the levels of CYP1A1 in mononuclear blood cells in the experimental animals were much lower than those recorded in river otters sampled in oiled areas of the Sound in 1998 (36.9 ± 16.7, mean ± SE; Ballachy *et al. in review*). The latter, however, may be a result of the quantification problems due to the anemia discussed above. If indeed expression of CYP1A1 in skin endothelium in response to hydrocarbon exposure is dose-dependent as we observed, then wild river otters in oiled areas of the Sound would have been exposed to levels in excess of 50mg/day/ kg body weight 7-9 years following *EVOS*. This, however, is unlikely. First, no differences in body mass, other biomarkers, or differences in diet selection and home-range sizes were detected between otters inhabiting oiled vs. nonoiled areas in 1996-1998

(Bowyer *et al. in review*). In addition, investigators did not record any signs of anemia and reduction in white blood cell counts in wild otters in 1996-1998 (Bowyer et al. *in review*), which were the most pronounced effects of oiling in the captive experimental otters (Ben-David *et al. in press*). Therefore, it is possible that other agents in addition to hydrocarbons may have elicited the induction of CYP1A1 in river otters in PWS. Although the nature of these agents has not been determined yet, PCBs, components of the diet, as well as secondary compounds from Sitka spruce (*Picea sitchensis*) and western hemlock (*Tsuga heterophylla*) trees have been suggested as potential inducers (for other examples see Förlin *et al.* 1985, Payne *et al.* 1987, Juchau 1990, Brunström 1992, Courtney *et al.* 1993, Lee *et al.* 1997). Evaluation of the occurrence of other potential inducers, as well as the relation between diet, body composition, and levels of CYP1A1 in these mustelids merits further investigation.

Alternatively, the high levels in both IHC and QRT-PCR during capture compared with the levels immediately after acclimation to captivity in our experimental river otters (figures 3 and 5) could be associated with trapping stress. Ben-David *et al.* (*in press*) identified a group of variables (AST, LDH, glucose, and BUN) that exhibited a similar pattern of a significant decrease between capture and the June sampling session followed by stable values throughout the experiment for the same individual otters. Although no other studies documented an increase in expression of CYP1A in response to trapping stress, several studies identified AST, LDH, glucose, and BUN as indicators of capture stress in a variety of wild animals (Seal and Hoskinson 1978, Williams *et al.* 1992, Boonstra *et al.* 1998, Keech *et al.*, 1998). Of these variables, only BUN and mononuclear blood cells CYP1A1 mRNA were significantly correlated ($r = 0.32$, $P <$

0.001; Ben-David *et al. in press*). Another anecdotal evidence suggesting that trapping stress may have been involved in induction of CYP1A is indicated by the observation that of 111 wild-caught river otters (Bowyer *et al. in review*), those 3 with the highest staining index of endothelial CYP1A1 either died shortly after capture from an unassociated disease process, or were detained in the trap for an extended period of time (> 48h) due to failure of the trap transmitter (G. M. Blundell, pers. comm.). Usually, the trapped otters spent less than 8 hours in the trap (G. M. Blundell, pers. comm.). Therefore, an association between trapping stress and CYP1A1 expression will depend on the interval between the onset of stress (as may be transmitted by excretion of cortisol and/or other adernocorticosteroids) and the initiation of induction. Interestingly, Engelhardt (1982) documented that the half-life of cortisol was significantly reduced in ringed seals (*Phoca hispida*) exposed to hydrocarbons. Nonetheless, which of the CYP hemoproteins is involved in the degradation of cortisol in both seals and river otters is unclear and merits further investigation.

In conclusion, this is the first study to document induction of CYP1A1 expression under conditions of chronic exposure to hydrocarbons. We observed increases in expression of CYP1A1 in skin endothelium in the oil-exposed groups during the oil administration period, followed by a significant decrease during rehabilitation in the river otters exposed to PBCO in the diet. The observation that in a longitudinal study with repeated sampling of the same individuals, such a pattern was observed provides encouragement for the use of cytochrome P450 1A1 as a biomarker for chronic hydrocarbon exposure in these animals. Nonetheless, our results, especially those involving the RT-PCR on mononuclear blood cells, also suggest that the induction

process of CYP1A1 may be complicated and interacting with other processes in vivo. Such interactions may obscure our ability to describe specific, quantitative, predictable, dose-response relations between exposure to hydrocarbons and induction of CYP1A1, which are required of a reliable biomarker. Evaluations of such interactions based on theoretical physiological models in live-animals merit further investigation and will assist in substantiating the potential utility of this approach of analyzing CYP1A as a marker of exposure to MC-type compounds.

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Table 1 – Concentrations of hydrocarbons in the two batches of weathered Prudhoe Bay Crude oil fed to river otters at the Alaska Sealife Center in Seward Alaska from 21 August to 28 November 1998.

	Analyte concentrations: (μg/g)	
Compound	Batch 1	Batch 2
naphthalene	283.14	60.16
2-methylnaphthalene	904.43	616.81
1-methylnaphthalene	753.80	531.44
2,6-dimethylnaphthalene	641.37	577.26
C-2 naphthalenes	2601.33	2359.91
2,3,5-trimethylnaphthalene	268.37	278.51
C-3 naphthalenes	2284.48	2305.84
C-4 naphthalenes	609.96	593.84
biphenyl	155.55	120.85
acenaphthylene	0.00	0.00
acenaphthene	13.42	12.43
fluorene	97.31	100.48
C-1 fluorenes	228.37	247.95
C-2 fluorenes	273.45	309.82
C-3 fluorenes	169.23	183.75
dibenzothiophene	177.90	188.40
C-1 dibenzothiophenes	359.69	378.64
C-2 dibenzothiophenes	473.14	511.84
C-3 dibenzothiophenes	371.25	387.41
phenanthrene	244.29	263.38
1-methylphenanthrene	182.57	196.85
C-1 phenanthrenes/anthracenes	759.29	820.86
C-2 phenanthrenes/anthracenes	874.31	940.14
C-3 phenanthrenes/anthracenes	485.21	540.15
C-4 phenanthrenes/anthracenes	80.23	85.26
anthracene	7.72	3.41

fluoranthene	4.34	4.46
pyrene	11.70	12.34
C-1 fluoranthenes/pyrenes	66.05	644.75
benz-a-anthracene	6.64	4.28
chrysene	44.48	45.27
C-1 chrysenes	66.13	67.56
C-2 chrysenes	66.78	71.22
C-3 chrysenes	15.75	33.08
C-4 chrysenes	2.76	3.25
benzo-b-fluoranthene	14.73	15.66
benzo-k-fluoranthene	0.00	0.00
benzo-e-pyrene	9.89	11.76
benzo-a-pyrene	2.90	2.21
perylene	1.03	11.09
indeno-123-cd-pyrene	1.03	0.58
dibenzo-a,h-anthracene	1.31	1.07
benzo-g,h,i-perylene	2.50	2.97

Figure Legends.

Figure 1 – Immunohistochemical staining of CYP1A1 in endothelium from skin biopsy of river otters. (A) An MAb 1-12-3 staining in captive river otter skin plug; F denotes follicle and V represents vascular endothelial staining. (B) An UPC10 control MAb staining of same tissue.

Figure 2 – Gel electrophoresis of CYP 1A1 PCR product from lymphocytes of river otters. Ethidium bromide-stained agarose gel containing PCR products resulting from amplification of river otter lymphocytes CYP1A1 cDNA (310 bp). Left lane represents standards for molecular weight markers.

Figure 3 – Mean (± SE) values of staining index of CYP1A1 from endothelial cells for control, low-dose and high-dose groups (n = 15; 5 in each group) of river otters held in captivity at the Alaska Sealife Center in Seward, Alaska, USA. Oil was administered between 21 August and 28 November 1998. Significant changes occurred in levels of the index throughout the experimental period (repeated measures ANOVA, $P < 0.001$; group effect $P = 0.288$; session effect $P = 0.001$). Levels significantly decreased between capture and transfer to captivity and significantly increased during the oiling period for all groups.

Figure 4 – Relation between cumulative dose of ingested weathered PBCO and values of staining index of CYP1A1 from endothelial cells of river otters from the low-dose (A) and high-dose (B) groups. No relation was detected for the low dose group,

but a significant near asymptotic relation was observed for the high dose group (non-linear curve estimation; $R^2 = 0.36$, $P = 0.005$; $y = 1.53*(x^{0.32})$).

Figure 5 - Mean (± SE) values of number of CYP1A1 mRNA molecules 10^6/100ng of total RNA in peripheral mononuclear blood cells of control, low-dose and high-dose groups ($n = 15$; 5 in each group) of river otters held in captivity at the Alaska Sealife Center in Seward, Alaska, USA. Oil was administered between 21 August and 28 November 1998. A significant decline in those levels occurred between capture and the experimental period (repeated measures ANOVA, $P = 0.002$; group effect $P = 0.681$; session effect $P < 0.001$). Note the change in values on axes. A significant increase occurred in all groups between the August sampling session and the second bleeding session in September (Sept II). Levels fluctuated through the period of oil administration and then significantly declined during rehabilitation (Tukey's multiple comparisons, $P < 0.05$).

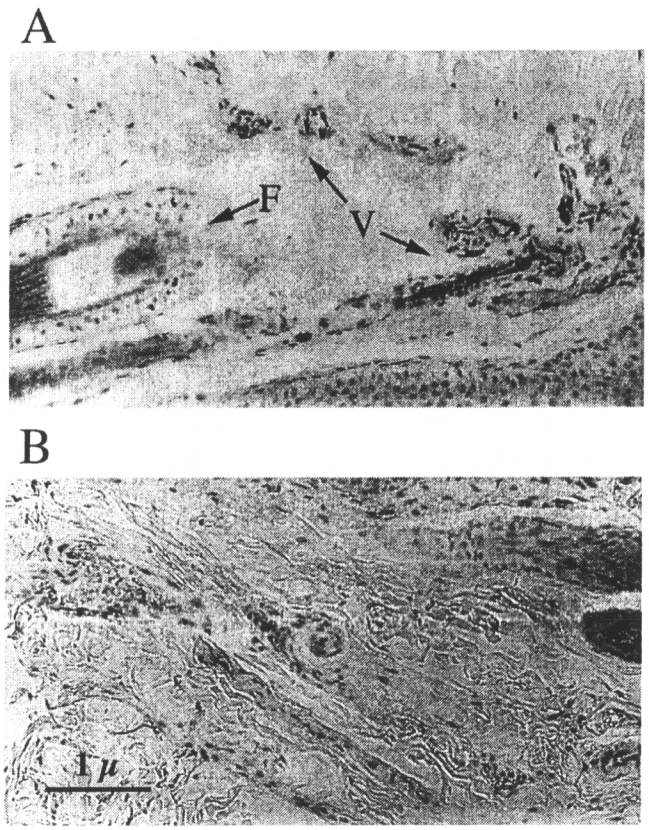
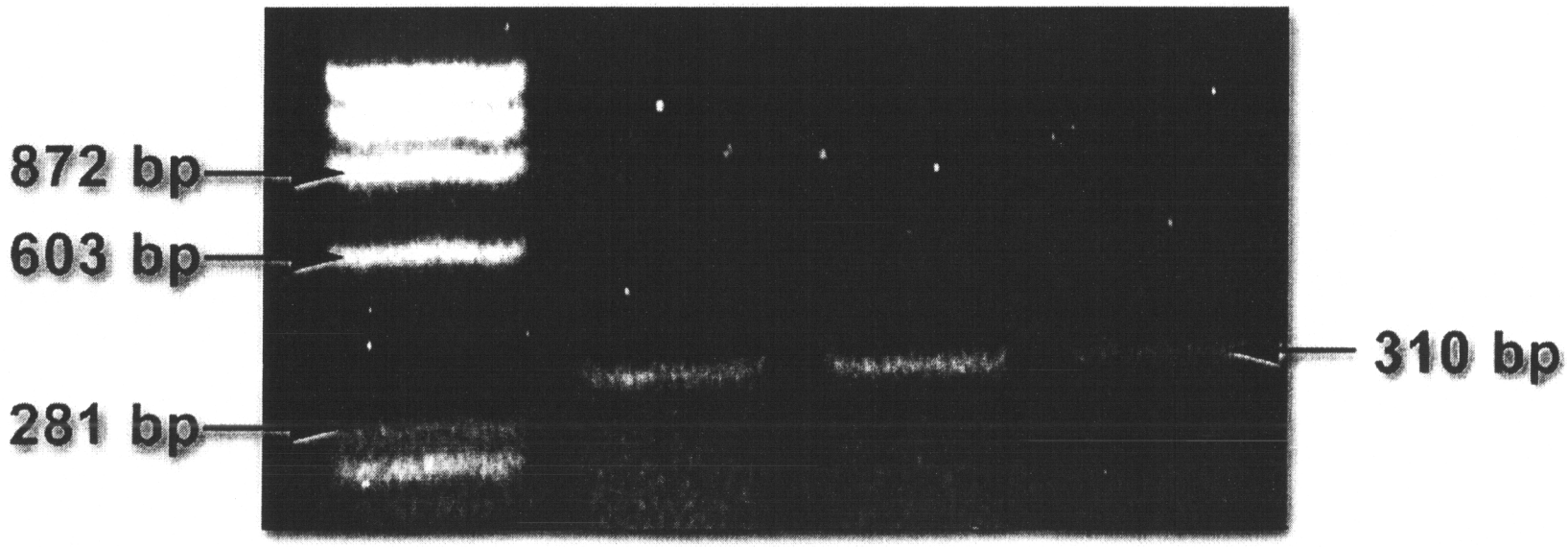


Figure 1 - Immunohistochemical staining of CYP1A1 in endothelium from skin biopsy of river otters. (A) An MAb 1-12-3 staining in captive river otter skin plug; F denotes follicle and V represents vascular endothelial staining. (B) An UPC10 control MAb staining of same tissue.

Ben-David et al. #263



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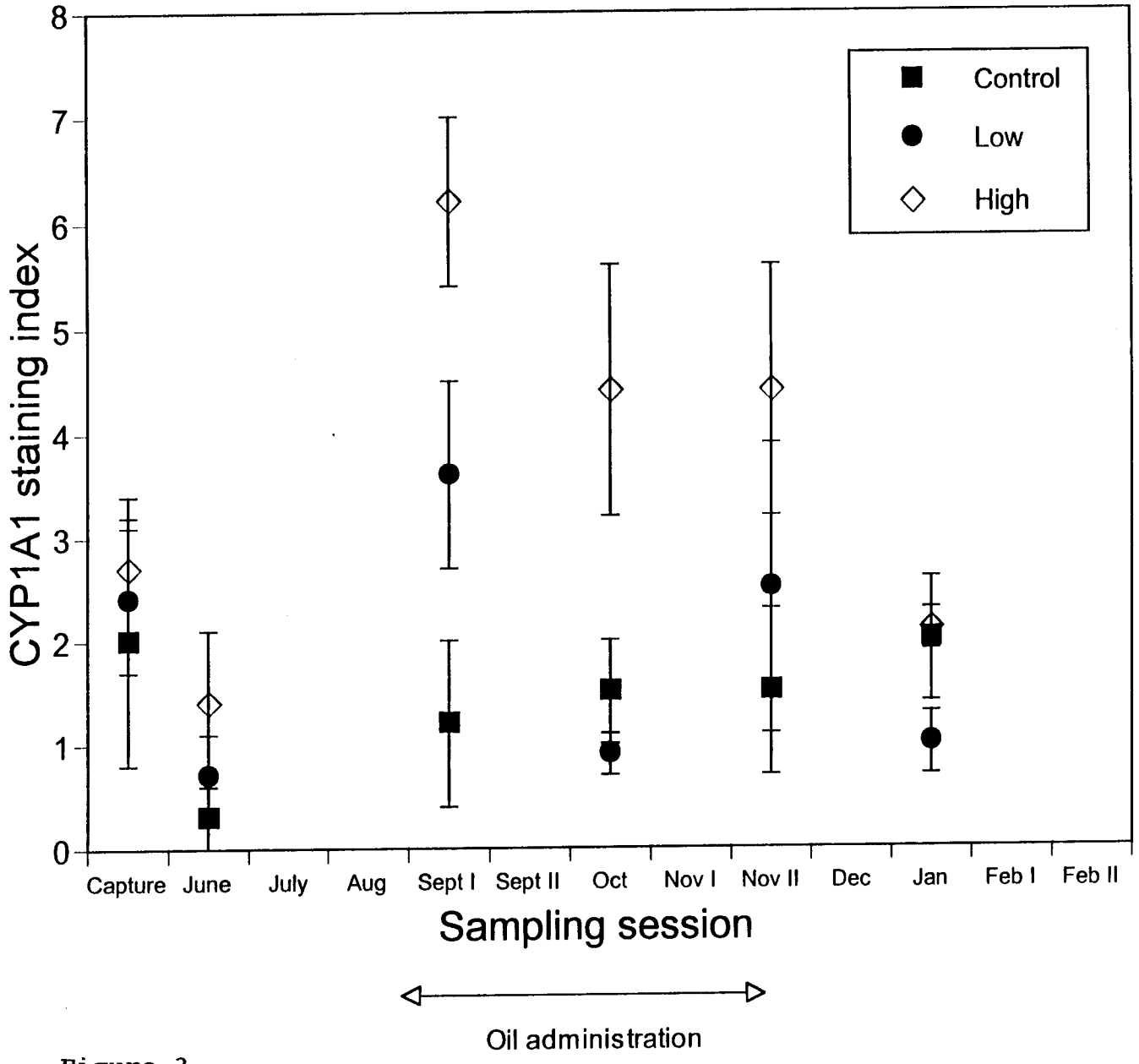
CYP1A1 staining index
0
1
2
3
4
5
6
7
8
Control
Low
High
Capture
June
July
Aug
Sept I
Sept II
Oct
Nov I
Nov II
Dec
Jan
Feb I
Feb II
Sampling session
Oil administration

Figure 3.

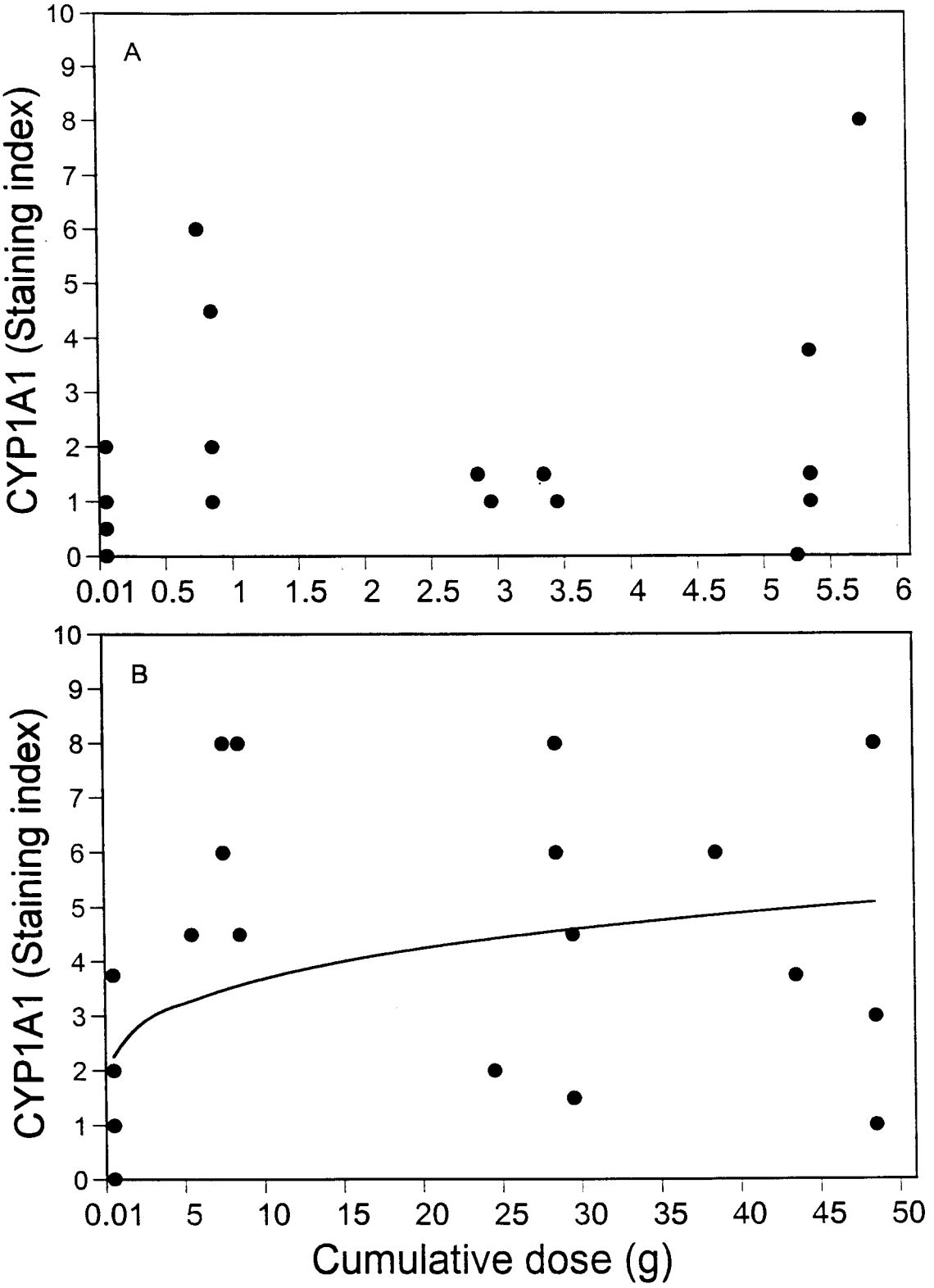
A
B
CYP1A1 (Staining index)
CYP1A1 (Staining index)
0
1
2
3
4
5
6
7
8
9
10
0.01
0.5
1
1.5
2
2.5
3
3.5
4
4.5
5
5.5
6
0.01
5
10
15
20
25
30
35
40
45
50
Cumulative dose (g)

Figure 7.

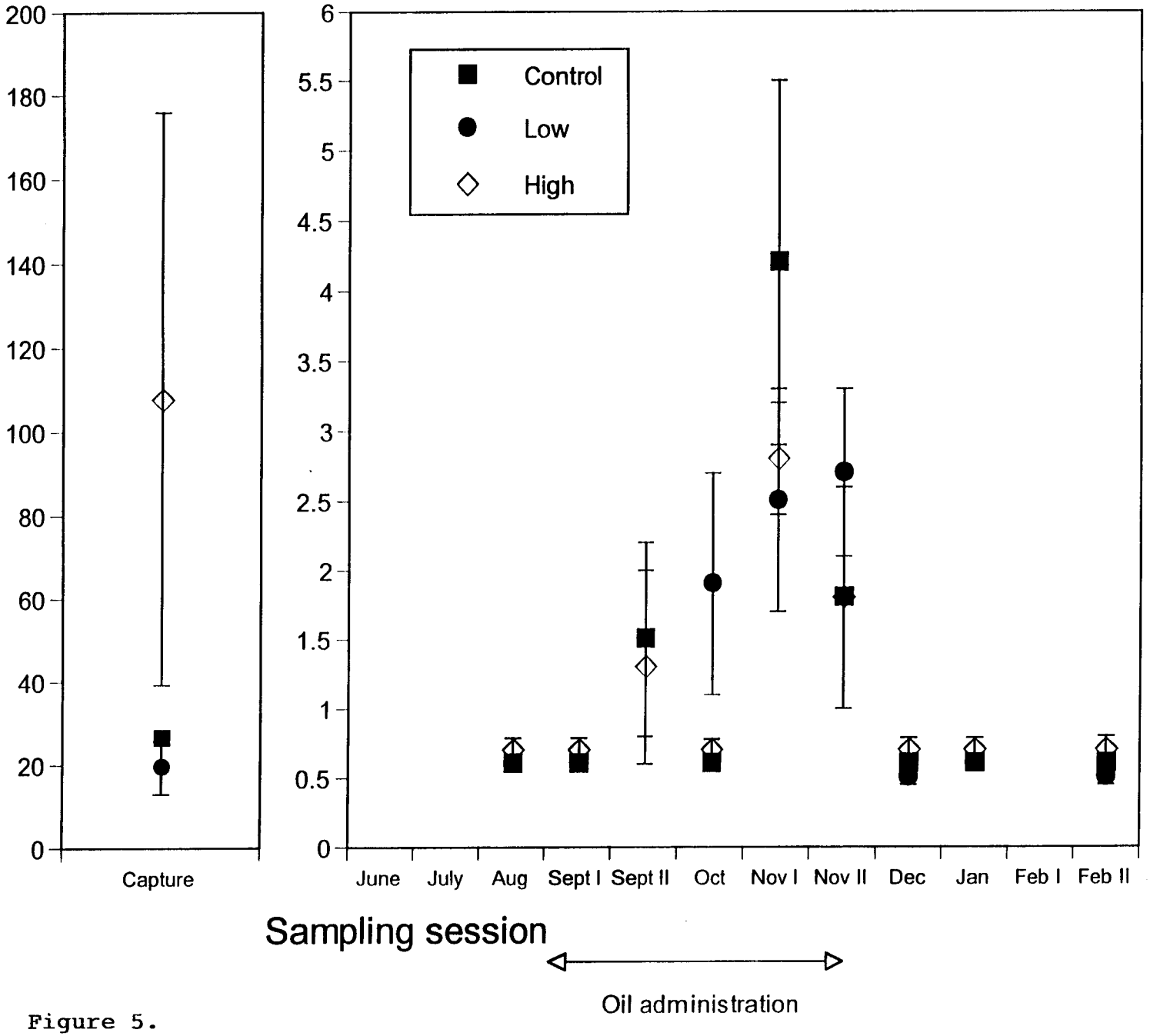
200
180
160
140
120
100
80
60
40
20
0
Capture
6
5.5
5
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
Control
Low
High
June
July
Aug
Sept I
Sept II
Oct
Nov I
Nov II
Dec
Jan
Feb I
Feb II
Sampling session
Oil administration

Figure 5.

Effects of oiling on exercise physiology and diving behavior of river otters: a captive study

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Effects of oiling on exercise physiology and diving behavior of river otters: a captive study

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Abstract: Following the *Exxon Valdez* oil spill (*EVOS*), river otters (*Lontra canadensis*) on oiled shores had lower body mass, selected different habitat characters, and had larger home ranges and less diverse diets than did otters living in nonoiled areas. We explored the possibility that these changes were due to the effect of crude oil contamination on physiological and behavioral processes in otters. Fifteen otters were exposed to two levels of oil contamination under captive, controlled conditions at the Alaska Sealife Center in Seward, Alaska, USA. We collected blood samples for hematological examinations and measured oxygen consumption in otters exercising on a motorized treadmill. We also observed diving and foraging behavior of otters offered live fish. Otters exposed to oil became anemic relative to controls. While oxygen consumption of resting river otters was not related to changes in hemoglobin concentration, exercising river otters with decreased hemoglobin levels displayed significantly increased oxygen consumption ($P = 0.042$). Oiled otters also performed fewer dives when chasing fish ($P = 0.04$), representing a potential decrease of 64% in capture rate of prey. Our data strongly support the hypothesis that changes in prey types and home range utilization by oiled river otters following *EVOS* were influenced by hematological changes, associated increases in energetic costs and reduced diving ability.

Keywords: Alaska, hemoglobin, hydrocarbons, *Lontra canadensis*, oxygen consumption, recovery times, running speed, schooling fishes, treadmill.

Introduction

Investigations in Prince William Sound (PWS), following the *Exxon Valdez* oil spill (*EVOS*), revealed that river otters (*Lontra canadensis*) on oiled shores had lower body mass and elevated levels of biomarkers (i.e., blood proteins indicative of physiological damage) relative to otters living on nonoiled shores (Blajeski et al. 1996; Bowyer et al. *in review*; Duffy et al. 1993; 1994*a*; 1994*b*; 1996). In addition, otters from oiled areas selected different habitat characters, had larger home ranges, and less diverse diets than those in nonoiled areas (Bowyer et al. 1994; Bowyer et al. 1995). These observed differences between river otters from oiled shores and those from nonoiled areas strongly suggest that oil contamination had an effect on physiological and behavioral processes in otters (Bowyer et al. *in review*).

Studies initiated following *EVOS* indicated that several other mammalian and avian predators displayed physiological stress related to oil toxicity. Oiled sea otters (*Enhydra lutris*), collected for rehabilitation, suffered from emphysema, ulcers, anemia, lesions and organ congestion (Williams et al. 1995). Similarly, free-ranging sea otters from oiled regions had greater antigenic stimulation than animals from nonoiled areas, and pups sampled in those regions had lower hemoglobin levels than pups from nonoiled areas (Rebar et al. 1994). Pigeon guillemots (*Cepphus columba*) had elevated levels of haptoglobins and blood proteins in specific locations and years, although dosing experiments in the field failed to demonstrate the connection between oiling and those parameters (Prichard et al. 1997). Similar changes in plasma proteins, abnormalities in white blood cells (leukocytes), reduction in the number of red blood cells (erythrocytes), and electrolyte imbalance, were previously observed in mink (*Mustela vison*) and polar

bears (*Ursus maritimus*) following exposure to hydrocarbons (Øritsland et al. 1981; J. Mazet, UC Davis, personal communication).

Such physiological stress may have a marked effect on the ability of animals to perform in the wild. Foraging behavior of semi-aquatic mammals such as river otters relies in part on their capacity to support aerobic metabolism while submerged (Dunstone and O'Connor 1979*a*; 1979*b*; Nolet et al. 1993). Kruuk et al. (1990) demonstrated that foraging success of European otters (*Lutra lutra*) in marine environments in Shetland was determined largely by behavior of both prey and predators. Although river otters are well adapted to diving (e.g., high surface-area of feet, high storage capacity for O_2, and good propulsion capabilities; Fish 1994; Nolet et al. 1993; Pfeiffer and Culik 1998; Tarasoff et al. 1972), limitations on aerobic capacity could affect the duration and depth of dives they perform (Nolet et al. 1993; Pfeiffer and Culik 1998) and consequent foraging efficiency. Thus, exposure to oil, associated chronic physiological stress, and reduction in numbers of red blood cells (i.e. lowered O_2 storage capacity; Øritsland et al. 1981) could have deleterious effects on the diving ability of coastal river otters.

In this study, we investigated the effects of exposure to oil on exercise physiology and diving behavior of river otters under controlled conditions. We hypothesized that exposure to oil would result in hematological changes that could lower aerobic capacity. Such changes will limit terrestrial and aquatic performance resulting in higher energetic costs as well as altered diving behavior of river otters. Because most dives performed by marine mammals are shorter than the aerobic breath-holding limit (Kooyman 1989; Kramer 1988; Nolet et al. 1993) we predicted that during the oiling period, foraging otters with reduced aerobic capacity would perform shorter dives than nonoiled controls.

Alternatively, if dive duration is largely dependent on stored oxygen in lungs, blood and muscles (Kooyman 1989), then recovery times would be longer in oiled animals as a consequence of prolonged time for oxygen replenishment. Thus, diving efficiency (duration of dive divided by the sum of dive and following recovery) should be lower for oiled otters.

Methods

General

Fifteen wild male river otters were live-captured in northwestern PWS using No. 11 Sleepy Creek® leg-hold traps (Blundell et al. 1999). Traps were placed on trails at latrine sites and monitored by trap transmitters (Telonics, Mesa, Arizona, USA) that signal when a trap is sprung (Bowyer et al. *in review*). Processing of otters began within 1 - 2 hours from capture. Otters were anesthetized with Telazol (9mg/kg; A. H. Robins, Richmond, Virginia, USA) administered using Telinject® darts and a blowgun. Blood and tissues were sampled from each individual otter at that time.

The fifteen wild-caught male river otters were transferred under sedation via air to the Alaska Sealife Center in Seward, Alaska, USA (ASLC). The number of subjects in this experiment was determined by an a-priori power analysis, and the need to minimize the impact of removing individuals from the wild population of river otters in PWS. The otters were held in captivity at ASLC from May 1998 to March 1999. The animals were housed as one large group in an area of 90 m^2 surrounding 6 pools (1 large salt-water pool - 4.5 m diameter by 3 m depth; 4 small salt-water pools - 2 by 1.5 by 1.5 m; and 1 small fresh-water tote - 1 by 1 by 1m). Otters were fed frozen fish on a daily basis (for

details see Ben-David et al. *in review a*) and diet was supplemented with live prey, vitamins, and minerals (Robbins 1993).

Experiments began in August allowing the animals 2.5 months to acclimate to the enclosure, feeding regimes, and handling. Following acclimation, otters were randomly assigned to 3 experimental groups of 5 individuals each: control group which received no oil; low dose group which received 50ppm of oil in their diet (i.e. 0.1g of oil every other day with an average individual daily consumption of 1kg food); and a high dose group which received 500 ppm of oil (i.e., 1.0g every other day). The exposure level for the low-dose group was determined based on levels of Prudhoe Bay crude oil (PBCO) found in mussel beds in PWS in 1995 (Short et al. 1996), in an attempt to simulate conditions of chronic exposure in the wild. The high-dose level was selected to simulate conditions in PWS immediately following *EVOS*. Weathered (comparable to 2 weeks weathering) Prudhoe Bay crude oil was administered to otters in gel capsules hidden in fish (Ben-David et al. *in review a*). Oil feeding lasted 100 days from August 21 to November 28, 1998. Data collection continued for an additional 100 days of rehabilitation. Animals were then fitted with radio transmitters and released back at the site of capture in PWS. Animals are currently being monitored to determine post-release survival (Ben-David unpublished data).

Hemoglobin levels

Prior to the exposure to oil (29-30 June, and 15-16 August 1998), a series of blood and tissue sampling was conducted on each individual otter (Ben-David et al. *in review a*). Blood and tissue sampling continued from August 15, 1998 until January 12, 1999 every three weeks. An additional sampling session occurred on February 24, 1999.

Otters were anesthetized with a combination of ketamine hydrochloride (100 mg/ml, Ketaset®, Aveco Co., Fort Dodge, IA., USA) at a dosage of 10 mg/kg, and midazolam hydrochloride (5 mg/ml, Versed®, Hoffman-LaRoche, Nutley, NJ, USA) at a dosage of 0.25 mg/kg (Spelman et al. 1993). The dosage was mixed in the same syringe and administered intramuscularly with Telinject® darts (Telinject U.S.A. Inc. Saugus, CA, USA) and a blowgun or hand injected while the otter was immobilized in a squeeze box. We drew blood from the jugular vein of each otter with care to keep samples sterile. A portion of the sample was preserved with EDTA (purple top Vacutainer®; Becton-Dickinson, Franklin Lakes, NJ, USA) for complete blood counts (CBC) and refrigerated until analysis (within 48 h). The remaining blood (approximately 10 ml) was collected in a red top Vacutainer® and allowed to clot; serum was removed (within 8 hr) following centrifugation at 3,000 rpm for 10 min, and refrigerated until analyses of serum chemistry. Three blood smears were made for each river otter at the time the blood was drawn. Serum-chemistry profiles were assayed with an Olympus 7000 (Olympus, Melville, NY, USA) and complete blood counts were performed with a Stack-S whole blood analyzer (Coulter, Miami, FL, USA). Samples were analyzed at Quest Diagnostics Incorporated (Portland, OR, USA).

Oxygen consumption

The rate of oxygen consumption was determined for ten of the fifteen male river otters (mean body mass = 11.1 ± 0.7 kg; 3 control, 3 low dose; and 4 high dose) during rest and running in October 1998, which coincided with the height of the oiling period. The animals were trained to run in a Plexiglas chamber (54 cm high x 31 cm wide x 138 cm long) mounted on a variable speed, motorized treadmill. Resting measurements were

taken for sedentary animals prior to each exercise test. Open flow respirometry was used with ambient air drawn in along the lower edge of the chamber according to Williams (1983). Air was drawn through the chamber by a vacuum pump at flow rates averaging 61 - 64 $l.min^{-1}$ to maintain the level of oxygen above 20% during the tests. Flow rates were monitored continuously with a dry gas meter (Singer, Inc.) that had been calibrated against a constant volume pump (Calibringe,Vacumed). Expired air exited a port located on top of the box and samples drawn through Drierite and sodasorb columns to remove water and carbon dioxide, respectively. The percentage of oxygen in the air samples were continuously monitored with an oxygen analyzer (Ametek S3-A) connected to a computer (Toshiba Satellite 100 CS). These values were converted to oxygen consumption (VO_2) using Sable Systems respirometry software and equations modified from Fedak et al. (1981) and Withers (1977). All values were corrected to STPD. The entire system was calibrated daily with dry, ambient air (20.94% O_2) and nitrogen gas (100% N_2) using nitrogen dilution techniques of Fedak et al. (1981).

The experiments were conducted at T_{air} = 2.9 - 9.4 °C which prevented overheating by the exercising otters. On each experimental day the otter was placed in the chamber and permitted to rest for approximately 10 - 15 minutes. The treadmill was turned on to the test speed, and $\%O_2$ monitored. During the experiments the otters maintained forward position at the front of the chamber. The animals ran for 10 - 20 min and were considered to be in a steady state when VO_2 varied by less than 4% over at least a 5-min period. Following the run the animals were released from the chamber and allowed to join the rest of the group in the enclosure; only one speed was tested on any experimental day. Test speeds were determined by the ability of the otters to keep up

with the tread at high speed while maintaining steady gaits. Speeds included: fast-walk at 0.72 $m.s^{-1}$, mixed gait of walk and gallop 1.05 – 1.2 $m.s^{-1}$, and gallop at 1.4 – 1.6 $m.s^{-1}$. Experiments were terminated if running performance was inconsistent or the animals turned around.

Diving Behavior

Dive duration, recovery times, and capture success (Table 1) were recorded for each individual otter (n = 12; 4 control, 3 low dose and 5 high dose) during experimental foraging sessions with a Newton MessagePad 2100 (Apple Inc., Austin, TX, USA) and Ethoscribe® software (Tima Scientific, New Brunswick, Canada). Otters were offered 2 types of live fish in the large salt-water pool: schooling fast fishes (Salmon or Sablefish) and nonschooling, slow intertidal fishes (Greenling or Rock fish; Table 2). The otters were allowed to forage for these fish for 30 minutes at a time (i.e., trial). The pool contained a PVC structure providing the fishes with hiding places from the chasing otter. In each trial, each otter was offered between 3 and 5 fishes of the same type, which were released in the pool together prior to the introduction of the otter. Fishes were allowed to explore the pool for several minutes before the beginning of the trial. Each session was also videotaped using an overhead remote camera and microwave transmission system. Data on chase intensity were extracted with frame by frame analysis using a Sony® SLV-998H VCR (Sony Corporation of America, Park Ridge, NJ, USA). Chase intensity was defined as the proportion of the dive time in which the otter actively chased the fishes. Data was coded 4 – if the otter spent 50 - 100% of the dive chasing the fishes; 3 – if the otter spent 10 – 50% of the time chasing; 2 – < 10%; and 1 – if no chase occurred. Diving

experiments were conducted prior to oiling (August 1998), at the height of oiling (October/November 1998) and at the end of the rehabilitation period (January/February 1999).

Statistical Analysis

To determine the effects of oiling on hemoglobin levels in river otters we used repeated measures ANOVA with oiling group (i.e., control, low dose, and high dose) and bleed session as factors. Analysis was followed by Tukey's multiple comparisons to establish where significant differences occurred.

Data on oxygen consumption was analyzed using linear regression with hemoglobin as the independent variable and oxygen consumption as the dependent one (Zar 1984). We conducted similar but separate analyses for animals at rest and during exercise.

Dive duration and recovery times were plotted sequentially for each foraging trial and analyzed for a fit to exponential distribution. Only cases where the r^2 was equal or greater than 0.9 and *P*-value was equal or less than 0.05 were included in subsequent analysis. For these sessions mean dive duration and mean recovery times were calculated and used in future analysis, because in these cases the mean equals the variance (Haccou and Meelis 1992). In addition, number of dives in each trial, and chase intensity during each dive was calculated. Mean dive duration, mean recovery times, mean chase intensity, and number of dives were tested for normality and were found to be normally distributed (W statistic > 0.9, SPSS for Windows). Differences in mean dive duration and mean recovery times for each trial, were analyzed with a 2-way ANOVA with number of dives used as a covariate to control for differences in number of dives between animals,

trials and sessions (Johnson and Wichern 1992; GLM - SPSS for Windows). The two factors were oiling group (control, low dose, and high dose) and sampling session (prior to oiling, height of oiling and rehabilitation). Data was blocked by individual to control for lack of independence among sessions. This corresponds to repeated measures analysis. We also calculated dive efficiency by dividing dive duration by the sum of dive length and the following recovery time (Kooyman et al. 1980; Nolet et al. 1993), and then established the mean dive efficiency in each trial. We used a 2-way ANOVA to investigate differences in dive efficiency between oiled and nonoiled otters with number of dives and chase intensity used as covariates to control for differences in number of dives and chase intensities between animals, trials and sessions (SPSS for Windows). The two factors were oiling group (control, low dose, and high dose) and sampling session (prior to oiling, height of oiling and rehabilitation). We used a curve-fitting approach to establish a relation between hemoglobin levels and dive efficiency, hemoglobin and number of dives as well as hemoglobin and chase intensity (Zar 1984).

Results

Hemoglobin levels

We found a significant reduction in hemoglobin (Hb) levels due to oiling in our experimental otters (Fig. 1; $P < 0.01$; group effect $P = 0.001$; session effect $P < 0.001$). Hemoglobin levels decreased in a similar fashion for all animals over the three months from capture to sampling in August. No significant differences ($P > 0.8$) were detected between groups for that period (Fig. 1). During the oiling period, levels of hemoglobin leveled off for the control group while values continued to decline for the oiled animals. No significant differences ($P > 0.2$) were detected between the low and high dose during

this period (Fig. 1). The lowest hemoglobin values were observed in the oiled otters in October, which coincided with both the treadmill and the diving experiments (i.e., height of oiling). After the administration of oil ended (Nov. 28, 1998), hemoglobin levels increased and no significant differences were detected between oiled and control animals in the December sampling. Similarly, no differences in hemoglobin levels were detected between the high dose and the control groups in January and February, but the low-dose group experienced a significant decline in hemoglobin levels during this time (Fig. 1). We were able to determine that this decline was caused by iron deficiency and reversed it with weekly iron injections before the animals were released.

Running performance and oxygen consumption

Oxygen consumption of our river otters resting on the treadmill averaged 12.84 ± 0.95 $mlO_2.kg^{-1}.min^{-1}$ (mean ± SE) and did not significantly vary ($P = 0.56$) with changes in hemoglobin concentration (Fig. 2). These values compare well with the resting values of 12.30 $mlO_2.kg^{-1}.min^{-1}$ (Pfeiffer and Culik 1998) and 9.60 $mlO_2.kg^{-1}.min^{-1}$ (Kruuk 1995) for the smaller European otter.

Nonoiled otters and oiled animals from both the low and high-dose groups exhibited different behavioral responses to running exercise. Oiled otters from both groups preferred to walk (0.72 $m.s^{-1}$) and avoided speeds that required mixed walk/bound gaits (1.05 – 1.2 $m.s^{-1}$) or bounding gaits (1.4 – 1.6 $m.s^{-1}$). All 3 control animals tested in the experiment performed in all tests without added encouragement. Of the oiled animals only one low-dose animal and one high-dose animal ran in the 1.4 – 1.6 $m.s^{-1}$ speed; the low dose animal only performed this trial once. The other 5 animals refused to run at 1.4

– 1.6 $m.s^{-1}$ although they performed well at a speed of 0.72 $m.s^{-1}$. Therefore, to compare performance of these individuals we used only the data from walking speed tests (0.72 $m.s^{-1}$).

Oxygen consumption of river otters running on the treadmill at 0.72 $m.s^{-1}$ was 2.3 to 3.1 times higher than rest. In contrast to the resting levels, oxygen consumption of exercising river otters decreased significantly ($P = 0.042$) with increases in levels of blood hemoglobin and was described by the equation,

$$VO_2 = 61.10 - 2.01(Hb) \qquad (r^2 = 0.35, n = 12) \qquad (1)$$

where VO_2 is in $mlO_2.kg^{-1}.min^{-1}$ and Hb is in g/dl .

Dive performance and behavior

Dive duration and duration of recovery were significantly correlated for the entire data set ($r = 0.501$, $P = 0.001$), suggesting that long dives were usually followed by long recovery times. Nonetheless, there may be a problem with our estimation of recovery times. Recovery was defined as all activities conducted by the animal following dives that were immediately followed by another dive. This includes surface swimming, climbing out of the pool and sitting on the edge of the pool (Table 1). Several animals spent long periods of time sitting on the edge between two consecutive dives. Those occurrences likely exceeded the time required for replenishing the stores of oxygen, and potentially reflect behavioral responses rather than physiological ones.

Dive duration for otters chasing fast schooling- fish was significantly lower than when they were chasing slow nonschooling-fish (Fig. 3 and 4; Wilcoxon, $P = 0.02$) suggesting higher levels of oxygen consumption during these chases. For comparison, time to first capture (i.e., time elapsed from the otter first dive to capture of the first fish) was significantly lower for otters foraging for slow fish ($n = 12$) than to that of fast fish (Fig. 5, Wilcoxon, $P = 0.013$).

We separated the subsequent analysis for the 2 types of fish to reduce the potential effects of diving speeds on dive duration. When number of dives was introduced as a covariate, dive duration and recovery times of otters did not significantly differ between the oiling treatment-groups and sessions for fast schooling-fish (Fig. 3; $P = 0.15$ and $P = 0.70$ respectively). For slow nonschooling-fish dive duration differed between oiling groups and sessions ($P = 0.042$) but no difference was detected for recovery times ($P = 0.17$; Fig. 4). The difference in dive duration between groups and sessions was largely a result of the different number of dives ($P = 0.04$). Number of dives during the height of oiling significantly differed (Tukey multiple comparisons, $P < 0.05$) between the control and highly oiled animals but not with the low dose ones (Tukey multiple comparisons, $P > 0.05$). In addition, while the number of dives between sessions did not change significantly for the control animals, it significantly did so for both low dose and high dose animals (Tukey multiple comparisons, $P < 0.05$; Fig. 4). When controlling for number of dives the effect of oiling on mean dive duration became non-significant ($P = 0.40$).

Dive efficiency did not significantly differ between groups and sessions (ANOVA, $P = 0.07$) for otters chasing both slow and fast fishes (Fig. 6). In addition,

number of dives or chase intensity had no significant effect on dive efficiency ($P > 0.2$). We were unable to detect a relation between hemoglobin levels and dive efficiency ($P = 0.36$). In addition, we were unable to establish a relation between hemoglobin levels and number of dives (Fig. 7; $P = 0.45$), but chase intensity exponentially decreased with decreasing hemoglobin concentrations (Fig. 7; $P = 0.027$):

$$\text{Chase intensity score} = 2.4 + 0.3 * \ln(\text{Hb}) \quad (r^2 = 0.19, n = 49 \text{ trials}) \qquad (2)$$

where chase intensity is coded 1 (none) to 4 (high) and Hb is in g/dl .

Discussion

Two factors seemed to cause a reduction in hemoglobin levels in our experimental otters. The transfer of these otters from the wild to captivity could explain the initial reduction we observed in all otters (Fig. 1). Whether this reduction was a result of a change in diet, change in availability of vitamins and minerals, reduction in levels of activity and exercise, or other environmental factors in this artificial setting is unclear. Nonetheless, the difference between the control and treatment groups throughout the oiling period suggests that oiling further aggravated the anemia. Similar results were previously observed in sea otters, mink, and polar bears following exposure to hydrocarbons (Mohn and Nordstoga 1975; Øritsland et al. 1981; Williams et al. 1995). A reduction in aerobic capacity was previously suggested for oiled sea otters (Williams et al. 1995). That the two oil treatment groups displayed comparable hemoglobin levels was probably due to the effects of oil ingestion on intestinal absorption of hydrocarbons. Ormseth and Ben-David (*in review*) documented that the high dose animals excreted

much of the ingested hydrocarbons, resulting in similar physiological exposure levels in the two groups.

Changes in aerobic capacity associated with the decline in hemoglobin concentration were most apparent during activity both on land and in water for the river otters in the present study. Based on a normal hemoglobin concentration of 15. 8 g/dl for wild river otters and extrapolation of the linear fit of the regression model (Fig. 2), the expected oxygen consumption for running at 0.72 $m.s^{-1}$ is 29.3 $mlO_2.kg^{-1}.min^{-1}$ (from equation 1). This compares with the measured VO_2 of 28.0-33.5 $mlO_2.kg^{-1}.min^{-1}$ for captive river otters with hemoglobin values of 14.4 g/dl and 40.4 $mlO_2.kg^{-1}.min^{-1}$ for river otters with the lowest hemoglobin values (10.9 - 11.1) in this study. Consequently, the decrease in hemoglobin, and presumably lower oxygen carrying capacity of the blood, resulted in a 37.6% increase in energetic cost of running in the severely anemic river otters. A potential mechanism for the observed elevation in costs may be increased respiratory intake of oxygen when energetic demands are high to compensate for the reduced circulating oxygen. Because captivity had an effect on hemoglobin levels, it would be difficult to directly assess the effects of oil contamination on wild river otters. Therefore, our discussion is limited to the relation between hemoglobin levels and metabolic costs rather than the direct effects of oil contamination. Nonetheless, wild oiled sea otters exhibited reductions in hemoglobin levels similar to those observed in our captive river otters (Williams et al. 1995), suggesting that our calculations are within reason.

That this relation between hemoglobin levels and oxygen consumption during running (equation 1) explained only 35% of the variability indicates that other factors

influenced oxygen consumption in the otters running on the treadmill. Such factors may be associated with habituation of animals to the handlers or alternatively to other effects of oiling. Ben-David et al. (*in review a*; *in review b*) documented elevation in several liver enzymes, reduction in white cell counts, and elevation in endothelial cytochrome P450-1A associated with oiling in these otters. These biomarker responses may indicate additional physiological damage, which affected exercise performance of the otters independent of the hemoglobin reduction.

An increase in aerobic cost of 37.6% in running oiled otters represents an additional metabolic demand on the naturally elevated energetic costs characteristic of aquatic or semi-aquatic mustelids. Both resting (Iverson 1972) and active (Dunstone and O'Connor 1979*a*, 1979*b*; Pfeiffer and Culik 1998; Stephenson et al. 1988; Williams 1983, 1989) metabolic rates of many species of mustelids (i.e. minks, river otters, sea otters) are higher than predicted for terrestrial mammals. In this study, the cost of transport, defined as the amount of fuel required to transport one unit of body mass over a unit distance (Schmidt-Neilsen 1972), for running river otters with normal hemoglobin levels was 13.6 $J.kg^{-1}.m^{-1}$. This value is nearly 2.7 times the predicted value for minimum transport costs for terrestrial mammals (Taylor et al. 1982). The difference between these values may be attributed in part to unusual gait patterns of semi-aquatic mustelids as described for running mink (Williams 1983). Alternatively, the otters in the present study may not have been tested at a speed that offers minimum transport cost (Williams *et al.* in prep.). Regardless, the extraordinarily high costs of running for this mammal appear to be exacerbated by the decrease in hemoglobin concentration. As a result, the

cost of transport for running river otters with the lowest hemoglobin levels in this study was 18.8 $J.kg^{-1}.m^{-1}$, 3.8 times the predicted level for running terrestrial mammals.

Similar decreases in aerobic capacity were apparent for aquatic activity in our river otters displaying decreased hemoglobin levels. Although a significant difference in number of dives existed for otters chasing slow fish (Fig. 4), such difference between groups and sessions could not be detected for fast fish (Fig. 3). That the number of dives did not differ between sessions and groups for the fast fish may be attributed to the fish behavior. These fish were more visible and may have induced the chase instinct in the otters more readily. Alternatively, the data for fast fish is less reliable than that for slow fish because we were forced to use different fish in each session (Table 3). For the pre-oiling session we used adult pink salmon; sable fish were used for the height of oiling; and juvenile pink salmon for the rehabilitation period. In addition, we did not have enough juvenile pink salmon to conduct the experiments on all 12 individuals. This greatly reduced our sample size and our power to detect differences because of missing values.

In contrast to our predictions, dive duration did not decrease, recovery times did not increase, and dive efficiency remained unchanged between sessions and treatment groups. Instead, the otters foraging after slow fish apparently corrected for impairment in diving ability by reducing the number of dives (i.e. submergence time) and chase intensity rather than by modifying dive characteristics during the height of oiling. This is further supported by the observation that during this session only one control animal was reluctant to dive after fishes (an animal that performed poorly in all sessions and perished of starvation soon after release; Ben-David, unpublished data). In comparison, three of

the oiled animals (one low dose and 2 high dose, all of which are currently thriving in the wild) were reluctant to do so (Fig. 3 and 4). Although the number of dives performed by the oiled otters during the height of oiling did not significantly differ from that of the controls, it is important to note that only in the oiled groups did the same individuals significantly alter their behavior between the three sessions as indicated by the repeated measures analysis. Nonetheless, number of dives was not directly related to reduction in hemoglobin levels (Fig. 7). Similarly, although chase intensity (or the work performance of the diving otters) was related to hemoglobin levels, this variable accounted for only 19% of the variation, suggesting that other factors including those associated with oiling may have affected the otters motivation to dive.

We attempted to evaluate the effects of hemoglobin reduction on oxygen stores by using a theoretical calculation of aerobic dive limit (ADL). ADL, which is defined as the maximum breath-hold possible without an increase in blood lactate concentration during or after a dive (Kooyman 1989), was likely reduced in these animals as a result of decreased oxygen carrying capacity of the blood. For this theoretical consideration we chose to compare hemoglobin levels of wild, free-ranging otters with a value representative of the most severely anemic otter in our experiment. For example, 1.34 ml of oxygen is carried with each g of Hb in the blood of healthy mammals (Guyton 1986). At capture, hemoglobin content for river otters in this study was 15.8 g Hb/100 ml blood, which corresponds to an oxygen content of 21.1 ml O_2 per 100 ml blood (15. 8 g Hb/100 ml blood x 1.34 ml O_2/g Hb). This compares with an O_2 content of only 14.9 ml O_2/ 100 ml blood for a river otter with a hemoglobin content of 11.1 g Hb/100 ml blood (Fig. 2). Using the calculations of Kooyman (1989) we find that the total blood oxygen stores of a

wild, free-ranging river otter is 110 ml O_2, while a river otter with low hemoglobin content (i.e., 11.1 g Hb/100 ml) has a blood oxygen store of 68 ml O_2.

The effect of these lower blood oxygen stores is a reduction in the total oxygen reserve available to the animal during submergence. When diving, aquatic mammals rely on oxygen stored in the blood, lungs, and muscles to support aerobic metabolic pathways (see Butler and Jones 1997 for a review). Calculated oxygen stores for wild river otters based on Kooyman (1989) are 27.2 mlO_2/kg body mass divided between the lungs (30.2%), blood (40.4%), and skeletal muscle (29.4%). This store is reduced to 23.0 mlO_2/kg body mass for river otters with hemoglobin content of 11.1 g Hb/100 ml blood. The aerobic dive limit of the river otter may be determined by dividing these oxygen stores by the metabolic rate of the submerged animal. In a study by Pfeiffer and Culik (1998) the average metabolic rate of submerged swimming European otters (body mass = 6.0 kg) was 0.51 ml $O_2.kg^{-1}.s^{-1}$. Because the river otters in the present study were larger (body mass = 11.1 kg), we corrected this metabolic rate for body size differences using body mass raised to the 0.75 power (Robbins 1993) and obtained a reasonable approximation for this discussion. This calculation predicts that the aerobic dive limit would be 54.1 sec for river otters with normal hemoglobin contents, and 45.7 sec for river otters with hemoglobin contents of 11.1 g Hb/100 ml blood. Thus, such a reduction in hemoglobin content of the blood could potentially reduce submergence time in a single dive by 8.35 sec or 15.4%. While these predictions are based on estimates of average metabolic rates and should be interpreted with caution, they provide a general framework for discussing the effects of decreased hemoglobin levels on diving ability of river otters.

Interestingly, mean dive duration for our captive river otters was well below the expected aerobic dive limit even before the exposure to oil, regardless of fish type (Fig. 3 and 4). The longest foraging dive we recorded was 88 sec, which exceeds the aerobic limit we calculated for wild otters, but such long dives were rare (0.3% of 2,293 dives performed by 12 individual otters). In addition, only 1.1% of all dives performed in our experiments were longer than the 45.7 sec limit we calculated for otters with hemoglobin level of 11.1 g/100 ml. This suggests that, like other diving mammals, river otters are capable of performing anaerobic dives although most dives will be within aerobic limits. These data correspond well with lengths of dives observed in a companion study on wild otters in PWS (21 ± 1 sec., mean ± SE; n = 441 dives; Ben-David, unpublished data) as well as for dives performed by European otters in Shetland (Kruuk 1995). Similar observations were made for other marine mammals. Kooyman (1989) reports that 90% of all dives performed by free-ranging marine mammals are below the aerobic dive limits of each species. That total submergence time and chase intensity were the only significant responses observed in this study, and the fact that most dives were well below our calculated ADL suggest that the otters altered their behavior to avoid the cumulative depletion of oxygen stores that may result from multiple dives.

Whether wild otters will respond similarly to captive well-fed animals is unclear. Nonetheless, if a reduction in stores of oxygen is translated into decreased submergence time within each specific foraging bout, then body condition, survival and reproduction of coastal river otters may be compromised. For example, the average number of dives for oiled otters chasing slow fish in 30-min trials decreased from 42 to 15. When multiplied by the average dive duration for slow fish (15 sec), this results in a reduction

from 10.5 to 3.75 min of total time of submergence in a 30 min. foraging bout. Thus, if total submergence time dictates the potential rate of capture of fishes, for an oiled otter, this potential may be reduced by nearly 64%. Kruuk (1995) demonstrated that the amount of time required for fishing in European otters increases exponentially with decreasing potential capture rate of prey. Using the model developed by Kruuk (1995), and a potential rate of capture of 600 fish in g per hour for nonoiled otters, we calculated that a 64% decrease in the potential capture rate will result in an increase from an estimated 1.5 hours to 5.2 hours of foraging for oiled otters. This, in turn, will result in additional energetic costs such as those associated with thermoregulation in water. Kruuk (1995) demonstrated that the exponential relation between the amount of time required for fishing and potential capture rate of prey, is dependent on water temperature, representing costs of thermoregulation.

The increase in foraging time (potentially 64%), increase in energetic costs associated with thermoregulation, as well as the potential increase of 37.6% in energetic costs of terrestrial locomotion (recorded in our treadmill experiment) are likely to cause a significant decrease in body condition in oiled free-ranging river otters. Indeed, Duffy et al. (1993) documented a significant reduction in body mass (controlled for age and sex classes) for otters live-captured in oiled areas in PWS. Furthermore, the constraints imposed by oiling on diving behavior of otters will likely alter the diet of otters. We would expect otters to concentrate on prey that are easy to capture and have high capture rate potential. Bowyer et al. (1994) documented that changes in diet of otters from oiled shores resulted mostly from a reduction in prey species. Between 1989 and 1990, perciform fishes (sand lances, gunnels, and ronquils) declined in diets of otters on oiled

areas whereas those groups increased in the diets of otters from nonoiled sites during the same period. Conversely, Malacostraca (crustaceans) increased in the diet of otters from oiled area but declined in the nonoiled area (Bowyer et al. 1994; *in review*). Yet, surveys of intertidal and subtidal organisms in PWS suggested that species composition and biomass did not differ between oiled and nonoiled areas (Thomas A. Dean, Coastal Resources Associates, Inc., pers. comm.). This suggests that these diet differences were a result of changes in foraging strategies by otters inhabiting oiled areas rather than from differences in prey availability.

To our knowledge, ours is the first study to document a relation between hematological changes due to oiling and increase in energetic costs for wild animals under controlled conditions. We were able to establish that even low doses of weathered oil caused significant reduction in aerobic capacity of river otters through anemia. This reduction, in turn, led to increase in energetic costs of terrestrial locomotion, a potential decrease in aerobic dive limit, and a potential increase in foraging time due to a decrease in total length of submergence during each foraging bout. These responses to oiling could have major implications to maintenance of body condition, survival, and reproduction of coastal river otters as well as other diving mammals and birds. In addition, our findings highlight the controversy associated with rehabilitation of oiled wildlife. Professionals should consider the relations between hematological profiles, aerobic capacity and foraging ability to increase the likelihood of post-release survival of their subjects. Although recovery from anemia by oiled animals may require several months (Williams et al. 1995), detaining animals in captivity for long periods can be detrimental to their subsequent survival (Ben-David unpublished data). Further studies investigating the

potential effects of reduction in hemoglobin levels on aerobic capacity of diving animals would greatly enhance our understanding of the long term effects of exposure to oil and may assist professionals in deciding on a course of action following future oil spills.

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adopted by the American Society of Mammalogists (Animal Care and Use Committee 1998).

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Table 1 – List and definitions of data collected for each individual otter during foraging trials at the Alaska Sealife Center, Seward Alaska.

Behavior	Computer code	Analysis code	Comments
Dive – from head submerged to head emerged	Dive	Dive	
Surface swimming – animal with head above water	Surf Swim	Recovery	
Climb out – animal is climbing out of the water	Climb Out	Recovery	
Edge – animal either sitting, walking or running around the edge	Edge	Recovery	If activity is immediately preceded and followed by a dive
		Non pool	If activity is preceded or followed by non pool activity
Eat – animal is feeding on fish	Eat	Non pool	
Non pool – all activities not related to pool (sleeping, scent marking, drinking, scratching door, etc.)	Non pool	Non pool	

Table 2 – Fish types and session schedules for diving experiments of captive river otters at the Alaska Sealife Center, Seward Alaska. Fish were obtained under permit from the Alaska Department of Fish and Game, Commercial Fish Division (# CF-98-024), and were collected in southcentral Alaska.

Fish type	Species	Number of fish	Session used
Fast /schooling	Adult pink salmon - *Oncorhynchus gorbuscha* (2-3kg)	220	Pre-oiling
	Pacific cod - *Gadus macrocephalus* (3-4kg)	8	Height of oiling
	Sable fish (black cod) – *Anoplopoma fimbria* (2-3kg)	28	Height of oiling
	Juvenile pink salmon - *Oncorhynchus gorbuscha* (0.3-0.5kg)	23	Rehabilitation
Slow / nonschooling (0.75 – 1.5kg)	Copper rockfish - *Sebastes caurinus*	11	All
	Red Irish lord - *Hemilepidotus spinosus*	8	All
	Yellowfin sole - *Limanda aspera*	16	All
	Kelp greenling - *Hexagrammos decagrammus*	201	All

Figure legends

Fig. 1 – Levels of hemoglobin in blood of captive river otters at the Alaska Sealife Center exposed to 3 levels of hydrocarbons in their diet (n = 15; five in each treatment group). Blood was collected every three weeks between August 15, 1998 and January 11, 1999. Six weeks elapsed between the June 29 and August 15 sessions and between the January 11 and February 22 sessions. For both low and high dose a significant decrease in hemoglobin occurred during the oiling period (Sept I to Nov II sessions; Tukey multiple comparisons; $\alpha = 0.05$). No such decrease was detected in control animals. A significant decrease in hemoglobin levels occurred in the low dose group in February, due to iron deficiency.

Fig. 2 – Oxygen consumption ($mlO_2.kg^{-1}.min^{-1}$) plotted against hemoglobin concentrations (g/dl) for captive river otters (n = 10 individuals) at rest and at a fast walk of 0.72 $m.s^{-1}$. No significant relation was detected between oxygen consumption and hemoglobin levels at rest ($P = 0.56$), but oxygen consumption increased significantly with decreasing hemoglobin ($P = 0.042$). Experiments were conducted at the Alaska Sealife Center in Seward.

Fig. 3 – Mean dive duration (sec), mean recovery time (sec), and number of dives for captive river otters diving after fast fish. No significant differences were detected for these variables (ANOVA, $P > 0.1$). Numbers above error bars represent sample sizes. Experiments were conducted at the Alaska Sealife Center in Seward.

Fig. 4 – Mean dive duration (sec), mean recovery time (sec), and number of dives for captive river otters diving after slow fish. No significant differences were detected for dive duration and recovery times (ANOVA, $P > 0.1$), but number of dives significantly differed between groups and sessions (ANOVA, $P = 0.04$). Number of dives during the height of oiling significantly differed between the control and highly oiled animals (Tukey multiple comparisons, $P < 0.05$) but not with the low dose ones (Tukey multiple comparisons, $P > 0.05$). In addition, while the number of dives between sessions did not change significantly for the control animals, it significantly did so for both low dose and high dose animals (Tukey multiple comparisons, $P < 0.05$) Numbers above error bars represent sample sizes.

Fig. 5 – Time to first capture (sec) for captive river otters foraging for fast and slow fish (Wilcoxon, $P = 0.013$; $n = 12$ trials by 12 individual otters for each fish type). Experiments were conducted at the Alaska Sealife Center in Seward, Alaska.

Fig. 6 – Mean dive efficiency (i.e., dive duration divided by the sum of dive length and the following recovery time; ± SE) for captive river otters diving after fast (top) and slow fish (bottom). No significant differences between groups and session were detected for dive efficiency (ANOVA, $P > 0.05$), regardless of fish type. Sample sizes are provided in Fig. 3 and 4.

Fig. 7 – Relation between hemoglobin levels (in g/dL) and mean chase intensity score (top; coded 1 – no chase, to 4 – high intensity; n = 49 trials) and number of dives (bottom; n = 67 trials). Chase intensity exponentially decreased with decreasing hemoglobin levels (P = 0.027), but no relation was detected between hemoglobin and number of dives (P = 0.45).

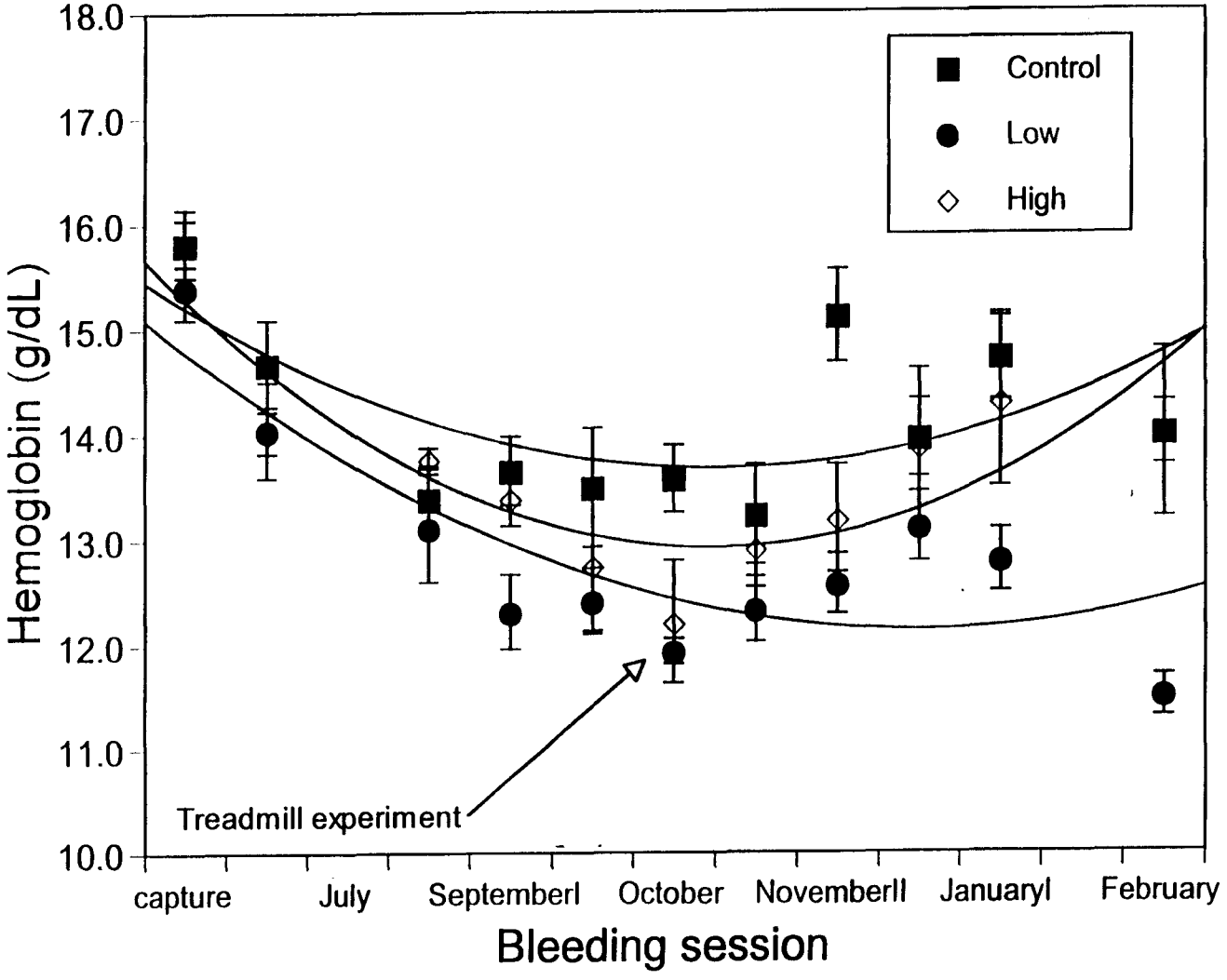
18.0
17.0
16.0
15.0
14.0
13.0
12.0
11.0
10.0
Hemoglobin (g/dL)
Control
Low
High
Treadmill experiment
capture
July
SeptemberI
October
NovemberII
JanuaryI
February
Bleeding session

Figure 1.

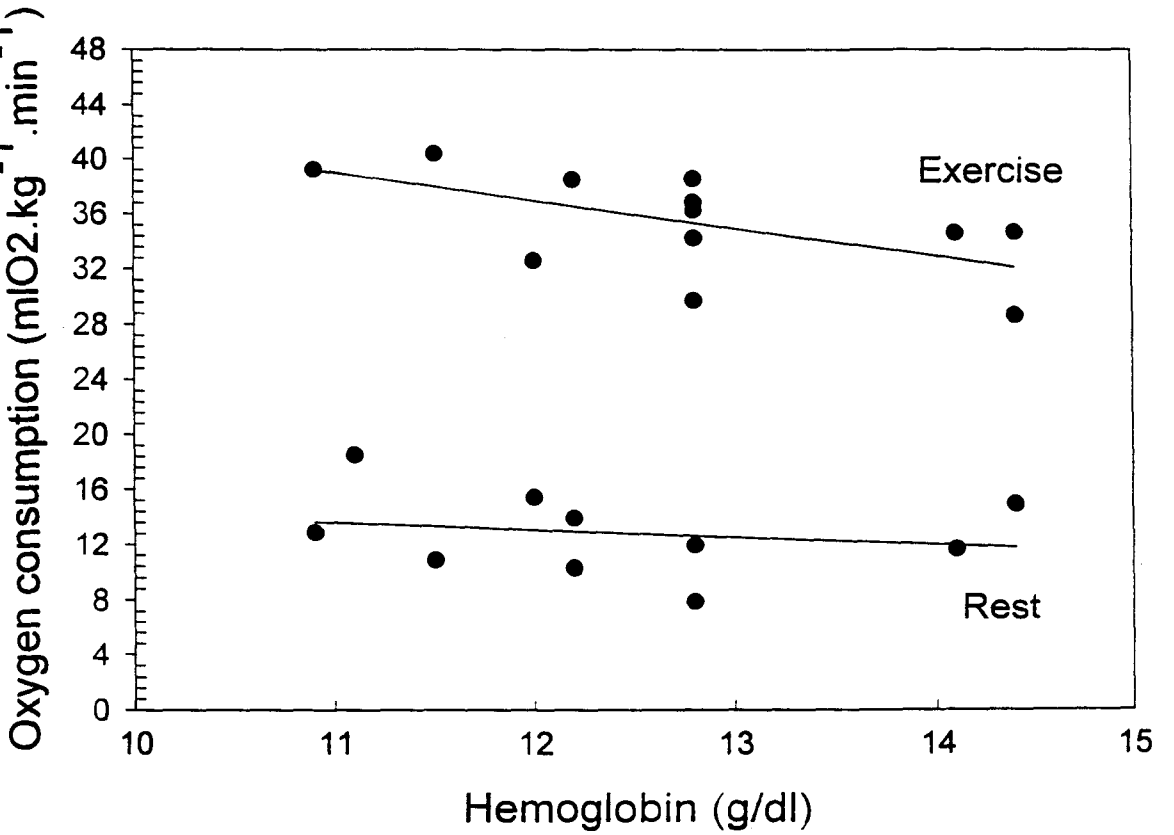
Oxygen consumption (mlO2.kg-1.min-1)
48
44
40
36
32
28
24
20
16
12
8
4
0
10
11
12
13
14
15
Hemoglobin (g/dl)
Exercise
Rest

Figure 2.

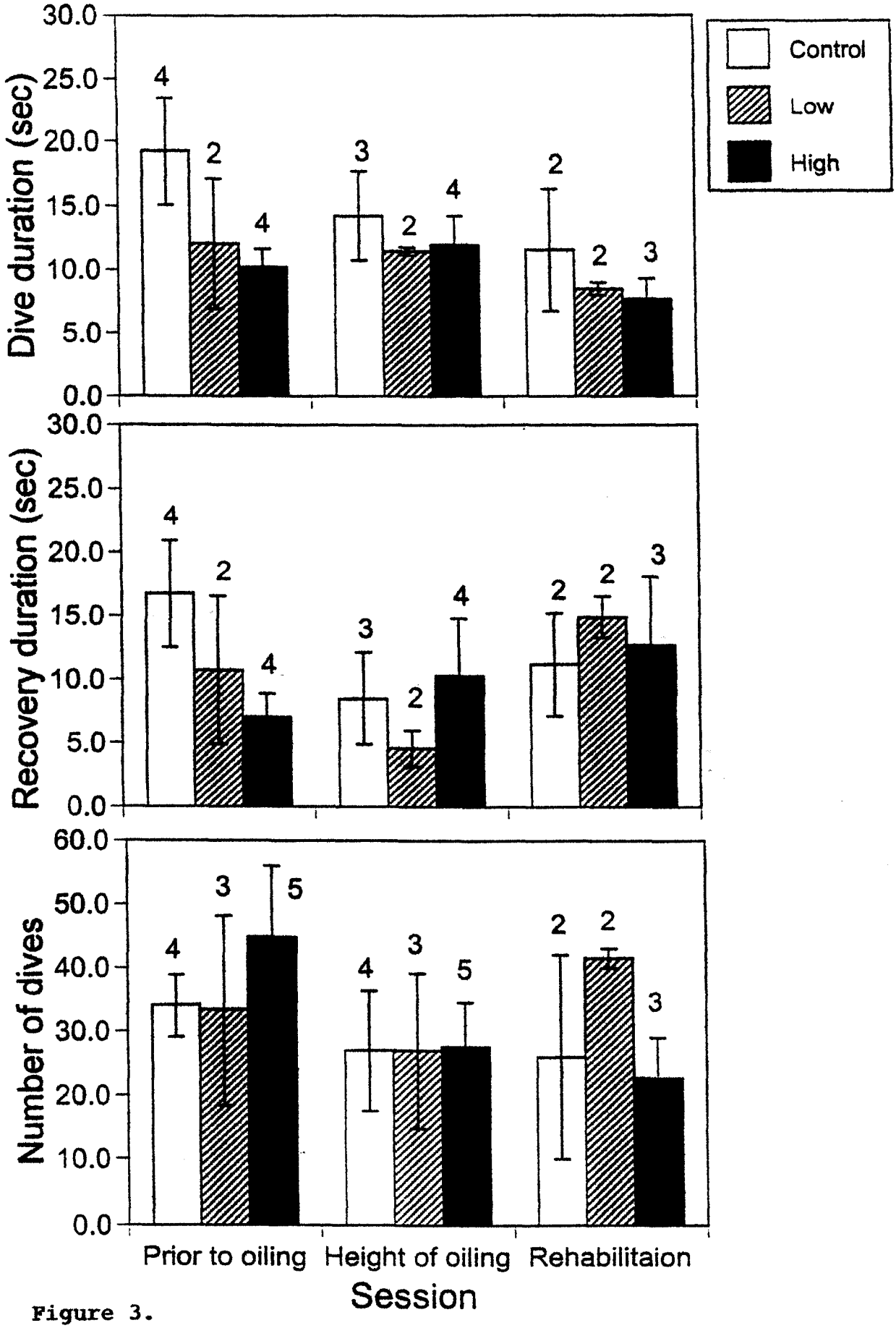
Control
Low
High
Dive duration (sec)
Recovery duration (sec)
Number of dives
Prior to oiling
Height of oiling
Rehabilitaion
Session

Figure 3.

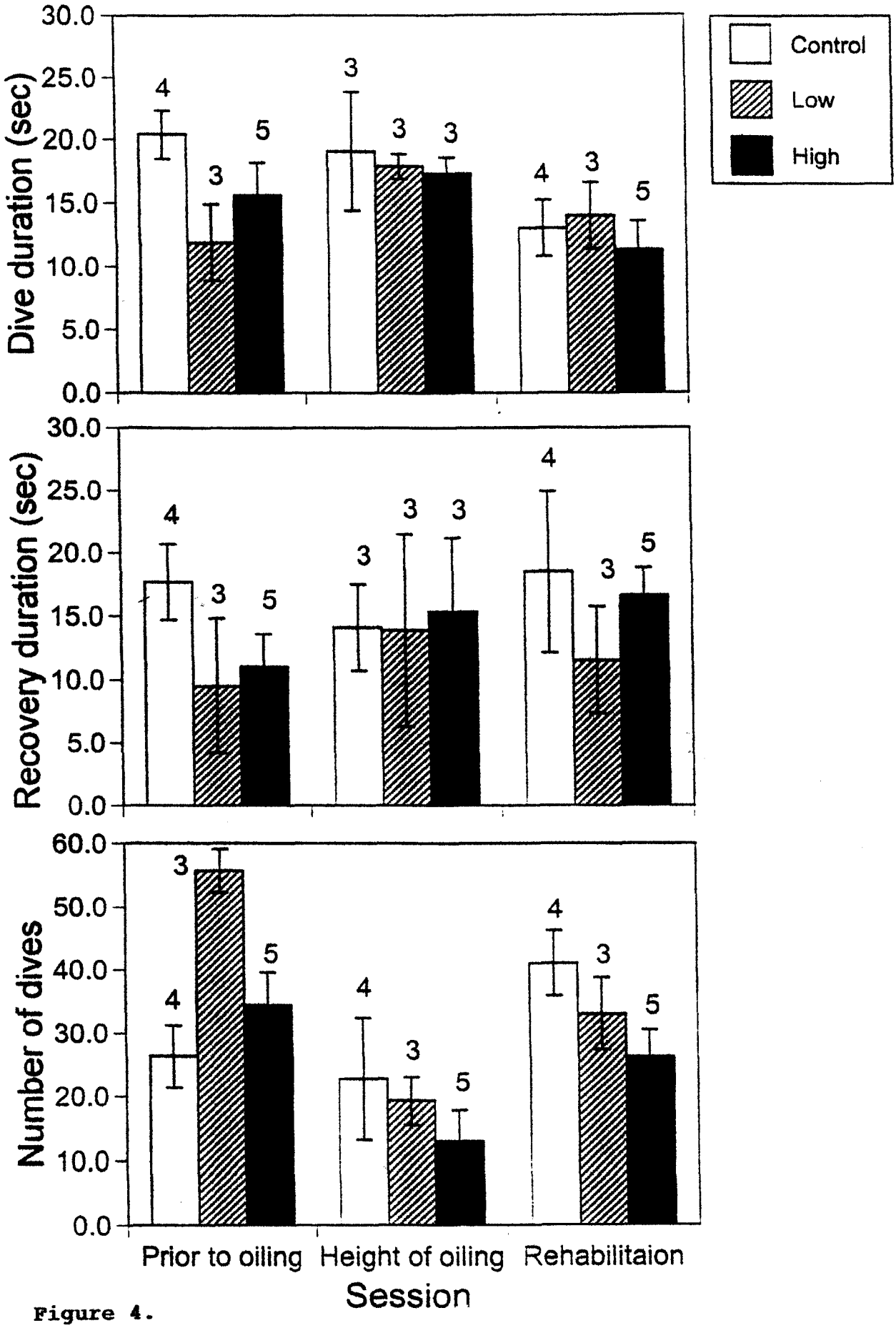
Control
Low
High
Dive duration (sec)
Recovery duration (sec)
Number of dives
30.0
25.0
20.0
15.0
10.0
5.0
0.0
60.0
50.0
40.0
30.0
20.0
10.0
0.0
Prior to oiling
Height of oiling
Rehabilitaion
Session

Figure 4.

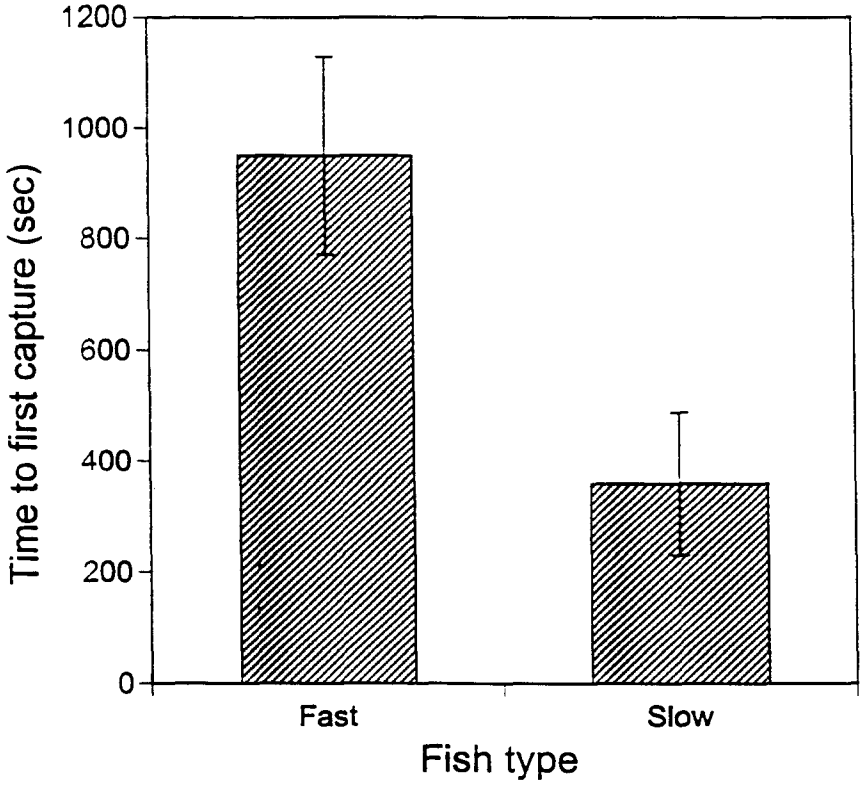
1200
1000
800
600
400
200
0
Time to first capture (sec)
Fast
Slow
Fish type

Figure 5.

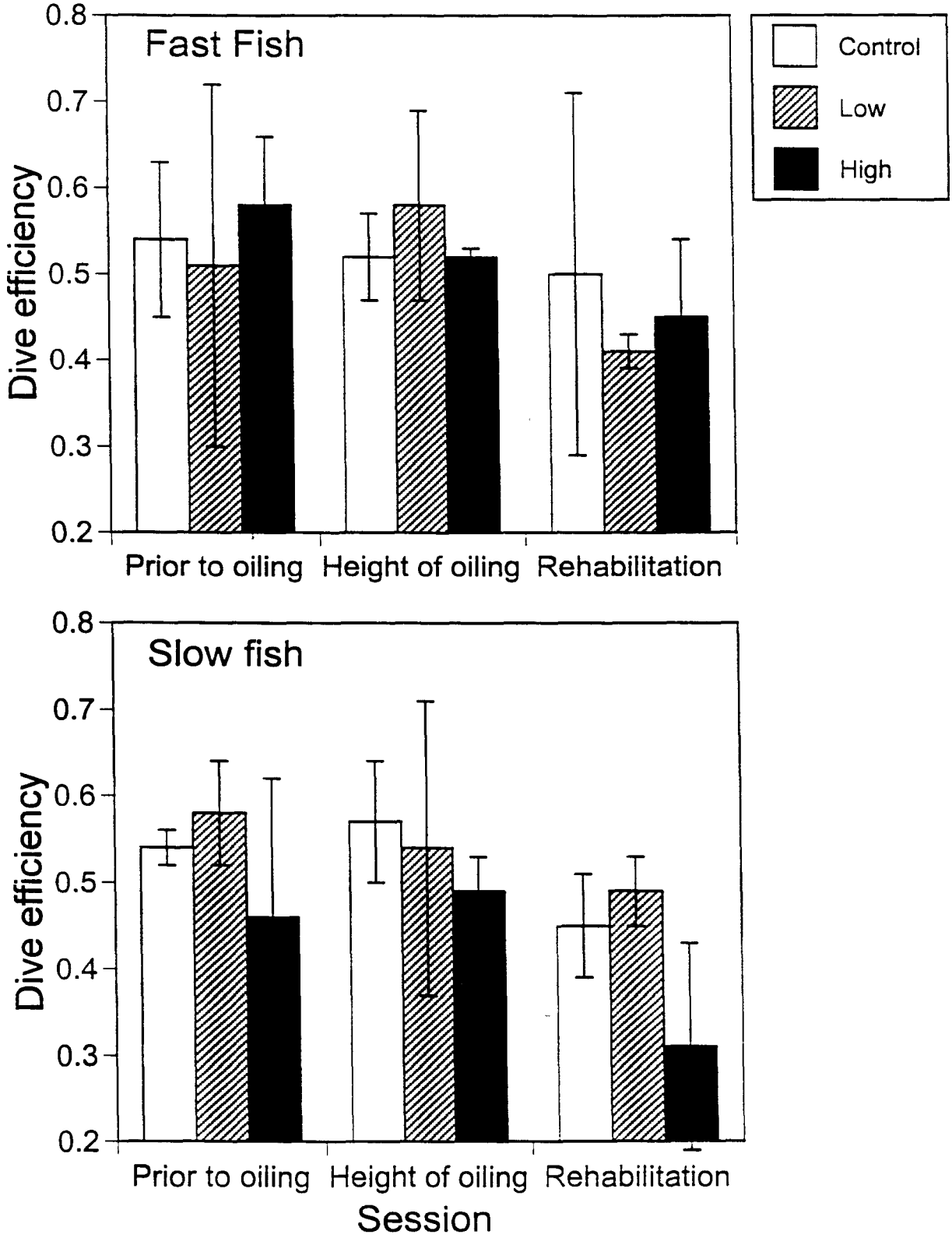
Fast Fish
Slow fish
Control
Low
High
Dive efficiency
0.8
0.7
0.6
0.5
0.4
0.3
0.2
Prior to oiling
Height of oiling
Rehabilitation
Session

Figure 6.

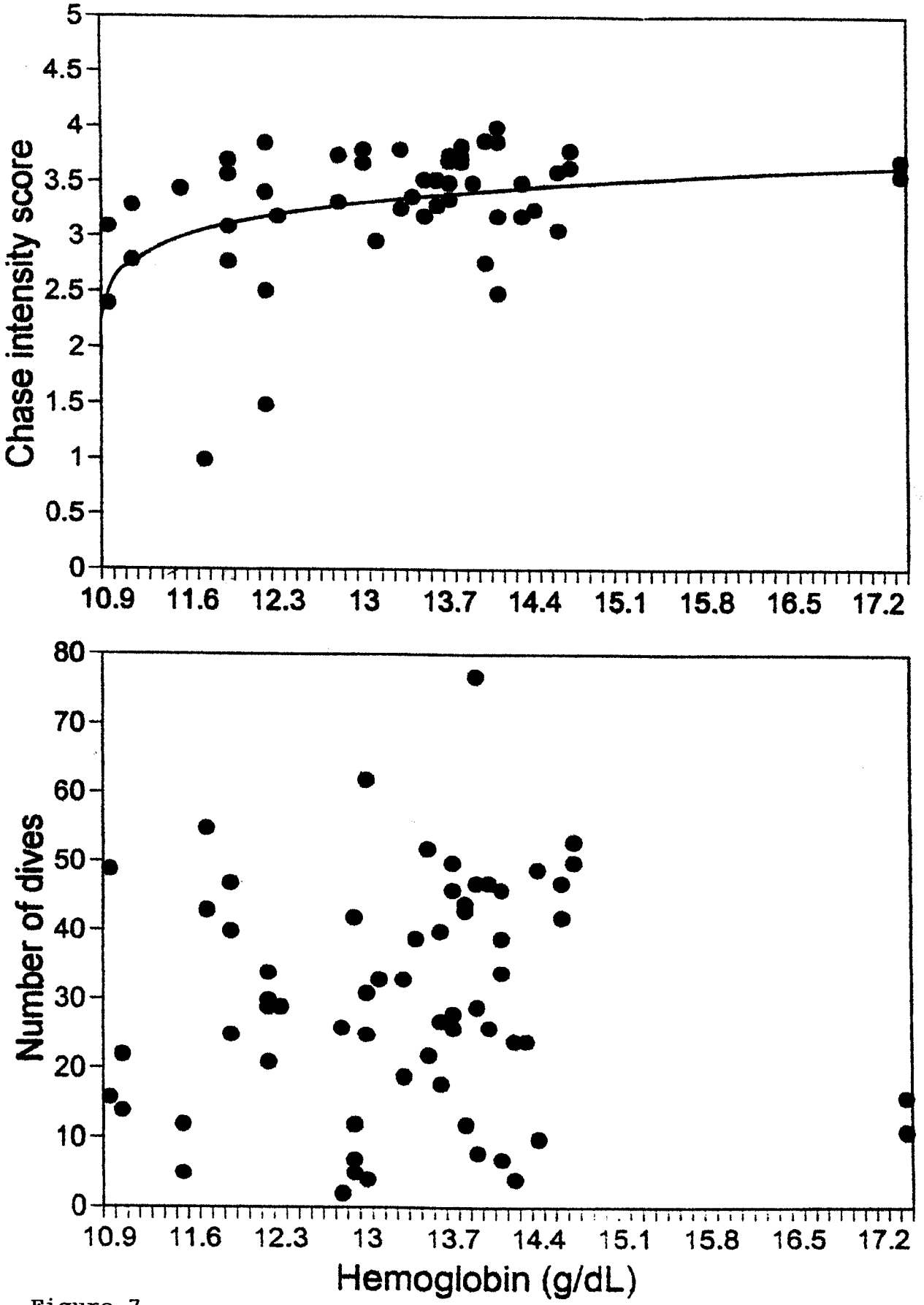
Chase intensity score
5
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
10.9
11.6
12.3
13
13.7
14.4
15.1
15.8
16.5
17.2
Number of dives
80
70
60
50
40
30
20
10
0
10.9
11.6
12.3
13
13.7
14.4
15.1
15.8
16.5
17.2
Hemoglobin (g/dL)

Figure 7.