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Response of River Otters to Experimental Exposure of Weathered Crude Oil: Fecal Porphyrin Profiles

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Profiles of porphyrins were characterized in fecal samples from river otters (*Lontra canadensis*) experimentally exposed to weathered crude oil to determine effects on heme synthesis. Fifteen male river otters were randomly assigned to three groups of five individuals each representing a control group, a low-dosage group that received 5 mg/kg body mass of oil per day, and a high-dosage group that received 50 mg/kg body mass of oil per day. Mean levels of coproporphyrin III (CoproIII) and protoporphyrin IX (ProtoIX) in fecal samples collected from all experimental river otters were higher throughout the experimental period than levels of CoproIII and ProtoIX in fecal samples collected previously at two field sites. No statistically significant differences in levels of CoproIII and ProtoIX were observed between treatment groups, although a trend of reduction in variability in CoproIII was observed in the low- and high-dose groups. We found no relation between levels of CoproIII and ProtoIX, suggesting that the process of disruption that leads to oxidation of the precursors of porphyrins is probably nonlinear. Our results also indicate that the interaction between oiled induced reduction in hemoglobin levels and induction of CYP1A1 corresponded with significantly lower levels of ProtoIX in the fecal samples, possibly representing high demand for ProtoIX. Therefore, while this experiment does not support the use of porphyrin profiles as an individual biomarker, it does suggest that the latter may be valuable when a weight of evidence is used in an ecotoxicological risk assessment in which the interactions between several biomarkers are explored.

Introduction

Porphyrins, oxidized intermediates of the heme biosynthetic pathway, are excreted in urine and feces when produced in excess (*1*). Alterations of enzymatic steps within the heme pathway may lead to accumulation of preceding porphyrin precursors and an elevation in their subsequent excretion (*2*). Such alterations may lead to significant changes in porphyrin profiles and patterns in animal excreta (*3*). Porphyrins following a biliary route of excretion (*4*) may be quantified readily in fecal samples with high-performance liquid chromatography (HPLC) separation and fluorescence detection (*5*). Changes in levels of porphyrins, including uroporphyrin (Uro), coproporphyrin III (CoproIII), and protoporphyrin IX (ProtoIX), can therefore serve as useful biomarkers of the effects of contaminants on heme metabolism and have been used in several toxicological studies in both laboratory and field settings (*6*−*13*).

Field studies designed to assess the effects of oil exposure in river otters (*Lontra canadensis*) following the *Exxon Valdez* oil spill in Prince William Sound, AK, demonstrated physiological and ecological responses consistent with contaminant exposure in animals inhabiting oiled areas of the Sound (*13*−*18*). Although those studies provided a wealth of information that contributed to a health assessment for river otters inhabiting oiled areas of Prince William Sound, as field studies, they could not establish a dose−response relationship. Such a relationship is central to any toxicological model linking specific contaminants with observed effects (*19*, *20*). Thus, in 1998, a controlled dosing experiment was designed to evaluate the effects of Alaska North Slope weathered crude oil administered to river otters through their diet. Analysis of a suite of biomarker measurements and behavioral observations were conducted to provide an assessment of the effects of weathered oil on the health of river otters (*21*−*23*). These studies documented a significant reduction in hemoglobin levels and induction of cytochrome P4501A1 (CYP1A1) in the oil-exposed river otters during the dosing period (*21*−*23*).

Information on the relation between weathered crude oil and disruption of the heme biosynthetic pathway is sparse. Studies on avian responses to PCBs demonstrated that exposure to this environmental pollutant resulted in accumulation of porphyrins in excreta of treated animals as compared with controls (*8*). Lorenzen et al. (*24*) described polyhalogenated aromatic hydrocarbon (PHAH)-mediated porphyrin accumulation and induction of cytochrome P4501A (CYP1A) in chicken (*Gallus domesticus*) embryos. Similarly, highly carboxylated porphyrins were documented in herring gull chicks (*Larus argentatus*) following exposure to PHAHs (*9*). In addition, several studies indicated changes in prophyrin profiles in invertebrates exposed to PHAH-type compounds (*3*, *25*). In our field studies in Prince William Sound from 1990 to 1996, we observed higher levels of CoproIII in fecal samples of river otters from oiled sites as compared with nonoiled sites (*13*, *18*). We designed the present study to evaluate the use of fecal porphyrin profiles as a nondestructive biomarker for exposure to weathered oil in river otters. We hypothesized that exposure to weathered oil would result in higher levels of CoproIII in fecal samples of river otters receiving doses of oil during the dosing period, similar to the higher levels we observed in our field study. In addition, we hypothesized that a disruption to the heme biosynthetic pathway would influence the relation between levels of CoproIII and ProtoIX in the excreta and would result in changes in profiles of excreted porphyrins. Additionally, because reduction in hemoglobin levels and induction of CYP1A1 would likely result in high demand on ProtoIX, we investigated the effects of these two variables on levels of ProtoIX in otter feces.

Methods

Experimental Conditions. Fifty-one river otters were live-captured in northwestern Prince William Sound, AK, from late April to late May 1998, with No. 11 Sleepy Creek leg-hold

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traps (*26*). Of these, 15 young adult males (1−4 yr old; body mass 10.2 ± 0.3 kg, mean ± SE) were transferred to the Alaska Sealife Center (ASLC), Seward, AK. The otters were held in captivity from May 1998 to March 1999. During this period, the animals were fed frozen fish on a daily basis, and their diet was supplemented with live fish (*21*). All frozen fish were obtained from commercial harvest in the Gulf of Alaska and from Eastern Greenland. Live fish were obtained from the inner waters of Resurrection Bay, AK (*21*). B-complex vitamins (0.5 mL) were injected intramuscularly during sampling sessions, which occurred every 3 weeks. Minerals were provided continuously in the form of a mineral block (*21*).

The experimental river otters were allowed to acclimate to the conditions of captivity for approximately 90 days prior to oil dosing. At the end of the acclimation period, otters were randomly assigned to three experimental groups of five individuals each: (i) a control group that received no oil; (ii) a low-dosage group that received 10 mg/kg every other day (5 mg/kg body mass per day); and (iii) a high-dosage group that received 100 mg/kg every other day (50 mg/kg body mass per day). North Slope crude oil obtained from Williams Alaska Petroleum, Inc., Fairbanks, AK, was weathered in the laboratory by continuous mixing with seawater for 10 days at 25 °C. The weathered oil was separated from the water and administered to the river otters in gel capsules hidden in fish every other day throughout the dosing period. Feeding of oil lasted 100 days from August 21 to November 28, 1998. Data collection continued for an additional 100 days of rehabilitation. Additional details on administration of oil are provided by Ben-David et al. (*21*−*23*).

Prior to exposure to oil, a series of blood and tissue samples were collected (June 29−30, and August 15−16, 1998) from each individual otter for analysis of several biomarkers including levels of hemoglobin and CYP1A1 (*22*, *23*). Blood and tissue sampling continued from August 15, 1998, until January 12, 1999, every 3 weeks. An additional sampling session occurred on February 22−24, 1999. Following anesthesia, a 10-mL blood sample was collected from the jugular vein for hemoglobin analysis, and a 3-mm skin sample was collected from the triceps on the left front limb for CYP1A1 evaluation (*22*, *23*). All methods were approved by Independent Animal Care and Use Committees at the University of Alaska Fairbanks (97-14) and the Alaska Sealife Center (98-002) and were in keeping with practices approved by the American Society of Mammalogists (*27*).

Sample Collection and Preparation. Fecal samples from each experimental river otter were collected during the week preceding a sampling session of blood and tissues. Fecal samples were collected every 3 weeks from June 29, 1998, until January 12, 1999. An additional sampling session occurred on February 22−24, 1999. Fecal samples deposited by river otters, individually identified through an observation window, were collected within 30 min of deposition; placed in Whirlpak bags; and frozen at −70 °C until analysis. Fecal samples representing the field sites in Prince William Sound and Southeast Alaska (Figure 1) were collected from specific locations termed latrines, where river otters socialize and deposit feces and anal secretions (*28*). The fecal samples were placed in plastic bags and stored in a freezer at −70 °C. Prior to porphyrin extraction, fecal samples were lyophilized for 24 h.

Expression of CYP1A1 was assessed with immunohistochemical analysis of CYP1A1 protein in skin biopsies at the Woods Hole Oceanographic Institution (*23*). Standard 5-μm sections of tissue were stained using an indirect peroxidase stain (Universal Immunoperoxidase Staining Kit (Murine), Signet Laboratories, Inc., Dedham, MA) with the monoclonal antibody MAb 1-12-3 and peroxidase-labeled goat anti-mouse IgG secondary antibody (*23*). 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. 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, Woods Hole Oceanographic Institution, personal communication). Specific staining by MAb 1-12-3 was evaluated by light microscopic examination of the stained sections scored for intensity of staining and for occurrence of staining. A stain index was computed by multiplying the intensity and occurrence for scale from 0 to 16 (*23*). Hemoglobin levels were determined as part of complete blood counts performed with a Stack-S whole blood analyzer (Coulter, Miami, FL). Samples were analyzed at Quest Diagnostics Incorporated (Portland, OR). For details see refs *21*−*23*.

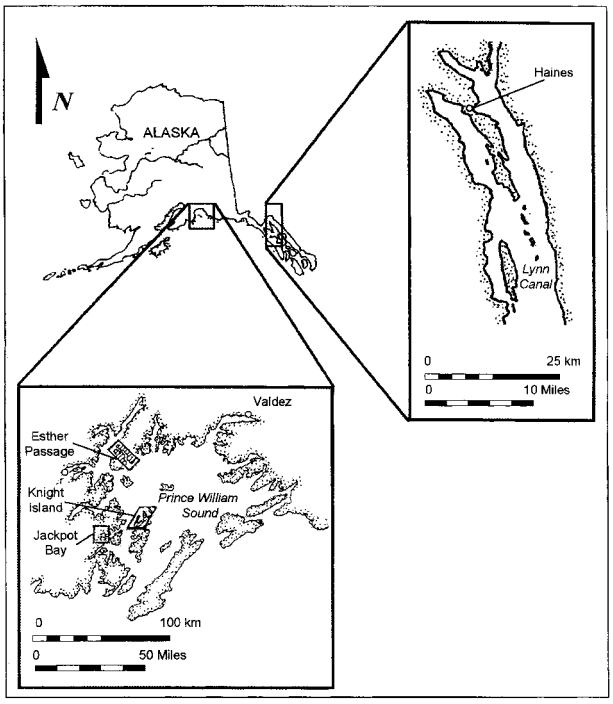

FIGURE 1. Field locations in Prince William Sound and Southeast Alaska where feces samples of river otters were collected at latrine sites for fecal porphyrin analysis.

Porphyrin Extraction and Quantification. Separation of porphyrins was facilitated by an HPLC system (Rheodyne Corp., Cotati, CA) described in detail by Taylor et al. (*18*, *29*). Porphyrin extraction from the fecal samples was accomplished with a modification of the procedure described by Bowers et al. (*30*). Porphyrins were extracted with HCl, concentrated on Sep-Pak cartridges (Waters Corp., Milford, MA), and eluted into cryogenic tubes with a polar solvent that was evaporated to produce a dry residue containing the porphyrins. The dry residues containing the porphyrins were stored frozen at −70 °C. For HPLC analysis, the dry residues were reconstituted in 100 μL of 6 N HCl and injected directly into the HPLC system. A Security Guard cartridge system (Phenomenex Corp., Torrance, CA) was relied upon to prevent deterioration of the analytical column. The HPLC method used (*29*) was a modification of the procedure described by Kennedy and James (*31*). Separation of porphyrins was facilitated with a 6-min gradient elution and a two-component mobile phase consisting of ammonium acetate (1.0 M, pH 5.16), and 100% methanol. The concentration of porphyrins in each fecal sample was calculated with a seven-point calibration curve (0.0−3.0 μM) developed with solutions

TABLE 1. Mean Concentrations (±SE) of Porphyrins Measured in Fecal Samples Collected from River Otters Experimentally Exposed to Weathered North Slope Crude Oil at the Alaska Sealife Center in Seward and the Mean Levels (±SE) of Porphyrins in Fecal Samples Collected from Three Different Field Sites in Alaska[a]

	concentration (nmol/g[b]; mean ± SE)					
porphyrin	control ($n = 5$)	low-dosage ($n = 5$)	high-dosage ($n = 4$)	Jackpot Bay[c] ($n = 23$)	Lynn Canal[d] ($n = 24$)	Knight Island[e] ($n = 22$)
UroI	0.03 ± 0.01	0.02 ± 0.01	0.02 ± 0.03	0.18 ± 0.04	0.04 ± 0.02	0.14 ± 0.03
CoproIII	1.31 ± 0.59	0.95 ± 0.15	1.13 ± 0.32	0.54 ± 0.11	0.50 ± 0.10	1.13 ± 0.20
ProtoIX	4.71 ± 0.83	4.95 ± 0.87	4.48 ± 1.65	2.31 ± 0.28	1.74 ± 0.35	0.64 ± 0.28

[a] Samples from the experimental otters were collected during the first three sampling sessions of the experimental period prior to the administration the oil. [b] Dry fecal weight. [c] Reference site in Prince William Sound, AK. [d] Reference site in northern Southeast Alaska. [e] Oiled site in Prince William Sound, AK.

of porphyrin standards (Porphyrin Products; Logan, UT) dissolved in 6 N HCl. For additional details see refs *18* and *29*.

Statistical Analysis. A repeated measures multivariate analysis of variance model (MANOVAR; PROC GLM; SAS, SAS Institute, Cary, NC; *32*) was used to evaluate differences in CoproIII and ProtoIX excretion between treatment groups of river otters throughout the experimental period. This test, however, may be conservative when multiple samples for each treatment (i.e., prior to oiling, during oil administration, and rehabilitation) are used. Therefore, we employed a *K* nearest-neighbors randomization test (*33*) to test for differences in distributions of CoproIII and ProtoIX excretion between the dosing periods and nondosing periods for all three treatment groups as well as between the control and high-dosage groups during the dosing period. One-tailed tests were conducted to test for differences in porphyrin excretion between treatment groups. Results obtained from one river otter from the high-dosage group were excluded from statistical analysis because that animal was involved in a surgical procedure during the middle of the dosing period. The high-dosage group was therefore represented by 4, instead of 5, individuals.

Regression analysis (PROC GLM; SAS; *32*) was used to evaluate relations between values of CoproIII and ProtoIX to test whether increases in one porphyrin will be associated with declines in another. Similarly, effects of hemoglobin levels and induction of CYP1A1 on levels of ProtoIX were evaluated using analysis of variance (ANOVA; PROC GLM; SAS) with hemoglobin and CYP1A1 values as factors and animal ID as covariate. For all statistical tests, significance level (α) was set at 0.05.

Results

Porphyrin profiles in fecal samples collected from the 15 experimental river otters were characterized primarily by UroI, CoproI, CoproIII, and ProtoIX (Table 1). Levels of CoproIII and ProtoIX were higher in fecal samples collected from the control group during the first three sampling sessions representing the acclimation period than in fecal samples collected from two reference field sites (Table 1; Figure 2). Similarly, levels of CoproIII and ProtoIX were higher in control group samples as compared with the two reference field sites during the entire experimental period (Table 1; Figure 2). Also, the mean levels of CoproIII in fecal samples collected from the control river otters during the first three sampling sessions were similar to the mean levels of CoproIII in fecal samples collected from an oiled field site in Prince William Sound 1 year following the *Exxon Valdez* oil spill (Knight Island; Table 1). Levels of UroI were lower in fecal samples collected from the experimental river otters than in fecal samples collected at one reference site (Jackpot Bay) but not from the second field location (Lynn Canal; Table 2).

High variability in porphyrin excretion characterized all treatment groups for each sampling session, particularly for ProtoIX (Figure 2). No statistically significant differences in levels of CoproIII or ProtoIX were detected between treatments groups (MANOVAR, $F = 0.177$, $P = 0.990$, df = 22, 2, and $F = 0.720$, $P = 0.730$, df = 22, 2, respectively). Likewise, a *K* nearest-neighbors randomization test detected no differences in distribution of CoproIII and ProtoIX between the dosing and nondosing periods for the control, low-dosage, and high-dosage groups ($P = 0.999$, 1.000, and 0.439, respectively) or between the control and high-dosage groups during the dosing period ($P = 0.715$). Regression analysis revealed no relation between levels of CoproIII and ProtoIX for any of the treatment groups (Figure 3; $P = 0.124$, $R^2 = 0.015$).

Despite the lack of significant differences in levels of CoproIII and ProtoIX between treatment groups, a trend of reduction in variability in values of CoproIII can be observed for both dosage groups during the dosing period (Figure 2). In addition, a trend of declining values of ProtoIX can be detected in the high dose group as compared with the low-dose and the control groups during the dosing period (Figure 2). Analysis of variance revealed that the interaction between an increase in expression of CYP1A1 and the decrease in circulating hemoglobin levels corresponded with a significant decline in excreted ProtoIX in feces of river otters (Figure 4; ANOVA, overall model, $P = 0.057$; hemoglobin, $P = 0.086$; CYP1A1, $P = 0.297$; interaction between hemoglobin and CYP1A1, $P = 0.039$).

Discussion

The development of effective biomarkers for environmental xenobiotics depends on the ability to describe quantitative, predictable, dose−response relations between exposure and the biological indicator (*20*). Although several studies documented accumulation of porphyrins in excreta of vertebrate and invertebrate animals in response to pollution by metals, PCBs, and PAH-type compounds (i.e., MC-type agents), and in areas with known spills of crude oil (*3*, *8*−*11*, *13*, *18*, *24*−*25*), some of the mechanisms involved in the disruption of the heme biosynthesis have not been unequivocally described. Several studies documented the involvement of CYP1A compounds in oxidation of uroporphyrogen to uroporphyrin, resulting in high uroporphyria following exposure to MC-type agents (*34*−*36*). In addition, administration of 5-aminolevulinic acid (ALA; the precursor of porphyrins) causes uropophyria in iron-treated mice (*37*). Such reaction was absent in CYP1A2(−/−) mice (*38*), suggesting that induction of CYP1As by MC-type agents has large effects on the heme biosynthetic pathway. Nonetheless, the mechanisms of inactivation of decarboxylases in the heme biosynthetic pathway following exposure to MC-type agents, the role of increases in body iron stores, or the effects of increases in demand for heme are still unknown (*38*−*39*), thus complicating the interpretation of results from field studies.

Higher levels of CoproIII and ProtoIX measured in the fecal samples collected from the experimental river otters but not observed in our field studies (Tables 1 and 2) were

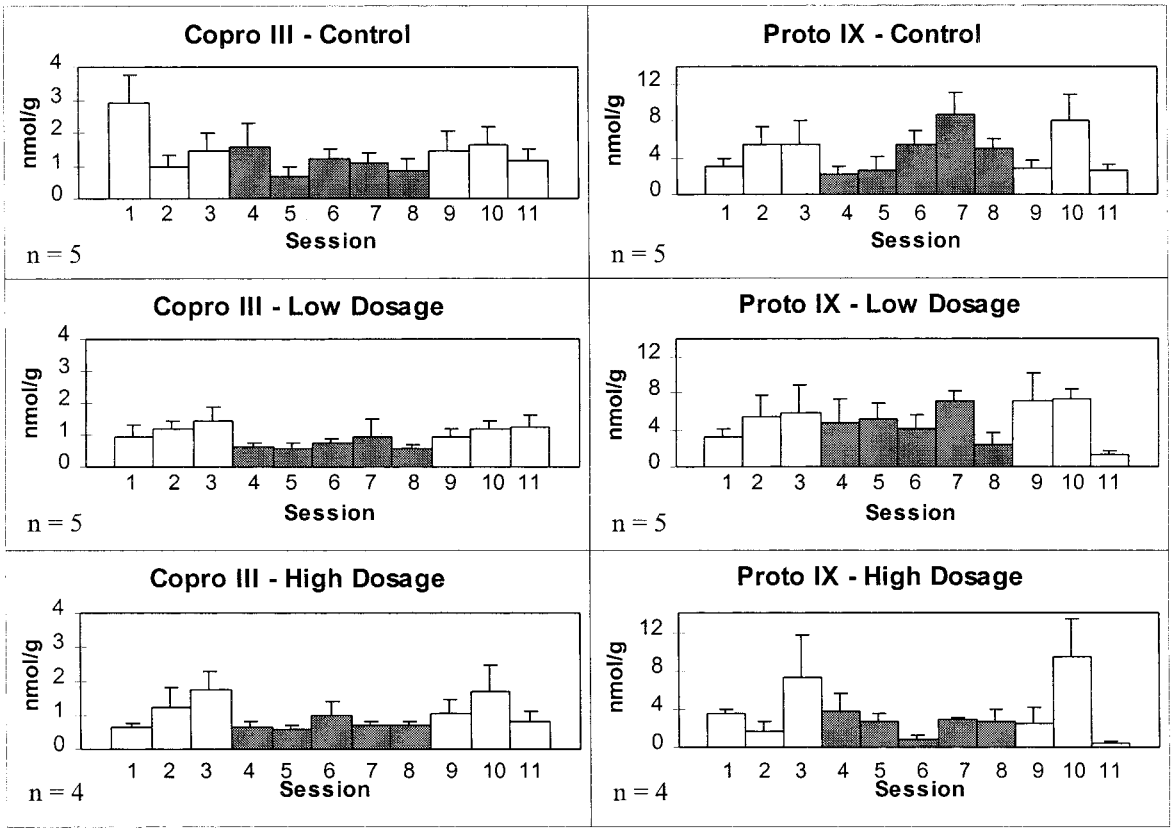

FIGURE 2. Mean levels (±SE) of coproporphyrin III (CoproIII) and protoporphyrin IX (ProtoIX) measured in fecal samples collected from river otters experimentally exposed to weathered North Slope crude oil. Otters were held in captivity at the Alaska Sealife Center in Seward, AK. Sessions 4−8 (gray bars) represent sampling sessions corresponding to the oil dosing phase of the experiment. No significant differences in CoproIII or ProtoIX levels were detected between treatment groups during the dosing period (MANOVAR, $P = 0.990$), although a trend of reduction in variability in levels of CoproIII in the dosed animals was observed.

TABLE 2. Percentage of Fecal Samples in Which Uroporphyrin I Was Detected and Concentration of Uroporphyrin I Detected in Fecal Samples Collected from River Otters Experimentally Exposed to Weathered North Slope Crude Oil and from Two Different Field Sites

location	fecal samples (%)	concn (nmol/g[a]; mean ± SE)
dosing experiment (n =161)	32	0.02 ± 0.01
Jackpot Bay[b] 1996 (n = 23)	78	0.18 ± 0.04
Lynn Canal[c] 1998 (n = 24)	38	0.04 ± 0.02

[a] Dry fecal weight. [b] Field site in Prince William Sound, AK. [c] Field site in northern Southeast Alaska.

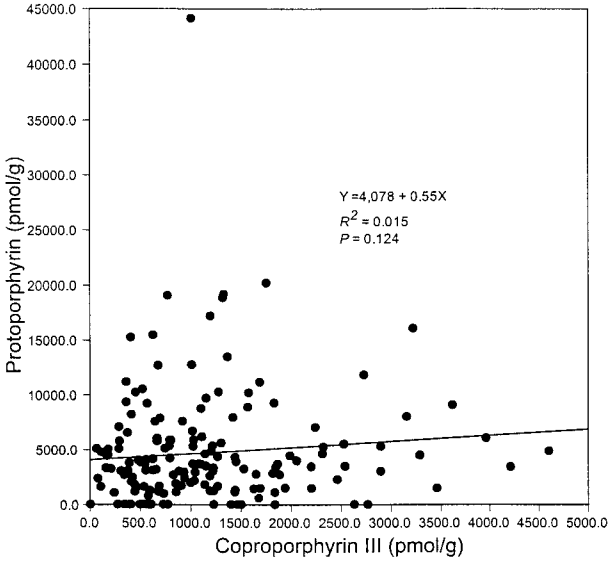

FIGURE 3. Relation between CoproIII and ProtoIX in fecal samples collected from river otters experimentally exposed to weathered North Slope crude oil. Regression analysis revealed no linear relation between levels of CoproIII and ProtoIX ($P = 0.124$, $R^2 = 0.015$), suggesting that a disruption to the heme biosynthetic pathway is nonlinear.

not likely caused by a higher quality of the experimental samples. The percentage of fecal samples containing detectable levels of ProtoIX, which is rapidly degraded by bacteria (*40*), was similar in samples from the experimental river otters (85%) and from the Lynn Canal reference site (79%, Figure 2; Table 1). Nearly 100% of the samples from all three sampling locations contained detectable levels of CoproIII, and the levels of CoproIII in samples collected from both the Lynn Canal and Jackpot Bay reference areas were similar. These results indicate that recovery of CoproIII may be less dependent on the age of the sample than ProtoIX. Conversely, the percent of fecal samples containing detectable levels of UroI as well as the mean levels of UroI in the experimental river otters were lower than those measured in the field studies (Tables 1 and 2).

Elevated excretion of CoproIII and ProtoIX in all the captive animals could have been influenced by dietary porphyrins. Given the stability of porphyrins to acid treatment, it is possible that the porphyrins detected in the otter feces originated in their food. Unfortunately, we did not examine porphyrin profiles in the fishes fed to the otters. Although the fishes we offered to the otters resembled their natural diet (*14*, *21*−*23*), the captive otters preferably

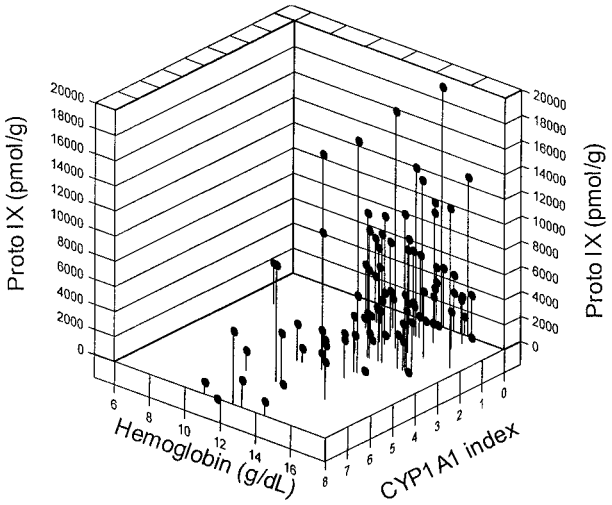

FIGURE 4. Relation between levels of hemoglobin (g/dL), CYP1A1 expression (staining index; *23*), and values of ProtoIX (pmol/g) in fecal samples collected from river otters experimentally exposed to weathered North Slope crude oil. Analysis of variance revealed that the increase demand for the heme molecule due to the interaction between an increase in expression of CYP1A1 and the decrease in circulating hemoglobin levels corresponded with a significant decline in excreted ProtoIX in feces of river otters (ANOVA, overall model, $P = 0.057$; hemoglobin, $P = 0.086$; CYP1A1, $P = 0.297$; interaction between hemoglobin and CYP1A1, $P = 0.039$).

consumed more of the pelagic fishes (i.e., salmon, *Oncorhynchus gorbuscha*; capelin, *Mallotus villosus*; and herring, *Clupea pallasii*), which are higher in lipids and proteins than intertidal fishes (*41*). Free-ranging river otters commonly consume more intertidal fishes then pelagic fishes (*14*). Thus, the influence of dietary porphyrins on fecal profiles in river otters is unclear and merits further investigation.

In contrast to our expectation, we detected no significant differences in porphyrin levels between treatment groups during the acclimation, dosing, or rehabilitation periods. Several factors may have contributed to the lack of observable differences in porphyrin levels between treatment groups in our study. It is possible that the diet we offered to the otters was contaminated with PAH-type xenobiotics because fishes were obtained from commercial harvests in the Gulf of Alaska and Eastern Greenland. Nonetheless, the likelihood that the diet, which was constant throughout the experiment, was contaminated is low. In a companion study, we documented low levels of CYP1A1 expression (near zero; *23*) in the captive river otters prior to the administration of oil as well as during the rehabilitation period, indicating that the diet was not contaminated. In contrast, levels of CYP1A1 in the dosed individuals during the exposure period were significantly higher and exhibited a dose−response (*23*).

Alternatively, we were unable to detect a porphyrin response to oil exposure because elevated excretion of CoproIII and ProtoIX, influenced by other factors such as a change in diet composition, reduction in activity levels, or other captivity-related physiological stress, may have masked more subtle changes in porphyrin excretion caused by effects of oil exposure. Indeed, changes in hepatic porphyrin excretion described by increases in the ratio of CoproIII to ProtoIX have been described in fasting animals (*42*). Also, the variability inherent in individual porphyrin excretion as indicated in Figure 2 and the individual variability in other biomarker responses to oil exposure in these captive otters (*22*, *23*) may have obscured the observation of any changes in porphyrin excretion. Thus, the high individual variability combined with the small sample sizes in each treatment group may have precluded the detection of oil-induced changes in porphyrin profiles in this study. The number of subjects in this experiment was determined by an a priori power analysis for other biomarkers in our companion study (*22*) and the need to minimize the impact of this project on wild populations of river otters in Prince William Sound. In retrospect, those sample sizes may have been too low for this experiment.

Interestingly, we found no relation between levels of CoproIII and ProtoIX. This lack of relation indicates that the process of disruption that leads to oxidation of the precursors of porphyrins is probably nonlinear. This nonlinearity may be caused in part by feedback induction of ALA synthase, which modulates the flux of intermediates through the heme biosynthetic pathway (*38*). In addition, this nonlinearity may be related to inhibition of decarboxylases activity and increased oxidation of porphyrin precursors. Although the negative effects of MC-type agents on activity of uroporphyrinogen decarboxylase are documented, the nature of the mechanism is still unclear (*39*). Similarly, little is known about the influence of MC-type agents on the activity of other enzymes in the pathway.

Our results indicate that the induction of CYP1A1 was not directly related to excretion of ProtoIX in otter feces (Figure 4). Jacobs et al. (*36*) determined that the catalytic effects of CYP1A2 on uroporphyrinogen oxidation were inhibited by specific antibodies against CYP1A2 but not antibodies against CYP1A1. This suggests that different CYP1As may have different oxidation functions in the heme biosynthetic pathway. Also, several studies documented species differences in effectiveness of CYP1As in uroporphyrinogen oxidation (*38*), suggesting that CYP1As may have different functions in different species. In addition, crude oil contains a mixture of PAH and non-PAH compounds (*22*) that in concert may have opposing effects on different enzymes in the heme pathway. Understanding of the interactions between PAH and non-PAH compounds of crude oil and the heme pathway in mustelids merits further investigation because animals belonging to this group are becoming recognized as sentinel species in the environment. Due to the potential complications from stress of captivity on wild animals, it may be beneficial to conduct such experiments on surrogate species such as domesticated mink (*Mustela vison*; *43*).

Our observation that an interaction between reduction in hemoglobin levels and induction of CYP1A1 was significantly related to levels of ProtoIX may represent an increased demand on ProtoIX, leading to lower levels of ProtoIX in the fecal samples (Figure 4). Custer et al. (*44*) documented a significant correlation between exposure to petroleum hydrocarbons, monoxygenases activity, and chromosomal damage to hematopoetic tissues in lesser scaup (*Aythya affinis*). Such chromosomal damage may be related to reduction in hemoglobin levels in animals exposed to petroleum hydrocarbons (*21*, *22*). Although we were unable to document a direct disruption in the heme biosynthetic pathway by crude oil, our data suggest that processes resulting in high demand for the heme molecule may have caused increased production of porphyrin precursors, potentially via ALAS modulation, and quick removal of compounds such as CoproIII. This is further supported by our observation that the percent of fecal samples containing detectable levels of UroI as well as the mean levels of UroI in the experimental river otters were lower than those measured in the field studies (Tables 1 and 2). Additional studies on the potential effects of reduction in hemoglobin levels and concurrent induction of CYP1A1 on porphyrin excretion in mammals will provide a better platform for interpretation of field data.

In conclusion, while this dosing experiment provided an unprecedented opportunity for evaluation of the effects of oil exposure on heme metabolism in river otters under

experimental conditions, our results emphasized the problems associated with high individual variability, low sample sizes, and study of complicated systems using single markers. This experiment does not support the use of porphyrin profiles as an individual biomarker because we were unable to demonstrate quantitative, predictable, dose−response relations between exposure and porphyrin profiles. Nonetheless, our results suggest that obtaining porphyrin profiles may be valuable when a weight of evidence approach is used in an ecotoxicological risk assessment in which the interactions between several biomarkers are explored. The establishment of a reliable baseline for porphyrin excretion in mustelids under different environmental and health conditions will be necessary for extrapolating from specific adverse health effects in individuals to effects observed at the population and ecosystem levels (*45*). The noninvasive nature of fecal sample collection, availability of rapid analytical techniques, and integration with measurements of other biomarkers warrants continued investigation.

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