



Profiles of Fecal Porphyrins in River Otters Following the *Exxon Valdez* Oil Spill

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Median levels of Coproporphyrin III (Copro III) in fecal samples of river otters (*Lontra canadensis*) collected from an oiled area in Prince William Sound, Alaska, USA, during 1990 were significantly higher than in samples collected from the same oiled area during 1996 ($p = 0.011$, one way analysis of variance), a nonoiled reference area in Prince William Sound during 1996 ($p = 0.002$) and a reference area in southeast Alaska during 1998 ($p = 0.004$). An overall test of significance that combined probabilities from the statistical analysis of this porphyrin study with those from other biomarker studies revealed a significant difference in physiological response of river otters between oiled and nonoiled areas of the Sound for 1990 ($p < 0.01$). We demonstrated that changes in levels of fecal porphyrins may serve as a biomarker that may contribute to a health assessment of wild river otters. © 2000 Elsevier Science Ltd. All rights reserved.

Keywords: porphyrins; biomarker; *Lontra canadensis*; Prince William Sound; Alaska; *Exxon Valdez*.

Introduction

Research following the *Exxon Valdez* oil spill in March 1989 has attempted to assess the biochemical and physiological effects within organisms resulting from chronic exposure to persistent levels of oil in the coastal marine environment. River otters (*Lontra canadensis*) serve as an excellent sentinel species for biomarker studies designed to assess effects of oil exposure because these mustelids occupy a high trophic position within the nearshore marine ecosystem and feed on intertidal and subtidal organisms (Ben-David *et al.*, 1998; Bowyer *et al.*, 1994). Furthermore, river otters are ubiquitous within Prince William Sound as well as the Gulf of Alaska (Bowyer *et al.*, 1995) and occur at densities of 20–80 animals per 100 km of shoreline (Testa *et al.*,

1994). River otters, therefore, may serve as effective indicators of the presence of oil in the coastal environment, its bioavailability and its potential effects on other coastal vertebrates.

River otters might be exposed to oil through their diet (Babcock *et al.*, 1990; Babcock and Short, 1996; Collier *et al.*, 1996; Meachum and Sullivan, 1990). River otters also may come in direct contact with oil remaining in sediments (Babcock *et al.*, 1990; Babcock and Short, 1996; Kvenvolden *et al.*, 1993) and subsurface seawater receiving oil from shoreline and sediment reservoirs of oil (Short and Harris, 1995). Consequently, river otters may be exposed to oil by grooming their pelage (Baker *et al.*, 1981); oil was recovered from the fur of river otters inhabiting oiled areas of Prince William Sound in 1997, eight years after the *Exxon Valdez* oil spill (Duffy *et al.*, 1999).

Several studies of biomarkers have demonstrated biochemical and physiological responses consistent with the effects of contaminant exposure in river otters inhabiting oiled sites within Prince William Sound. Levels of blood haptoglobins, interleukin-6 ir and a series of blood enzymes were elevated in river otters inhabiting oiled sites compared with nonoiled sites in 1991 (Duffy *et al.*, 1993, 1994a). River otters from oiled sites also had lower body mass compared with nonoiled sites in 1991 (Duffy *et al.*, 1993). Total porphyrins, measured spectrophotometrically in whole fecal extracts from river otters, were elevated in samples collected from oiled sites compared with nonoiled sites during 1990 (Blajeski *et al.*, 1996).

This study was designed to evaluate the effects of crude oil exposure on heme biosynthesis in river otters in Prince William Sound through the quantification of individual porphyrins in fecal samples. Porphyrins are oxidized intermediates of the heme biosynthetic pathway (Fig. 1) and follow a biliary or urinary route of excretion when produced in excess, depending on the specific porphyrin (Duffus and Worth, 1996). Porphyrins may subsequently be quantified in fecal samples with high-performance liquid chromatography (HPLC) separation and fluorescence detection (Lim, 1991). A

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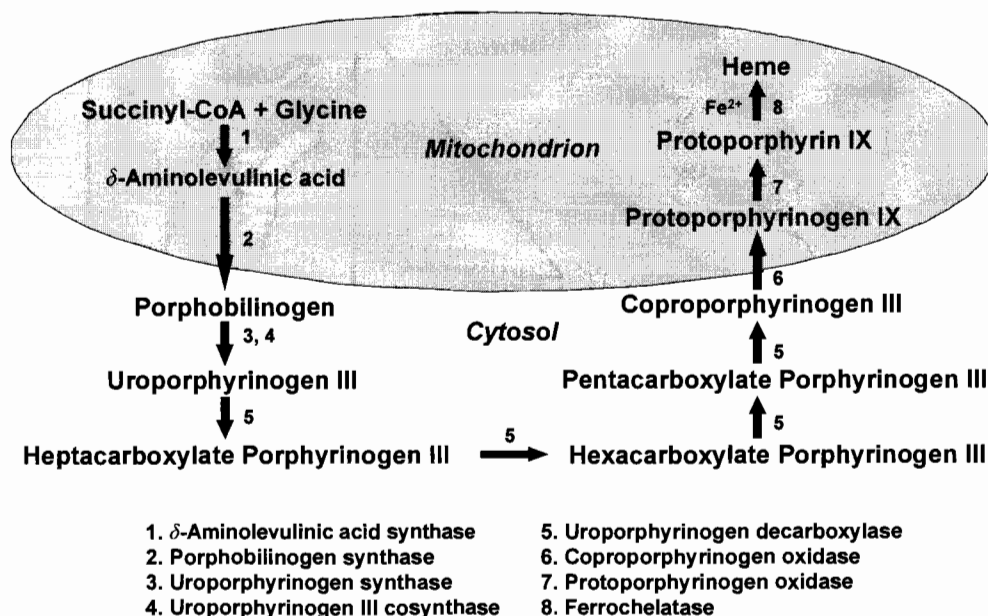


Fig. 1 The heme biosynthetic pathway.

change in the profile of porphyrin excretion, therefore, may serve as a useful biomarker indicating possible alteration of heme metabolism as a result of contaminant exposure (De Matteis and Lim, 1994). We hypothesized that the elevated levels of total porphyrins observed previously in river otters inhabiting oiled sites compared with nonoiled sites in Prince William Sound during 1990 (Blajeski *et al.*, 1996) could be attributed to an increase in the excretion of individual porphyrins, which may be caused by specific alterations of the heme biosynthetic pathway. We quantified levels of individual porphyrins with HPLC in river otters to determine if differences in patterns of fecal porphyrin excretion existed between river otters inhabiting oiled and nonoiled sites. We also hypothesized that as the availability of oil decreased over time, the patterns of porphyrin excretion would become similar in fecal samples from river otters inhabiting oiled and nonoiled sites. To determine if recovery was occurring over time, we compared patterns of porphyrin excretion in fecal samples of river otters collected from the same oiled area during 1990 and 1996. We also assessed recovery by combining the results of this porphyrin analysis with those of other biomarker studies to characterize temporal trends in physiological response.

Methods

Sample collection and preparation

Fecal samples were taken from the frozen archive at the Institute of Arctic Biology, University of Alaska Fairbanks, Fairbanks, Alaska, USA. The fecal samples were collected in Prince William Sound from the same oiled site on northern Knight Island (60°30'N, 147°40'W) during 1990 and 1996, from a nonoiled ref-

erence site in Esther Passage (60°53'N, 147°55'W) during 1990, a nonoiled reference site in Jackpot Bay (60°25'N, 147°30'W) during 1996 and in Lynn Canal, south-east Alaska (59°10'N, 135°20'W) during 1998 (Fig. 2). One hundred twenty fecal samples representing specific locations called latrine sites, where river otters socialize and deposit feces and anal secretions (Ben-David *et al.*, 1998), were analysed; 22 from Knight Island during 1990, 28 from Knight Island during 1996, 23 from Esther Passage during 1990, 23 from Jackpot Bay during 1996 and 24 from Lynn Canal during 1998. Although river otters will deposit feces at several latrines (Testa *et al.*, 1994), we minimized the likelihood of obtaining multiple samples from the same individual by spreading our sampling across large areas (80 km of shoreline at Knight Island and Esther Passage, 55 km at Jackpot Bay and 50 km at Lynn Canal). Moreover, only one fecal sample was analyzed from each latrine site and samples were collected over a short period of time (24–48 h). Thus, we assumed that each sample represented a separate individual, or that any remaining bias was small. The fecal samples were placed in plastic bags and stored in a freezer at -70°C . Prior to porphyrin extraction and HPLC analysis, the fecal samples were lyophilized for 24 h.

Porphyrin analysis

Extraction of porphyrins from the fecal samples was accomplished with a modification of the procedure described by Bowers *et al.* (1992). Porphyrins were extracted with HCl, concentrated on 500 mg trifunctional C-18 (tC-18) solid-phase extraction columns (Sep Paks®; Waters Corp.) attached to a Supelco Visiprep vacuum manifold (Supelco Corp., Bellefonte, PA, USA) and eluted into cryogenic tubes with a polar solvent that

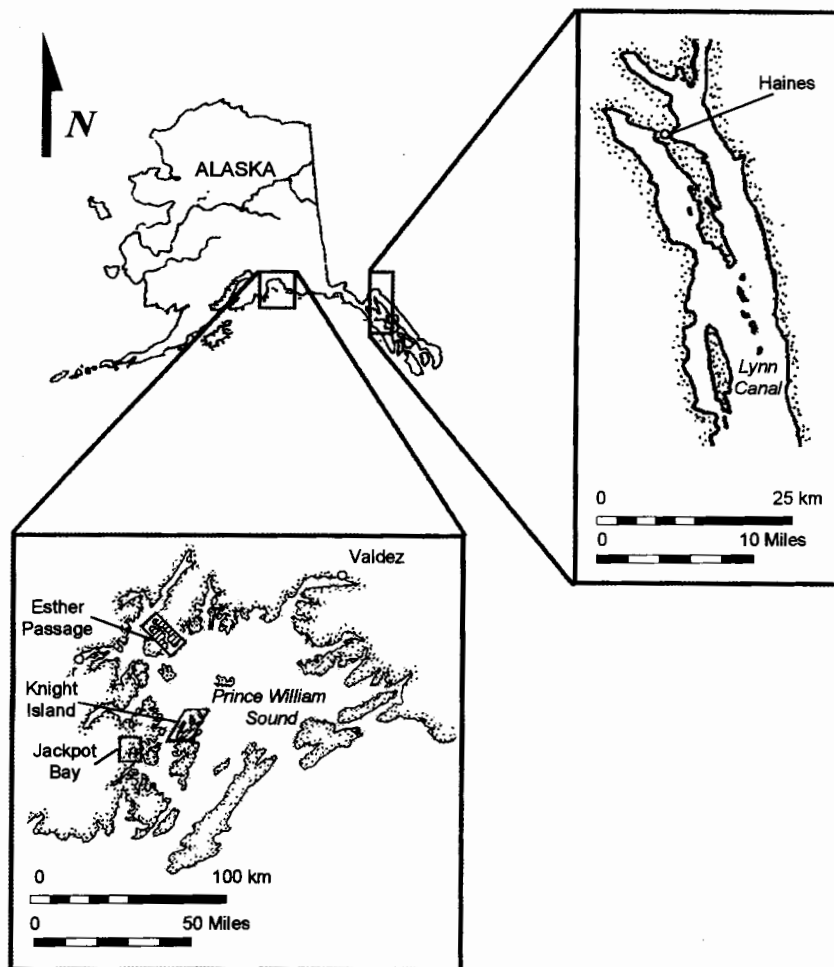


Fig. 2 Sampling locations in Prince William Sound, Alaska, USA, and Lynn Canal, Alaska, USA, for fecal porphyrin analysis in river otters.

was evaporated to produce a dry residue containing the porphyrins. Five ml of 6 N HCl were added to 1.0 g of dry fecal material and the sample was macerated with a glass rod for approximately 1 min. The sample was mixed with a bench top vortex for 1 min, sonicated in a water bath for 5 min and mixed again for 1 min. Five ml of sodium phosphate buffer (0.01 M, pH 3.5) were added to the sample. The sample was mixed again for 1 min and centrifuged for 10 min at 4000 rpm. The coarse pellet was removed from the centrifuge tube and the sample was centrifuged again for 10 min at 4000 rpm.

Sep Pak® cartridges were prepared by washing first with 7 ml of acetonitrile followed by 7 ml of sodium phosphate buffer (0.01 M, pH 3.5). Eight ml of the fecal supernatant were delivered to the Sep Paks® and allowed to gravity feed. The Sep Paks® were washed with 3 ml of sodium phosphate buffer (0.01 M, pH 3.5) to facilitate complete delivery of the supernatant to the Sep Pak®, followed by 7 ml of sodium phosphate buffer (0.01 M, pH 7.5) to enhance recovery of the porphyrins. The concentrate containing the porphyrins at the top of the Sep Paks® was eluted into 5 ml cryogenic centrifuge tubes with 1 ml of acetonitrile, followed by 0.5 ml of

acetonitrile: 1.0 N HCl (1:1, v/v) and finally 1 ml of acetone under vacuum. The eluates were placed in a water bath at 55°C and evaporated to a dry residue under a stream of air. The dry residues containing the porphyrins were stored frozen at -70°C. For HPLC analysis, the dry residues were reconstituted in 500 µl of 6 N HCl and injected directly into the HPLC system. Although the recovery varied with the individual porphyrin, the recovery or average extraction efficiency for all porphyrins was 75%. Sample spike recovery averaged 89%.

HPLC analysis

Separation of porphyrins was facilitated by an HPLC system consisting of two Waters 510 pumps (Waters Corp., Milford, MA, USA) a Rheodyne 7125 injector valve equipped with a 5 µl injection loop (Rheodyne Corp., Cotati, CA, USA) and a 3 cm by 0.46 cm Luna C-18 column equipped with a Security Guard cartridge system (Phenomenex Corp., Torrance, CA, USA). Porphyrins were detected with a McPherson FL-748 fluorescence detector equipped with a 405 nm excitation cut-off filter, a 620 nm interference emission filter with a

10-nm bandwidth and a red sensitive photomultiplier tube (McPherson Corp., Chelmsford, MA, USA). Baseline 810 and Maxima 820 chromatographic software (Waters Corp.) were used for system control and peak integration. The HPLC method used was a modification of the procedure described by Kennedy and James (1993). 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) as solvent A and 100% methanol as solvent B. Gradient elution commenced upon injection and proceeded from 25% B at time zero to 50% at 1.0 min, then to 95% B at 4.0 min, remained at 95% B for 1.5 min and returned to 25% B at 6 min. The column was allowed to re-equilibrate for 5 min at 25% B before the next injection. The concentration of porphyrins in each fecal sample was calculated with a seven-point calibration curve (0.0–3.0 μM) developed with solutions of porphyrin standards (Porphyrin Products, Logan, Utah, USA) dissolved in 6 N HCl.

Statistical analysis

A one-tailed analysis of variance (ANOVA; PROC GLM/CONTRASTS, SAS, SAS Institute, Cary, NC, USA) was used to evaluate Copro III concentrations between oiled and nonoiled locations and between sampling years. Data on Copro III was ranked (Conover and Iman, 1981) prior to performing the ANOVA (PROC RANK, SAS). To test for overall significance in biomarker response described by levels of Copro III as well as levels of haptoglobins and body mass measurements reported in previous studies of river otters inhabiting oiled and nonoiled areas of Prince William Sound during 1990, a combined test of probabilities was performed as described by Sokal and Rohlf (1981).

Results

The porphyrins detected in the fecal samples from river otters were primarily Uroporphyrin I (Uro I), Heptacarboxylate porphyrin I (Hepta I), Coproporphyrin I (Copro I), Coproporphyrin III (Copro III) and Protoporphyrin IX (Proto IX) (Fig. 3). Proto IX was detected in 30% of the samples collected in Prince

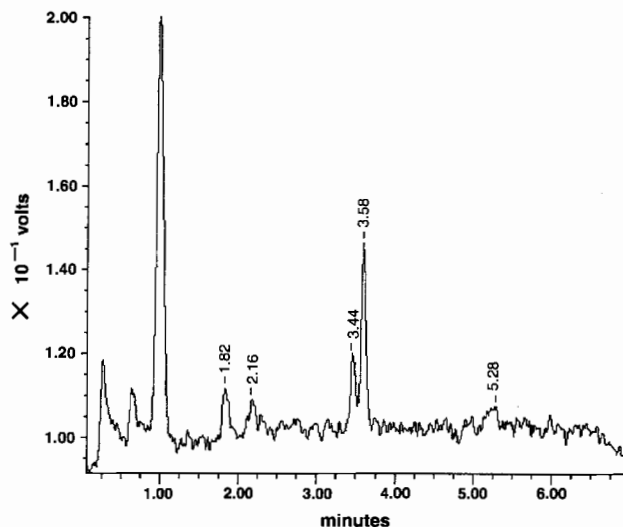


Fig. 3 Chromatogram of the porphyrin profile in a fecal sample of a river otter collected in the oiled area (Knight Island) of Prince William Sound, Alaska, USA, during 1990. Both Coproporphyrin I and Coproporphyrin III as well as Uroporphyrin I, Heptacarboxylate porphyrin I, and Protoporphyrin IX are discriminated.

William Sound and 79% of the samples collected in Lynn Canal. The mean levels of porphyrins detected ranged from 0.03 nmol/g for Uro I and Hepta I in the Knight Island 1996 oiled area to 1.74 nmol/g for Proto IX in the Lynn Canal reference area (Table 1). The median value for Copro III was highest at Knight Island 1990 (an oiled area) (Fig. 4). The overall ANOVA model was significant for the location-year effect (one-tailed; $F=2.96$; d.f. = 4, 115; $p=0.011$) indicating that Copro III levels were significantly different among locations and years. Individual one-tailed contrasts for year and location effects revealed Copro III levels at Knight Island in 1990 were significantly higher than those at Knight Island in 1996 ($p=0.012$), the Jackpot Bay reference area in 1996 ($p=0.002$) and the Lynn Canal reference area in 1998 ($p=0.004$). Although the median level of Copro III was higher at Knight Island in 1990 than the median level of Copro III at Esther Passage in 1990, the difference was marginally non-significant ($p=0.086$). A test to evaluate differences in biomarker response that combined probabilities of separate tests of

TABLE 1

Mean concentrations of porphyrins detected in fecal samples of river otters from Prince William Sound, Alaska, USA and Lynn Canal, Alaska, USA.

Porphyrin	Concentration (nmol/g*; Mean \pm S.E.)				
	Knight Island 1990 (n = 22)	Knight Island 1996 (n = 28)	Esther Passage 1990 (n = 23)	Jackpot Bay 1996 (n = 23)	Lynn Canal 1998 (n = 24)
Uro I	0.14 \pm 0.03	0.03 \pm 0.01	0.09 \pm 0.02	0.18 \pm 0.04	0.04 \pm 0.02
Hepta I	0.20 \pm 0.06	0.03 \pm 0.01	0.08 \pm 0.04	0.06 \pm 0.02	0.05 \pm 0.02
Copro I	0.23 \pm 0.06	0.37 \pm 0.14	0.21 \pm 0.06	0.09 \pm 0.03	0.54 \pm 0.20
Copro III	1.13 \pm 0.20	0.43 \pm 0.08	0.93 \pm 0.20	0.54 \pm 0.11	0.50 \pm 0.10
Proto IX	0.64 \pm 0.28	0.42 \pm 0.14	0.97 \pm 0.33	0.70 \pm 0.28	1.74 \pm 0.35

* Dry fecal weight.

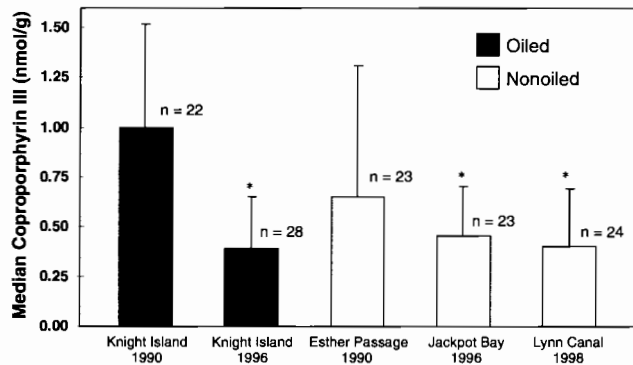


Fig. 4 Median concentrations of Coproporphyrin III for each year and location. Error bars represent one-half the interquartile distance. * Significantly different from Knight Island 1990.

significance for differences in Copro III levels, haptoglobin levels and body mass revealed an overall significant difference in biomarker response between oiled and nonoiled areas during 1990 ($p < 0.01$).

Discussion

The potential for certain chemical exposures to result in an alteration of the heme biosynthetic pathway leading to the excretion of porphyrin precursors, particularly in humans and laboratory animals, has been well established (Marks, 1985; Silbergeld and Fowler, 1987). Several studies have focused on the use of patterns of porphyrin excretion as a biomarker of contaminant exposure in wildlife. In a controlled field study, methylmercury administration resulted in significantly elevated levels of renal and hepatic porphyrins in European starlings (*Sturnus vulgaris*) (Akins *et al.*, 1993). Mediterranean crabs (*Carcinus aestuarii*) collected in the wild and studied in a laboratory setting had decreased levels of fecal porphyrins following exposure to benzo(a)pyrene, polychlorobiphenyls and methylmercury (Fossi *et al.*, 1997). Highly carboxylated porphyrins were elevated in herring gull chicks (*Larus argentatus*) from a site contaminated with polyhalogenated aromatic hydrocarbons compared with a noncontaminated site within the Great Lakes (Kennedy and Fox, 1990).

In a previous study using a spectrophotometric method, river otters in an oiled area of Prince William Sound had higher levels of total porphyrins in fecal samples than river otters in a nonoiled area during 1990. Qualitative HPLC analysis of extracted samples indicated that elevated coproporphyrin excretion was most prevalent in the fecal samples of river otters inhabiting the oiled area (Blajeski *et al.*, 1996). Moreover, low but significantly different concentrations of oil-related hydrocarbons occurred on the pelage of river otters inhabiting oiled relative to nonoiled areas of Prince William Sound, suggesting that chronic exposure to oil was occurring eight years following the oil spill (Duffy *et al.*, 1999).

Samples for this study had been archived from previous phases of this research (early phase, 1989–92; late phase, 1996–99) in which we used two approaches: intensive studies in 1 oiled and 1 nonoiled area and Sound-wide sampling of multiple oiled and nonoiled sites. Our initial design, intensive studies conducted in 1 location for each treatment (e.g., oiled and nonoiled), allowed us to collect more types of data over a long period. That approach, however, created the potential for bias relative to site-specific phenomena. To overcome that problem, we employed Sound-wide surveys that provided replicate data from several sites for each treatment. Our intensive sites of study from 1989 to 1991 were Herring Bay and surrounding areas (90 km of shoreline) on northern Knight Island as the oiled area and Esther Passage 82 km of shoreline between the mainland and Esther Island as our nonoiled site. The distance between those two areas is approximately 60 km. In 1991–92, Sound-wide sampling included many oiled areas and nonoiled areas; however, we only collected blood samples for haptoglobin and IL-6 analysis. In 1996, our intensive sites were (oiled) Herring Bay and surrounding areas (45 km of shoreline) and (nonoiled) Jackpot Bay (55 km of shoreline along Dangerous Passage). Study sites in 1996 are located approximately 30 km apart. We replaced our original intensive nonoiled area (Esther Passage) with a new site (Jackpot Bay area) in the latter phase of our studies to accommodate the overall needs of the multi-species approach for the Nearshore Vertebrate Research Project.

In contrast to our previous study, the refined extraction and HPLC techniques described herein allowed discrimination between the number I and III isomers of coproporphyrin (Fig. 1) and indicated that Copro III may have been responsible for the higher total porphyrin levels observed previously (Table 1). Although our initial intent was to analyze the entire porphyrin profile in the river otter fecal samples, low recovery of Proto IX in the samples collected in Prince William Sound, which apparently had deteriorated over time and with previous handling, precluded a more diagnostic multivariate statistical analysis designed to focus on changes in the levels of porphyrins relative to each other. None the less, recovery of Copro III was approximately 95% for all sampling areas and our results indicate that elevated levels of porphyrins in fecal samples of river otters inhabiting oiled areas of Prince William Sound reported previously may have been attributed to elevated levels of Copro III. Levels of Copro III were significantly higher in river otters from Knight Island in 1990 than that same area in 1996, the Jackpot Bay reference area in 1996 and the Lynn Canal reference area in 1998. Copro III levels also were higher in river otters at Knight Island in 1990 than at Esther Passage in 1990, however, that difference was marginally not significant. A LANDSAT thematic Mapper image indicated the presence of oil at the southern terminus of Esther passage 2 weeks after the *Exxon Valdez* oil spill (Stringer *et al.*, 1992). Our data

would suggest that some of the Esther Passage river otter may have been exposed to oil in 1989.

As noted earlier, there are few field studies where porphyrin profiles from field studies were measured in which a comparison to this study would allow evaluation for biological significance. Our approximate two-fold change in magnitude is lower than that found by laboratory studies in which a chemical exposure was used to disrupt the heme biosynthetic pathway. Also, we cannot rule out the possibility that compensatory changes in this complex pathway allowed adequate heme synthesis. In fact, anemia was never detected in our field studies. However, the data reported here can help create a weight of evidence which is an approach to deal with the uncertainty common in ecosystem studies which deal with populations that have high individual variability (Newman, 1998).

Levels of Copro III in fecal samples collected from the oiled area during 1990 and 1996 followed a similar temporal pattern of physiological response as that observed with other biomarkers measured in river otters at the same oiled area from 1990 to 1992. River otters inhabiting the oiled area during 1990 had higher levels of haptoglobin, an acute phase protein indicative of tissue damage and lower body mass than river otters inhabiting the nonoiled area (Duffy *et al.*, 1993). A follow-up study, however, reported no significant differences in haptoglobin levels and body mass of river otters between the same oiled and nonoiled sites by 1992 (Duffy *et al.*, 1994b).

These same studies also reported higher haptoglobin levels and lower body mass in river otters inhabiting the Esther Passage reference area during 1990 than during 1992. The observation of higher levels of haptoglobins and lower body mass in river otters at Esther Passage during 1990 corresponds to the observation of Copro III levels in fecal samples collected from Esther passage during 1990 similar to Copro III levels in fecal samples collected from the oiled area during 1990. With a test of combined probabilities (Sokal and Rohlf, 1981), however, an overall significant difference in biomarker response (Copro III, haptoglobins, body mass) occurred between the Knight Island oiled area and the Esther Passage nonoiled area during 1990.

Results of this study correspond to results of other biomarker studies of river otters of Prince William Sound following the *Exxon Valdez* oil spill and provide evidence that levels of fecal porphyrins may serve as an effective biomarker of environmental stress. When coupled with ecological observations of loss of diet diversity (Bowyer *et al.*, 1994) and differences in habitat selection and sites of home ranges (Bowyer *et al.*, 1995) biomarkers such as porphyrin profiles can provide a powerful methodology to assess health and recovery of wild river otters. Further studies will be necessary to establish a dose-response relationship between oil exposure and changes in the profile of fecal porphyrins in wild river otters.

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