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Cytochrome P-450 Induction and Histopathology in Preemergent Pink Salmon from Oiled Spawning Sites in Prince William Sound

MICHAEL WIEDMER AND MARK J. FINK
Alaska Department of Fish and Game, Habitat and Restoration Division
333 Raspberry Road, Anchorage, Alaska 99518, USA

JOHN J. STEGEMAN AND ROXANNA SMOLOWITZ
Woods Hole Oceanographic Institution, Biology Department
Woods Hole, Massachusetts 02543, USA

GARY D. MARTY[1] AND DAVID E. HINTON
University of California, Department of Medicine, Davis, California 95616, USA

Abstract.—The March 1989 *Exxon Valdez* oil spill contaminated intertidal pink salmon *Oncorhynchus gorbuscha* spawning areas in Prince William Sound and the Gulf of Alaska. To determine if 8- to 26-month old oil remaining in some spawning areas produced physiological responses in developing pink salmon eggs and alevins, we conducted an initial assessment of cytochrome P-4501A induction and histopathologic lesion occurrence in preemergent pink salmon collected from oiled spawning substrates. Egg and alevin samples were collected from four oiled and five reference sites in Prince William Sound, Alaska, between December 1989 and May 1991. Immunohistochemical staining for cytochrome P-4501A was increased in alevins from 13 of 16 samples from oiled sites, but was not increased in any of the 7 samples from the reference sites. Cytochrome P-4501A induction was not detected in egg samples from either oiled or control sites. Persistent P-4501A staining through the end of the study was evidence for chronic exposure of two year-classes of pink salmon to hydrocarbon contamination. Histopathologic lesions were more frequent in alevins from oiled sites, but differences were not statistically significant, and lesion occurrence seemed dependent on developmental stage. These results provide evidence that pink salmon alevins developing in heavily oiled sites were exposed to hydrocarbons more than 2 years after the initial spill and that the hydrocarbons induced detectable physiological changes. Results of this study were used to develop appropriate treatments for oiled anadromous fish streams.

The 24 March 1989 grounding of the TV *Exxon Valdez* spilled 43.9 million liters of Prudhoe Bay crude oil into the waters of Prince William Sound. Currents and winds carried the oil through the islands of the southwestern Sound and into the Gulf of Alaska. The spill eventually oiled almost 2,000 km of shoreline, including a minimum of 213 streams supporting anadromous fish.

The state of Alaska Department of Fish and Game (ADFG) cooperated in the spill response program. In this effort, we focused on the need for, and the adequacy of, treatment of intertidal areas in and around anadromous fish streams. Because up to 75% of wild Prince William Sound pink salmon *Oncorhynchus gorbuscha* spawn intertidally, the ADFG was concerned that oiled beach sediments might affect developing pink salmon eggs and larvae.

In Prince William Sound, adult pink salmon spawn in inter- and supratidal stream channels from late July through early October. All pink salmon die within a few days of spawning. Eggs typically hatch in December and January, and alevins remain within the spawning substrate, absorbing their yolk sac, until emerging as free-swimming fry between April and June.

Fish are generally most sensitive to environmental pollutants as juveniles (McKim 1977; Moles et al. 1979). While oil remained in and near some spawning streams by fall of 1989, some individuals involved in the spill response believed that any remaining oil would be biologically inert. If remaining weathered oil was biologically inert, additional treatment would not be necessary. To determine if remaining oil was biologically inert, the ADFG began the initial assessment reported here. We analyzed wild preemergent pink salmon collected from oiled spawning substrates by (1) measuring cytochrome P-450 induction in eggs and alevins as an indicator of exposure to hydrocarbons and (2) documenting histopathological changes in alevins from oiled sites.

[1]Present address: University of California, Department of Anatomy, Physiology, and Cell Biology, School of Veterinary Medicine, Davis, California 95616, USA.

Cytochrome P-450 is a particular group of monooxygenase (MO) enzymes that mediates oxidation of petroleum hydrocarbons and other xenobiotics, thereby facilitating their excretion (Jimenez and Stegeman 1990). Studies of free-ranging fish exposed to petroleum contamination (Burns 1976; Payne 1976; Kurelec et al. 1977) and to general industrial contamination (Spies et al. 1982; Foureman et al. 1983; Spies et al. 1988) showed elevated MO activity associated with the cytochrome P-4501A, the hydrocarbon-inducible P-450.

Despite the potential of using histopathologic lesions as biological indicators of exposure to toxic environmental contaminants (Hinton and Lauren 1990), and the relatively common occurrence of large oil spills, comparatively few studies have been done on field-exposed fish. Histopathologic lesions have been demonstrated in free-ranging adult fish up to 2 years after an oil spill (Haensly et al. 1982). For larval fish, lesions have been reported after exposure to crude oil in the laboratory (Cameron and Smith 1980; Hawkes and Stehr 1982), but lesions in naturally exposed larvae have not previously been reported.

Study sites

Preemergent pink salmon were collected from intertidal stream spawning substrates at four oiled sites and five reference sites in southwestern Prince William Sound (Figure 1). Oiled sites were located in unnamed streams at Herring Point and Marsha Bay on Knight Island, Shelter Bay on Evans Island, and Sleepy Bay on Latouche Island. Reference sites were located in unnamed streams of Herring Bay on Knight Island, Kake Cove and the east side of Chenega Island, Shelter Bay, and north of Eshamy Bay. Fish samples designated "oiled" were collected in close proximity to visible oil while samples designated "reference" were collected from areas with no visible sources of potential contamination. Heavy oil deposition at the contaminated sites occurred primarily during late March and early April, 1989. Site observations indicated that potential exposure of developing pink salmon embryos to hydrocarbons was attributable primarily to chronic leaching from and direct exposure to contaminated sediments, rather than to reoiling from refloated oil.

Methods

Eggs and alevins were collected from spawning substrates in mid- to upper intertidal zones between December 1989 and May 1991 (two brood years). Eggs were collected in December 1989 and September 1990. Alevins were collected in December 1989, May and June 1990, and March and May 1991. Eggs averaged 6–7 mm in diameter; alevins averaged 30 mm long. Because of subfreezing temperatures, hand shovels, rather than egg pumps, were used to extract specimens from spawning gravels. Excavated substrates were placed and agitated immediately upstream of a 0.7-m × 1.0-m funnel net, where stream currents flushed the eggs and alevins into the net. Specimens were separated from debris and immediately placed in 10% phosphate-buffered formalin. Specimens collected in 1991 were anesthetized in tricaine methanesulfonate (MS-222) before fixation. Overall, 1–6 alevin samples from each oiled site (16 total samples) and 1 or 2 samples from each reference site (7 total samples) were collected. Two egg samples each from two oiled sites and one sample each from two reference sites were also collected. Each sample consisted of 4–6 alevins or 8–12 eggs. Four egg samples were incidently collected at three other sites. Two egg samples were collected from an oiled site near Point Countess, and one egg sample each was collected at non-oiled sites in Thumb Cove and at Point Countess (not identified in Figure 1).

Representative sediment samples (one per site) collected at the oiled sites in Marsha Bay, Shelter Bay, and Sleepy Bay in 1989, and at Herring Point in 1990, were analyzed for *n*-alkanes and isoprenoids with gas chromatography using flame ionization detection (GC–FID) and for aromatic hydrocarbons using gas chromatography with mass spectrometry (GC–MS) to verify that the oil source was North Slope crude. Hydrocarbon analysis of sediments was conducted by the Geotechnical and Environmental Research Group at Texas A&M University (College Station, Texas). No sediment samples from reference sites were analyzed. Lack of contamination at these sites was visually determined.

Alevin and egg samples were analyzed immunohistochemically at Woods Hole Oceanographic Institution (WHOI), Massachusetts, for induction of cytochrome P-4501A, the specific P-450 induced by polycyclic aromatic hydrocarbons (PAHs) (Stegeman and Lech 1991). One to six alevin samples from each of the four oiled sites (94 total alevins) and one to two alevin samples from each of the five reference sites (42 total alevins) were analyzed. One to three egg samples from each of the oiled sites and one egg sample from each of three reference sites were also analyzed. Tissues were processed routinely into paraffin. Eggs and alevins were em-

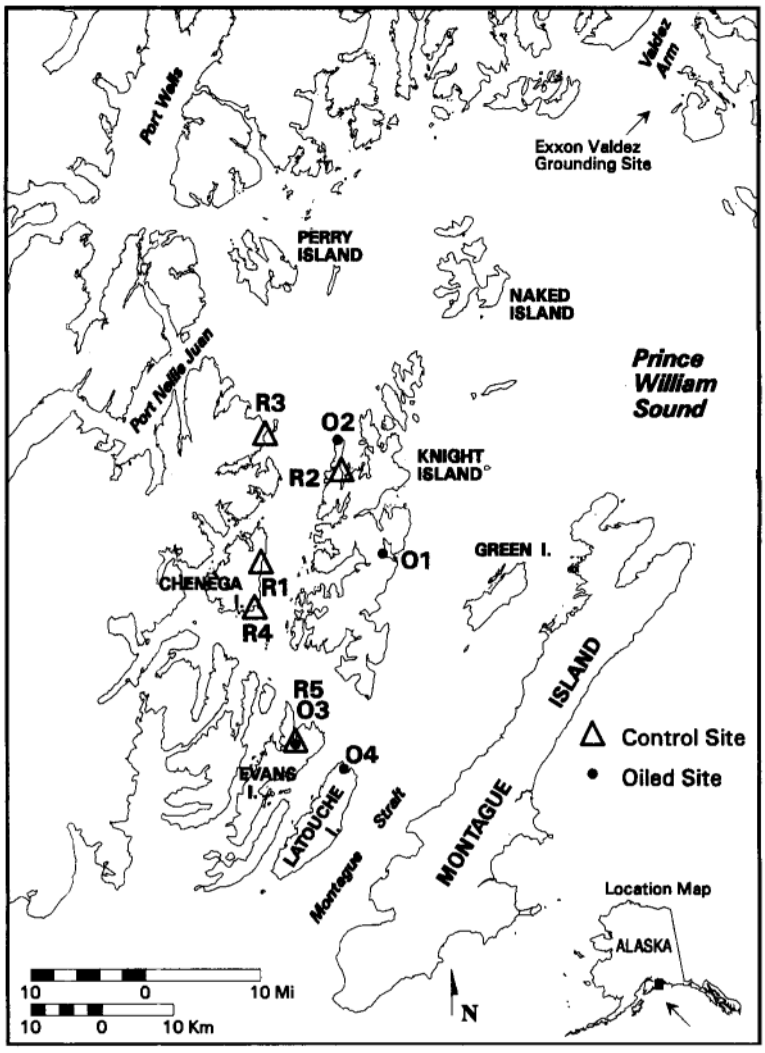

FIGURE 1.—Pink salmon egg and alevin collection sites in Prince William Sound, Alaska. Oiled sites are O1 = Marsha Bay, O2 = Herring point, O3 = Shelter Bay, O4 = Sleepy Bay. Reference sites are R1 = East side Chenega Island, R2 = Herring Bay, R3 = Eshamy Bay, R4 = Kake Cove, R5 = Shelter Bay. Sites O3 and R5 are located in two separate streams approximately 600 m apart.

bedded whole, in lateral-to-dorsolateral recumbency, with two fish or 8–12 eggs per paraffin block. Resultant blocks were trimmed and sections discarded until the approximate midsection was reached. Five-micron-thick sections were stained with monoclonal antibody (MAb) 1-12-3 and a peroxidase-labeled secondary antibody (Park et al. 1986; Smolowitz et al. 1991). Monoclonal antibody 1-12-3 is specific for P-4501A proteins and cross-reacts with P-4501A in all vertebrate species tested to date. For quality control, serial sections of at least one block per sample were stained identically except for substitution of the primary antibody with a nonspecific monoclonal antibody (of the same immunoglobulin G subclass). All sections were treated under identical conditions and duration. Staining intensity and occurrence of P-4501A was determined for tissues in each egg or alevin, and an overall, relative level of induction was determined for each sample: negative (0), very mild (1), mild (2), moderate (3), and strong or severe (4). After P-450 induction analysis, duplicate slides and em-

bedded alevins were sent to the University of California at Davis (UC Davis) for histopathological analyses. Where liver was missing from duplicate slides, additional sections were cut at UC Davis. To avoid possible bias, background information on samples and collection sites was not provided to workers at WHOI or UC Davis until after analyses were completed.

Individual glass microscope slides containing two or three serial or near-serial longitudinal sections (two alevins per slide) were examined to determine the extent of autolysis, the presence of major organs, the relative abundance of liver glycogen and yolk stores (scored as none [0], minimal or moderate [1], or abundant [2]), and the presence of lesions. Frequently occurring lesions were ranked and scored relative to other similar lesions as follows: none (0), mild (1), moderate (2), or severe (3).

Egg and alevin samples were stratified by time and pooled for each site. The Wilcoxon–Mann–Whitney two-sample test (Conover 1980) was used to test for statistical differences between the median of cytochrome P-4501A induction and lesion scores from oiled and reference sites. Statistical tests were one-tailed and performed at the $\alpha = 0.10$ level. The null hypothesis for all tests was that oiled and reference sites were affected equally. Lesions used in scoring included epidermal atrophy (EA) and myofiber degeneration and/or necrosis (MDN). Scores for individual hepatocellular degeneration (IHD) and vacuolar degeneration of gastric glands (VDGG) were not used because of several missing values.

Results

Cytochrome P-4501A induction was observed in endothelial and epithelial cells of several organs from alevin samples from each of the oiled sites (13 of 16 samples) and appeared to be independent of posthatch developmental stage. All seven alevin samples from reference sites were negative for induction. In samples from oiled sites, organs exhibiting positive immunohistochemical staining included kidney, gill, liver, intestine, heart, brain, yolk sac, skin, peritoneal connective tissue, and pharyngeal epithelium (Table 1). Because all tissues were not visible in sections from every individual fish, some induced tissues may have gone undetected. Cytochrome P-4501A induction was not observed in any of the 10 egg samples, regardless of hydrocarbon exposure. In one instance where both eggs and alevins were simultaneously collected from the same microsite (Marsha Bay [O1], December 1989), alevins had mild-to-moderate induction while eggs had none.

Cytochrome P-4501A content was significantly elevated in alevin samples from oiled sites in May and June 1990 ($P < 0.067$) and in March 1991 ($P < 0.10$). Because only one oiled-site (O1) sample and one reference-site (R1) sample were collected in December 1989, no statistical tests were performed; however, the oiled site had mild staining for P-4501A expression, whereas the reference site had no staining (Figure 2). No statistical difference was detected in May 1991 ($P < 0.20$) (Figure 2). There was no significant change in induction intensity between 1990 and 1991.

Lesions were observed in 6 of 13 samples from oiled sites exhibiting elevated P-4501A (Table 1). In two samples collected from Marsha Bay (O1) in May 1991, 11 of 12 alevins had EA and VDGG, but P-4501A induction was negative. In three of seven samples from reference sites, alevins had lesions but no increase in P-4501A (Table 1). In 1990, EA was found in one of three reference samples (3 of 18 alevins); in 1991, VDGG or EA or both were found in two of four reference samples (4 of 24 alevins).

Contamination of oiled-site sediments by North Slope crude oil was confirmed.

In 1989–1990 and 1990–1991 brood years, histopathological lesions were observed in 8 of 16 samples (35 of 93 alevins, 38%) from oiled sites and 3 of 7 samples (7 of 42 alevins, 17%) from reference sites. Lesions occurring at more than one study site included EA (most common), MDN, and VDGG. Lesions were moderate or severe in 17 of 21 fish from oiled sites in 1990 compared with 7 of 14 fish from oiled sites in 1991. Lesions were absent in December 1989 and March 1991 samples. No statistically significant differences were detected in the occurrence of lesions between oiled and reference sites in May–June 1990 ($P < 0.27$) or May 1991 ($P < 0.40$). However, because of small sample size, there was insufficient power to provide for meaningful statistical analysis.

The occurrence of lesions appeared to correlate with absorption of the yolk sac. Lesions were absent in samples with abundant yolk stores from both oiled and reference sites in December 1989 and March 1991 (Table 1). However, yolk stores were scored as absent or minimal in 96% (23 of 24) and 72% (13 of 18) of alevins with lesions from oiled sites in May–June 1990 and May 1991 samples, respectively. Yolk stores were not significantly different between oiled and reference sites in either 1990 or 1991 ($P < 0.40$ and $P < 0.60$, respectively).

Some infrequent lesions occurred only in alevins

TABLE 1.—Cytochrome P-4501A induction and histopathology in preemergent pink salmon alevins collected from within intertidal sediments of the streams at study sites in Prince William Sound, 1989–1991.

Location	Date	P-4501A score[a]	Tissue induced[b]	Lesions[c]	Median yolk score[d]
			1989–1990 brood year		
Reference					
Chenega Island R1	Dec 9	0	None	None	2.0
Herring Bay R2	May 30	0	None	None	2.0
Eshamy Bay R3	Jun 2	0	None	EA	1.0
Oiled					
Marsha Bay O1	Dec 8	2.5	K_t	None	2.0
Sleepy Bay O4	May 25	0	None	None	2.0
Sleepy Bay O4	May 25	1	VCC	None	2.0
Sleepy Bay O4	May 25	1	VCC	EA	2.0
Marsha Bay O1	May 30	2	VCC, $I_{m,c}$	EA, MDN, IHD	0.5
Herring Point O2	Jun 8	3	G_e, Sk, VCC	EA, MDN	0.5
Shelter Bay O3	Jun 8	4	G_e, $K_{s,t}$, H, B, PC, L_h, VCC, $I_{c,e,m}$	EA, MDN	1.0
			1990–1991 brood year		
Reference					
Shelter Bay R5	Mar 21	0	None	None	2.0
Kake Cove R4	Mar 22	0	None	VDGG	2.0
Shelter Bay R5	May 4	0	None	EA, VDGG	1.5
Kake Cove R4	May 5	0	None	None	2.0
Oiled					
Sleepy Bay O4	Mar 17	3	$G_{e,b}$, Y, $K_{c,s,v}$, H	None	2.0
Marsha Bay O1	Mar 21	3	L_h, P, G_e, I_e, $K_{s,v}$	None	2.0
Marsha Bay O1	Mar 21	2	I_e, K_c	None	2.0
Shelter Bay O3	Mar 21	2.5	Y, I_e, L_s, $K_{s,v}$	None	2.0
Shelter Bay O3	Mar 21	3	I_e, $K_{s,v}$, G_b, H, L_h, Y	None	2.0
Sleepy Bay O4	May 4	3	G_e, P, H, $K_{c,s,v}$, Y, $L_{h,c}$	EA	2.0
Shelter Bay O3	May 4	2	L_h, $K_{s,v}$	EA	2.0
Marsha Bay O1	May 14	0	None	EA, VDGG	1.0
Marsha Bay O1	May 14	0	None	EA, VDGG	1.0

[a] Sample scores of 0 (negative), 1 (very mild), 2 (mild), 3 (moderate), and 4 (strong) were based on the extent of occurrence and intensity of immunochemical staining of P-4501A in histological sections of six fish per sample.

[b] Tissue types that stained immunochemically for cytochrome P-4501A: $K_{c,s,t,v}$ = kidney collecting ducts, sinusoidal endothelium, tubular epithelium, and vascular endothelium; VCC = vertebral cord cartilage; $I_{e,m,c}$ = intestine enterocytes, midgut and cecal epithelium; $G_{b,e}$ = gill buds and epithelium; Sk = skin; H = atrial or ventricular endothelium; B = brain vessel endothelium; PC = peritoneal connective tissue; $L_{c,h,s}$ = liver central veins, hepatocytes, and sinusoidal endothelium; Y = yolk sac endothelium; P = pharyngeal epithelium.

[c] Indicates the presence of any of four lesion types in one or more of the fry in a sample: EA = epidermal atrophy; IHD = individual hepatocellular degeneration; MDN = myofiber degeneration and/or necrosis; VDGG = vacuolar degeneration of gastric glands.

[d] Yolk stores were qualitatively scored as 0 (none), 1 (minimal), or 2 (abundant).

from oiled sites. All six alevins from Marsha Bay (O1, May 1990) had mild to moderate vacuolar changes in the liver; spherical spaces, each about 25 μm in diameter and partly filled by basophilic wispy material, were scattered throughout the liver and were probably of hepatocellular or Ito cell origin (they were scored as IHD). Three of the six alevins from the same Marsha Bay sample (O1, May 1990) had mild to moderate amounts of individual necrotic cells (origin unknown) in the subepithelial connective tissue of the stomach. Two fish from Herring Point (O2, June 1990) had lesions: one had mild renal tubular necrosis; the other had mild thrombosis of a branchial vessel. One fish from Shelter Bay (O3, May 1991) had a 200-μm-diameter focus of bile duct hyperplasia in the liver.

Discussion

Immunohistochemical staining results from this study indicate that preemergent pink salmon from oiled sites in Prince William Sound responded to chronic PAH exposure by induction of cytochrome P-4501A up to 26 months after initial oiling of intertidal spawning sediments. Other studies have demonstrated that P-450 content drops to basal levels 3 weeks after removal of the inducing agent, although this rate may be temperature dependent (Kloepper-Sams and Stegeman 1989). By fall 1989, many argued that any *Exxon Valdez* oil residues remaining in and near salmon spawning sites were biologically inert. This study clearly shows that 2 years after the spill, oil residues contaminating in-

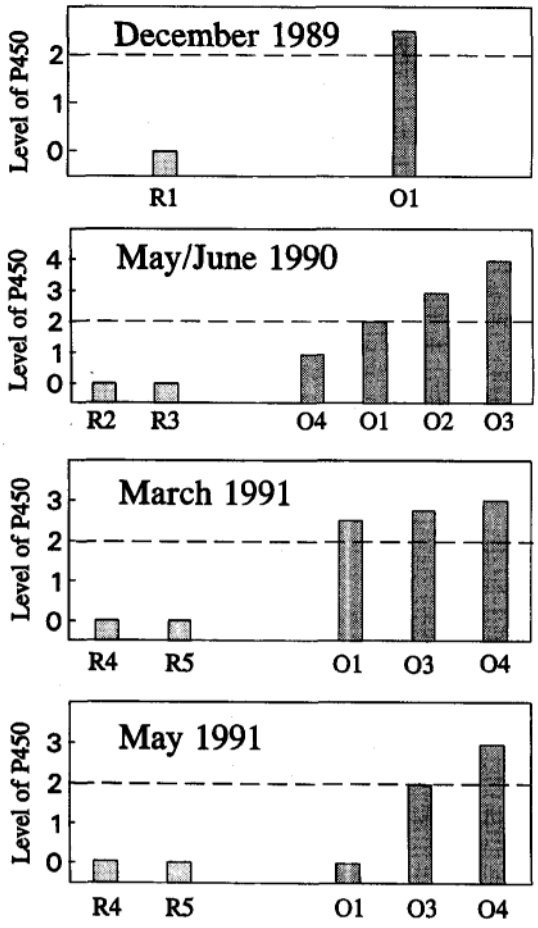

FIGURE 2.—Median cytochrome P-4501A induction scores for pink salmon alevin samples collected in December 1989, May–June 1990, March 1991, and May 1991 from reference (left) and oiled (right) sites. Induction scores are 0 = negative, 1 = very mild, 2 = mild, 3 = moderate, and 4 = severe. See Figure 1 for site locations.

tertidal substrates were not biologically inert and were still inducing measurable physiological responses in wild salmon.

Physiological systems of preemergent pink salmon alevins are competent to synthesize cytochrome P-4501A in response to oil contamination. Not only was P-4501A induced in hepatic tissues, but at least 11 tissues of nine organ systems were also inductively competent. Induction in the vascular endothelium of kidney, brain, heart, and ceca suggests a response to blood-borne xenobiotics. These results correspond well with reports of P-4501A localization in other fish species (Smolowitz et al. 1991). Induction in gill epithelium may indicate exposure to oil via water. However, P-4501A was also induced in gill epithelia when fish were exposed to xenobiotics via intraperitoneal injection (Smolowitz et al. 1991), indicating that the route of exposure may involve passage of PAHs via blood. Induction in cecal epithelium was observed by Smolowitz et al. (1991) in fish not exposed to food-borne xenobiotics but to intraperitoneal injection; inducers were thought to have entered the gut with the bile, followed by induction in mucosal tissue.

Elevated P-450 levels were not detected in eggs, not even in those collected at the same time and location as alevins with elevated P-450 levels. The absence of induction in eggs is consistent with other findings (Binder and Stegeman 1984; Goksøyr and Solberg 1987).

Studies have demonstrated that as a result of P-450-mediated metabolism, toxicity of PAHs greatly increases (Nebert and Gelboin 1968; Diamond and Clark 1970; Malaveille et al. 1975; Wood et al. 1976). The products of P-450-mediated metabolism bind to RNA (Blobstein et al. 1976), and DNA (Glover and Sims 1968; Sims et al. 1974) via arene oxide intermediates (Jerina and Daly 1974). The degree of metabolite binding is positively correlated to carcinogenic (Brookes and Lawley 1964) and mutagenic (Jerina and Daly 1974; Stegeman and Lech 1991) potential. These studies suggest that elevation of cytochrome P-4501A in preemergent pink salmon from oiled sites may create an increased potential for mutagenesis and carcinogenesis.

Fish exposed to xenobiotics have demonstrated an inverse correlation between MO activity and androgen, estrogen, and corticoid concentrations (Sivirajah et al. 1978), and testicular development (Truscott et al. 1983). Spies et al. (1988) found an inverse correlation between MO activity and parameters of female reproductive success (e.g., decreased proportion of viable eggs, decreased fertilization success, and decreased embryological success). Bue et al. (1996, this volume) found that pink salmon egg mortality was elevated in oil-contaminated streams during the falls of 1989, 1990, 1991, and 1992. These observations suggest that the reproductive potential of salmon developing in contaminated sediments may be diminished. As part of ongoing oil spill studies, continuing effort is being directed to evaluate the long-term reproductive impact to pink salmon resulting from exposure to oil-contaminated spawning substrates.

Unlike induction of P-4501A, the relationship of most histopathologic lesions was less clearly related to oil exposure. Lesions such as IHD, subepithelial

gastric cell necrosis, renal tubular epithelial necrosis, and biliary epithelial hyperplasia occurred only in alevins from oiled sites. Because none of these lesions occurred at more than one site, the lesions cannot be unequivocally related to oil exposure. However, similar lesions have previously been related to oil exposure in fish: (1) hepatocellular microvesicles, a type of degenerative change, in Atlantic cod *Gadus morhua* exposed to Hibernian crude oil in the laboratory (Khan and Kiceniuk 1984) and (2) necrosis of renal tubular epithelial cells and interrenal tissue in the mummichog *Fundulus heteroclitus* exposed to naphthalene, a component of crude oil, in the laboratory (DiMichele and Taylor 1978).

Lesions occurring at more than one site were even less related to oil exposure. First, myodegeneration and necrosis were peracute lesions and probably were related to physical trauma of removing alevins from the gravel during collection. Second, VDGG occurred in alevins from both oiled and reference sites and was thought to represent a type of artifact or unrelated lesion rather than an oil-associated lesion. However, hydropic degeneration of gastric gland epithelial cells was described in plaice *Pleuronectes platessa* associated with the *Amoco Cadiz* oil spill (Haensly et al. 1982). And third, EA also occurred in alevins from both oiled and reference sites. Literature reports vary on the effects of oil exposure on epidermal thickness in fish. Studies with Venezuelan crude oil found decreased epidermal thickness (Burton et al. 1984, 1985), whereas studies with Hibernia crude oil (Khan 1991) and Louisiana crude oil (Solangi and Overstreet 1982) found gill lamellar hyperplasia. Decreased epidermal thickness and decreased mucous cell abundance, the primary components of EA, may be developmental changes associated with absorption of the yolk that are unrelated to oil exposure. This subject deserves further study.

Of interest was the absence of EA or other lesions in all alevins with abundant yolk stores. Lesion prevalence might have increased had all samples been collected after alevins had absorbed most of their yolk sacs. Korn and Rice (1981) found that fish with abundant yolk were most tolerant to aromatic hydrocarbons. The amount of yolk influenced sensitivity because the lipophilic, aromatic hydrocarbons were partitioned into the yolk, reducing the availability of the hydrocarbons to other embryonic tissues. Oil exposure may not, however, result in a pathognomonic lesion. As evidence, alevins from both oiled and reference streams had lesions when their yolk reserves were minimal.

In previous studies with the water-soluble fraction of Prudhoe Bay crude oil, sensitivity (mortality) to aromatic hydrocarbons in pink salmon increased from egg to fry (Rice et al. 1975; Moles et al. 1979; Korn and Rice 1981). Emergent fry were the most sensitive. However, no histopathology data is available on the effects of hydrocarbons on the early life stages of salmon. It should be reemphasized that all fish observed in the current study were collected early in their development and before emergence from spawning gravels. No attempt was made to collect older fish that were exposed as juveniles to contaminated substrates. Therefore, long-term effects of oil exposure on lesion development were not assessed. Laboratory studies are underway to determine more definitively the role of developmental age at oil exposure on the generation of histopathologic lesions in pink salmon alevins.

The current study demonstrates that pink salmon alevins in some heavily oiled sites continued to be exposed to physiologically relevant levels of hydrocarbons more than 2 years after the initial spill. Furthermore, this study demonstrates the utility of immunohistochemical detection of cytochrome P-4501A in fish as a marker of hydrocarbon exposure (Stegeman et al. 1991). Finally, these results should assist in developing appropriate standards for monitoring treatment of streams used by anadromous fish after major oil contamination.

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