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Evidence of Damage to Pink Salmon Populations Inhabiting Prince William Sound, Alaska, Two Generations after the *Exxon Valdez* Oil Spill

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Abstract.—Our investigations into the effects of the 1989 *Exxon Valdez* oil spill in Prince William Sound, Alaska, suggest that chronic damage occurred to some populations of pink salmon *Oncorhynchus gorbuscha*. Significantly elevated embryo mortalities were observed from 1989 through 1993 in populations inhabiting streams previously contaminated by oil. No statistically detectable difference in embryo mortality was observed in 1994 and 1995. We assessed the possible influence of the natural environment on these findings by collecting gametes from adults returning to contaminated and to uncontaminated streams, transporting the gametes to a hatchery where intrastream crosses were made, and incubating the resulting embryos under identical environmental conditions. Significantly increased embryo mortality was detected for embryos originating from the oil-contaminated lineages in 1993 but not in 1994, which indicated that the significant differences detected in the field in 1989–1993 were not induced by naturally occurring environmental variables.

On March 24, 1989, the supertanker *Exxon Valdez* ran aground on Bligh Reef in Prince William Sound, Alaska, spilling approximately 41 million liters of crude oil (Bragg et al. 1994). The resulting slick moved through western Prince William Sound and the western Gulf of Alaska, contaminating approximately 2,000 km of coastal habitat (Bragg et al. 1994), killing an estimated 250,000 seabirds (Piatt and Ford 1996) and 4,000 sea otters *Enhydra lutris* (Garrott et al. 1993; Degange et al. 1994). Sublethal effects were also documented (Hose et al. 1996; Wiedmer et al. 1996; Marty et al. 1997). Despite a US$2 billion cleanup and restoration effort, subsurface oil remains in some of the beaches (Wolfe et al. 1994; Babcock et al. 1996; Spies et al. 1996).

One of the most abundant vertebrate species in the area is pink salmon *Oncorhynchus gorbuscha* of both wild and hatchery origin. Up to 75% of wild pink salmon that spawn within the Sound do so in intertidal areas (Helle et al. 1964). Unfortunately, their extensive use of intertidal spawning areas and the use of nearshore marine areas by juveniles made pink salmon vulnerable to oil exposure from the spill.

Mortality of pink salmon embryos was examined annually in 10 oil-contaminated (oiled) and 15 nearby, uncontaminated (reference), streams from 1989 through 1992 (Bue et al. 1996). In that work, stream oiling was assessed through visual observations of the stream and the adjacent area during the spring of 1989. The observations were reviewed and adjusted if needed according to the results of anadromous stream surveys conducted in southwestern Prince William Sound by the Alaska Department of Fish and Game, Habitat Division (Middleton et al. 1992). The oiling classifications of the streams correlated with the findings of the fall of 1989 shoreline surveys (ADEC–SRS 1989; Neff et al. 1995) and similar pink salmon work by Brannon et al. (1995). Each fall, live and dead embryos were collected from the stream gravel along transects established in three intertidal zones (1.8–2.4 m, 2.4–3.0 m, and 3.0–3.7 m above mean low water) and the area above mean high water (>3.7 m above mean low water). More than 2,500

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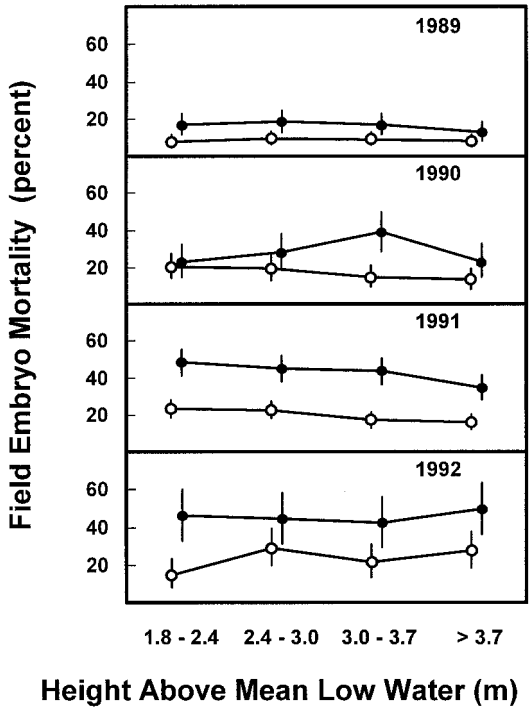

FIGURE 1.—Mean pink salmon embryo mortality observed during fall field sampling in 1989 through 1992 (Bue et al. 1996). Solid circles indicate oil-contaminated streams ($N = 10$); open circles identify reference streams ($N = 15$); error bars represent 90% confidence intervals.

embryos were examined on average from each stream zone to estimate embryo mortality.

Bue et al. (1996) measured significantly greater embryo mortality in oiled streams than in reference streams in 1989 ($P = 0.004$) and 1990 ($P = 0.023$); significant differences were recorded in all intertidal areas in 1989 and in the upper intertidal zone in 1990 (Figure 1). These results were consistent with the observed patterns of oil contamination and the results of controlled oiling experiments. Wolfe et al. (1994) found that among oiled streams, the intertidal areas were contaminated in 1989, and much of the remaining oil was deposited in the upper intertidal zone in 1990. In controlled oiling experiments, Marty et al. (1997) and Heintz et al. (1995) found that pink salmon embryos experienced significantly higher mortality when incubated in oiled gravel than in clean gravel. Heintz et al. (1995) also detected significantly elevated mortalities in pink salmon embryos incubated in oiled gravel that had weathered for a year.

In 1991 we observed a larger difference in embryo mortality between oil-contaminated and reference streams than was previously recorded ($P = 0.003$; Figure 1); this dissimilarity was observed across all stream zones, even in the area above that directly influenced by oil. A similar, but less extreme, pattern of embryo mortality was observed again in 1992 ($P = 0.010$; Figure 1). Evidence of oil contamination in the intertidal areas was dramatically reduced by 1991 (Wolfe et al. 1996), yet elevated mortality of embryos in oiled streams continued (Bue et al. 1996).

The 1991 and 1992 evaluations demonstrated significant differences in embryo mortality between oil-contaminated and reference streams in both the intertidal and upstream zones. These findings were unexpected because the presence of oil was dramatically reduced in all areas for these years. We developed three hypotheses that could explain these findings: (1) that oil-induced damage to the 1989 and 1990 broods included deleterious mutations in the germ line, (2) that incubating embryos continued to be damaged in a physiological manner by an oiled environment even after visually observable oil was gone and that this impact was expressed as functional sterility, (3) that the observed differences in embryo mortality were due to naturally occurring environmental factors that differed between oiled and reference streams.

All three hypotheses were supportable. Both the genetic-damage and physiological-damage hypotheses seemed credible. Past studies had confirmed that pink salmon embryos take up polycyclic aromatic hydrocarbons (PAHs; Moles et al. 1987), a major component of crude oil, and that these compounds were capable of inducing chromosomal lesions (McBee and Bickham 1988) and influencing endocrine function (Thomas and Budiantara 1995). Pink salmon have an obligate 2-year life cycle that results in two genetically isolated lineages, one produced during odd years and the other during even years (Heard 1991). Therefore, genetic or physiological damage induced in one brood year would be expressed in that lineage 2 years later. The environmental-difference hypothesis seemed credible because environmental factors (wind and currents) determined the distribution of the oil, and such factors might also influence the survivability of salmon embryos incubating intertidally.

In this study we continued to monitor pink salmon embryo mortality in oiled and reference streams and tested the environmental-difference hypothesis with a controlled incubation experiment.

Methods

Field monitoring.—We followed methods for pink salmon embryo sampling described by Bue et al. (1996), which were modeled after procedures described by Pirtle and McCurdy (1977). The 10 oil-contaminated and 15 reference streams sampled for pink salmon embryos each fall from 1993 through 1995 were the same ones studied by Bue et al. (1996) from 1989 through 1992.

On each study stream, four zones, three intertidal (1.8–2.4 m, 2.4–3.0 m, 3.0–3.7 m above mean low water) and one that was above most tidal influence (>3.7 m) were measured from the mean low tide mark and marked with stakes. A linear transect approximately 30.5 m in length was established in each zone. The transect ran diagonally across the stream, and its location was staked to ensure continuity of transects between years. Fourteen 0.3-m^2, circular digs were systematically made along each transect with a high-pressure hose and a specially designed net to flush and capture embryos. Numbers of live and dead embryos and recently hatched alevins were used to estimate embryo mortalities by stream zone.

Differences in embryo mortality were evaluated with a mixed-effects two-factor experiment with repeated measures on one factor (Neter et al. 1990). The two factors were (1) extent of oiling (two levels: oil-contaminated and reference) and (2) the height in the intertidal zone (four levels). The data were blocked by stream, a random effect nested within extent of oiling.

Controlled incubation experiment.—Intrastream crosses were made from gametes from 30 male and 30 female pink salmon collected from each of eight oiled and eight reference streams in southwestern Prince William Sound in 1993 and 1994 (Figure 2). The resulting embryos were incubated in a common environment, after which mortality was assessed. Care was taken to select oil-contaminated and reference streams with similar geographic locations, physical characteristics, and pink salmon spawning times. Streams selected for this study were a subset of those included in the field sampling described in Bue et al. (1996, Figure 1).

Before the experiment, we estimated that gamete collection and the subsequent crosses for four streams would constitute 1 d of work; consequently, we estimated it would take 4 d to complete the experiment. Therefore, the experiment was designed in a blocked fashion in which each day of gamete collection and fertilization constituted a block. All gamete collections, matings, and incubator loadings were conducted in an identical fashion for all streams.

Adults were captured in the stream mouth by means of a beach seine and held in shallow water. Only gametes from ripe individuals (adults that readily extruded eggs or sperm when gently massaged) were taken. Eggs (approximately 1,500) from each female were removed by excising the abdominal wall and allowing them to flow directly into a 1-L Zip-Lock plastic bag. The 30 bags of eggs were then sealed and packed on cotton towels over a 10-cm layer of wet ice in insulated ice chests. Sperm samples from each male (2–3 mL) were placed into a 15-mL plastic centrifuge tube and capped; the 30 tubes were placed on ice in the same chest as the eggs for that stream. When all gametes were collected from a stream, the ice chest was flown to the Armin F. Koernig Hatchery (an average 10-min flight time; Figure 2).

Construction of a stream-specific embryo pool consisting of all single-pair crosses ($30 \times 30 = 900$) began immediately after the gametes arrived at the hatchery. Crosses were made by first placing 5-mL of eggs (approximately 30 eggs) from each female into each of 30 cups (0.47 L each). After this step, each cup contained approximately an equal number of eggs from each female. Each cup of eggs was fertilized by a different male with 1-mL of sperm, followed by 100-mL of freshwater to initiate fertilization. This procedure provided each male an equal opportunity to fertilize eggs from each female. The fertilized eggs were allowed to sit for approximately 3-min, after which they were recombined into a 3-L plastic container and gently rinsed and mixed with freshwater three times.

Embryos from each day of stream sampling were placed into one of four vertical stacks of incubator trays (one stack for each day of collection). Six trays within each stack were divided into 16 equal compartments each with plastic strips (four rows by four columns). Each strip was sealed to the tray to prevent mixing of eggs and larvae between compartments. Twenty-four 100-mL samples of embryos (approximately 580 embryos) were randomly collected from each stream-specific embryo pool and loaded into separate compartments by using a randomized loading scheme.

Dead eggs in each compartment were counted and removed 36 h postfertilization, after which the trays were undisturbed for 4 weeks. Water flow to each of the four incubator stacks was maintained at 15 L/min. Each incubator stack received a so-

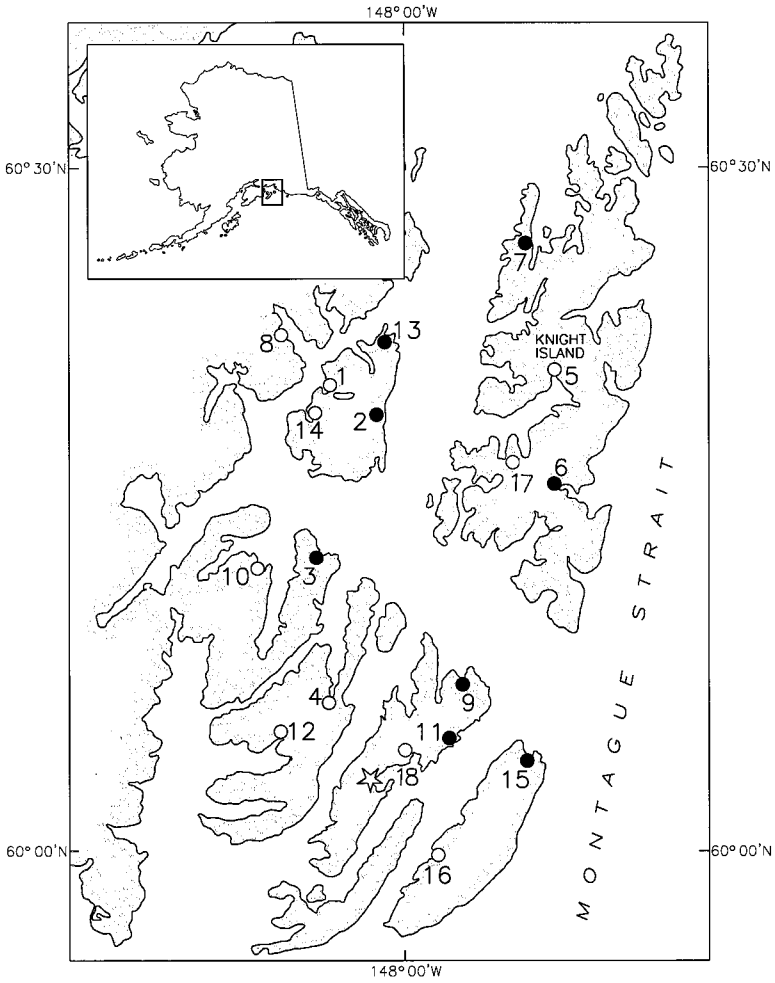

FIGURE 2.—The study area in southwestern Prince William Sound, Alaska, including approximate positions of oil-contaminated (solid circles) and reference (open circles) streams and the Armin F. Koernig Hatchery (open star).

dium chloride bath (20‰) for 20 min twice per week to control fungus.

Mortality of eyed embryos was determined and recorded when a distinct embryo eye could be seen through the chorion. Embryos at this stage were siphoned out of their compartments with clear flexible tubing (10-mm inside diameter) and allowed to drop 10–12 cm into a container of freshwater. The resulting physical shock caused coagulation of yolk material in dead embryos that allowed easier identification and removal. Live and dead embryos were gently placed back into their original compartments after siphoning. Both live and dead embryos were counted; the dead were removed and discarded. All larvae were destroyed after hatching.

A technician, who was stationed at the hatchery during the 3 months of the experiment, performed normal fish culture duties and collected mortality data. The technician was made aware of the day of collection for record keeping but did not know which incubator compartments represented oiled or unoiled streams. The statistical difference in mortality due to oil contamination was evaluated with a blocked analysis of variance.

Results

Field Monitoring

Elevated embryo mortalities were detected in oiled streams in 1993 ($P = 0.010$; Figure 3). A significant stream zone effect was also evident ($P = 0.006$), although no oil-by-zone interaction was found ($P = 0.320$). Estimated contrasts indicated

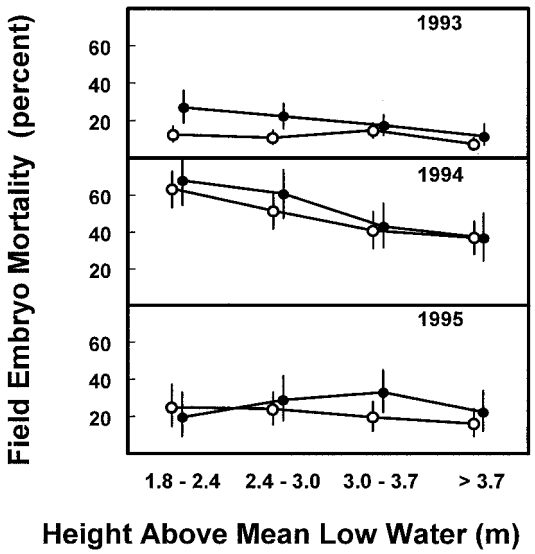

FIGURE 3.—Mean pink salmon embryo mortality observed during fall field sampling in 1993 through 1995. Solid circles indicate oil-contaminated streams ($N = 10$); open circles identify reference streams ($N = 15$); error bars represent 90% confidence intervals.

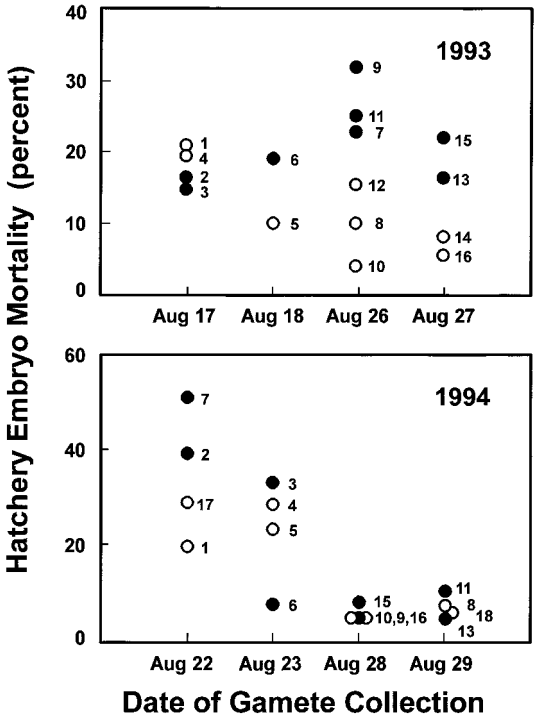

FIGURE 4.—Mean mortality of pink salmon embryos observed in the controlled incubation experiment in 1993 and 1994. Embryos were from oil-contaminated streams (solid circles) and reference streams (open circles); the number next to the circle identifies the stream location (see Figure 2).

the differences were in the two lower intertidal zones. No statistically significant difference in embryo mortality was detected in 1994 or 1995 between the oiled and reference streams ($P = 0.675$ and 0.4894, respectively; Figure 3). A significant zone effect was detected in 1994 ($P = 0.001$) but not in 1995 ($P = 0.280$), and there was no evidence of an oil-by-zone interaction for either year ($P = 0.801$ and 0.318, respectively).

Controlled Incubation Experiment

In 1993, gamete collection and subsequent fertilizations began on August 17, when four streams were sampled. Only two streams were sampled the following day due to the low number of ripe fish in the remaining study streams. Sampling was postponed until August 26, at which time ripe fish were plentiful, and six streams were sampled. Four streams were sampled the following day to complete the mating scheme. A modification of the incubator loading scheme was made for the August 26 sampling to accommodate the change from four streams to six streams. The randomized loading design was maintained, but only 18 replicate samples from the embryo pool were collected for four streams and 12 replicate samples for two streams. Embryo mortality was scored at the eyed stage on September 17, 20, 28, and October 2 for the 4 d of sampling, respectively.

Significantly elevated embryo mortalities were observed for the oil-contaminated streams ($P = 0.012$; Figure 4). Stream-specific estimates of embryo mortality were precise (Table 1), and average mortalities were 0.21 for oiled and 0.12 for reference streams.

In 1994, four streams were sampled each day (August 22, 23, 28, and 29), and embryo mortality was scored at the eyed stage on September 22, 25, 27, and 29, respectively. No significant difference in embryo mortality was observed ($P = 0.308$; Figure 4). Stream-specific estimates of embryo mortality were again precise (Table 1), and average mortalities were 0.20 for oiled and 0.15 for reference streams.

Discussion

The lack of an accurate and precise estimate of oil exposure was common to many field studies designed to evaluate the effect of the *Exxon Valdez* oil spill on animal populations. Streambed oiling was patchy rather than uniform. This observation

TABLE 1.—Estimated mean embryo mortality and corresponding SE for pink salmon embryos incubated at the Armin F. Koernig hatchery in 1993 and 1994; *N* is the number of embryo samples (about 580 embryos/sample).

Date of collection	Stream[a]	Treatment[b]	Embryo mortality		*N*
			Mean	SE	
1993 incubation experiment					
Aug 17	1	R	0.20	0.005	24
	2	O	0.16	0.006	24
	3	O	0.15	0.029	24
	4	R	0.20	0.036	24
Aug 18	5	R	0.10	0.006	24
	6	O	0.19	0.009	24
Aug 26	7	O	0.22	0.005	18
	8	R	0.11	0.006	18
	9	O	0.32	0.010	18
	10	R	0.04	0.004	18
	11	O	0.25	0.013	12
	12	R	0.16	0.007	12
Aug 27	13	O	0.17	0.011	24
	14	R	0.08	0.005	24
	15	O	0.12	0.023	24
	16	R	0.06	0.005	24
1994 incubation experiment					
Aug 22	7	O	0.51	0.004	24
	17	R	0.29	0.005	24
	2	O	0.39	0.005	24
	1	R	0.20	0.003	24
Aug 23	3	O	0.33	0.004	24
	4	R	0.28	0.005	24
	5	R	0.23	0.004	24
	6	O	0.08	0.003	24
Aug 28	16	R	0.04	0.002	24
	15	O	0.08	0.003	24
	10	R	0.04	0.002	24
	9	O	0.04	0.002	24
Aug 29	13	O	0.05	0.002	24
	8	R	0.07	0.003	24
	11	O	0.10	0.004	24
	18	R	0.06	0.003	24

[a] Stream locations are depicted by stream number from Figure 2.
[b] Treatment R indicates reference streams; treatment O indicates oil-contaminated streams.

is supported by the results of Brannon et al. (1995), in which measured PAHs fluctuated dramatically over time within oiled streams. Although they attempted to do so, Brannon et al. (1995) did not obtain a reliable estimate of field exposure. Such a measurement would have been difficult and extremely expensive to obtain.

We dealt with the lack of a quantitative estimate of streambed oiling by assigning streams to either oil-contaminated or reference categories. While our classifications were initially based on visual observations, they were reevaluated in the fall of 1989 with the results of the anadromous stream surveys conducted in southwestern Prince William Sound (Middleton et al. 1992) as well as with the data collected by the Alaska Department of Environmental Conservation–Spill Response Staff (ADEC–SRS 1989; Neff et al. 1995). With one exception, our characterization of contamination is identical to that of Brannon et al. (1995) for the nine streams present in both studies.

Field Monitoring

Elevated pink salmon embryo mortality observed in oil-contaminated streams in 1993 was consistent with previous significant differences observed annually from 1989, the year of the oil spill, through 1992 (Bue et al. 1996). No statistically detectable difference in embryo mortality was observed in 1994 or 1995, suggesting that the influence responsible for the elevated mortality was reduced.

Controlled Incubation Experiment

In our controlled incubation experiment, we detected elevated embryo mortalities in 1993 but not in 1994 for populations of pink salmon from oil-contaminated lineages. Because the field data agree with data from the controlled incubation, we concluded that naturally occurring variation in the environment could not explain the systematic significant differences in embryo mortality that persisted in post–oil spill generations.

Embryo mortalities observed in the controlled incubation experiment were slightly higher than would be expected in a production hatchery (average mortalities for the controls in 1993 and 1994 of 12% and 15%, respectively). We attributed this higher mortality to the increased handling of gametes required to make the crosses. Both oiled and reference groups were treated identically and replicated. Consequently, the difference between oiled and reference groups was of interest rather than the level of overall mortality.

Long-Term Effects

Pink salmon that spawned during the fall of 1991 were from the 1989 brood year, the brood year that incubated in oiled gravels during the fall of 1989 and spring of 1990. The 1993 and 1994 embryos were the progeny of the 1991 and 1992 broods, respectively. Continuing embryo mortality through 1993 suggests that exposed pink salmon either experienced damage to their germ line in 1989 and 1990 or that the toxicity of the oil persisted through 1991 at a level capable of causing physiological dysfunction.

That genetic damage to pink salmon populations

may have occurred as a result of the *Exxon Valdez* oil spill should not be surprising. Major chromosomal aberrations were observed in rodents inhabiting a petrochemical-polluted site (McBee and Bickham 1988). Polycyclic aromatic hydrocarbons are known to cause a variety of genotoxic responses in a variety of organisms including teleosts (Kocan and Powell 1985; Fong et al. 1993; reviewed in Van Beneden and Ostrander 1994). The link between oil pollution and damage to somatic genes is of concern for the immediate generation of the oiled population (Longwell 1977; Daniels and Means 1989; Brown et al. 1996; Hose et al. 1996). But until now, the connection has not been made between the detection of somatic damage and the possible occurrence of germ line genetic damage that may affect the viability of affected populations generations after a pollution event.

Interestingly, germ line genetic damage would probably persist in populations of pink salmon for more generations than it would in other vertebrates. Salmonids share a recent tetraploid ancestry through a gene duplication event approximately 25–100 million years ago (Ohno et al. 1969; Allendorf and Thorgaard 1984). Although some duplicate loci in salmon have been lost (Allendorf 1978; Allendorf et al. 1984), many loci are redundant, thereby masking deleterious recessive alleles. Putative lesions caused by crude-oil constituents might fail to express phenotypically until genetic assortment occurs in subsequent generations (Ohno 1970).

The possibility that the elevated embryo mortalities were due to physiological changes in pink salmon exposed to crude oil remaining in sediments in and around streams has not been assessed. Oil has been shown to have adverse effects on fish reproduction (Truscott et al. 1983; Thomas and Budiantara 1995), although these studies were conducted by treating mature fish with oil and then evaluating for differences in sexual maturation, levels of reproductive hormones, and oocyte development between treated and control fish. We found no completed studies in which embryos were treated and later evaluated for reproductive success. There is evidence that oil was in the intertidal environment in Prince William Sound in 1991 (Babcock et al. 1996), and cytochrome P-450 induction in pink salmon alevins was detected during the spring of 1991 in areas of streams oiled in 1989 (Wiedmer et al. 1996). These two studies indicate that oil was available to pink salmon in 1991 and that some exhibited a physiological response to an oiled environment (Tuvikene 1995).

We would like to reiterate that the field work described in this study and in Bue et al. (1996) was based on observational data, and we cannot definitively prove that crude oil was directly responsible for the elevated mortalities in oil-contaminated streams. We do believe there is strong evidence to suggest that the significant differences in embryo mortalities observed in 1993 were due to a parental effect. This work raises many questions concerning the effect of crude oil on reproductive potential that should be evaluated through controlled experiments. Finally, we also believe this work points to the need for long-term monitoring, beyond the generation immediately affected by a pollution event.

Acknowledgments

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