Sensitivity of Fish Embryos to Weathered Crude Oil: Part I. Low-Level Exposure During Incubation Causes Malformations, Genetic Damage, and Mortality in Larval Pacific Herring (Clupea pallasi)

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Abstract—Pacific herring eggs were exposed for 16 d to weathered Alaska North Slope crude oil. Exposure to an initial aqueous concentration of 0.7 parts per billion (ppb) polynuclear aromatic hydrocarbons (PAHs) caused malformations, genetic damage, mortality, and decreased size and inhibited swimming. Total aqueous PAH concentrations as low as 0.4 ppb caused sublethal responses such as yolk sac edema and immaturity consistent with premature hatching. Responses to less weathered oil, which had relatively lower proportions of high molecular weight PAH, generally paralleled those of more weathered oil, but lowest observed effective concentrations (LOECs) were higher (9.1 ppb), demonstrating the importance of composition. The LOEC for more weathered oil (0.4 ppb) was similar to that observed in pink salmon (1.0 ppb), a species with a very different development rate; by inference, other species may be similarly sensitive to weathered oil. Our methods simulated conditions observed in Prince William Sound (PWS) following the Exxon Valdez Oil Spill (EVO) spill. Biological effects were identical to those observed in embryolarval herring from PWS in 1989 and support the conclusion that EVO caused significant damage to herring in PWS. Previous demonstration by our laboratory that most malformed or precocious larvae die corroborates the decreased larval production measured after the spill.

Keywords—Herring larvae Morphological damage Genetic damage Polynuclear aromatic hydrocarbons Exxon Valdez oil spill

INTRODUCTION

On March 24, 1989, the T/V Exxon Valdez grounded in Prince William Sound (PWS), Alaska; the resultant oil spill was the largest in U.S. history. In the short-term aftermath of the spill, damage attributed to oil was measured in several species of fish, including herring, salmonids, and rockfish [1–4]. Herring were just beginning to spawn when the spill occurred, and about half of the spawned biomass was exposed to Exxon Valdez oil (EVO) [1]. Exposure of herring eggs to petroleum hydrocarbons frequently results in small, abnormal larvae with poor survival potential [5–7]. Herring larvae collected throughout PWS (May–July 1989) exhibited conditions associated with oil exposure, including morphologic malformations, genetic damage, and small size [8]. It is likely that such severely affected larvae die because site-specific instantaneous mortality rates were highest near oiled beaches [1]. One Natural Resource Damage Assessment (NRDA) study calculated a 52% loss in larval herring productivity for PWS in 1989 as a result of the spill [1].

However, the existence of toxic effects in herring eggs and larvae due to oil exposure remains contentious because a study funded by Exxon did not find oil-related impacts in PWS [9]. Differences in methodology may primarily explain the divergent conclusions. This investigation was undertaken to mimic post-EVO spill conditions and determine the effects of oil on herring development in a laboratory setting free from the confounding variables inevitable in field studies. We studied the responses of herring eggs exposed to weathered oil similar in composition and concentration to that observed in PWS and compared the effects on larval survival, morphology, and development with the effects reported in the NRDA studies. Specific experimental objectives were to determine if exposure of incubating eggs to polynuclear aromatic hydrocarbons (PAHs) leached from spilled oil would cause mortality, morphological effects, behavioral effects, and changes in hatch timing; if exposure of incubating herring eggs to PAH would cause genetic defects in somatic cells, effective PAH concentrations and exposure durations; and if oil weathering influenced toxicity. Experimental results support the conclusion that EVO caused significant damage to herring eggs and larvae in PWS.

MATERIALS AND METHODS

Experimental overview

Total PAH (TPAH) concentrations in water were chosen to bracket maximum mean TPAH concentrations observed in open water of PWS following the EVO spill (EVOS) (up to 6.24 ± 0.63 ppb [10]). Water was contaminated by passage through oiled gravel; eggs were exposed to PAH dissolved from oil, not oil droplets. To test weathering effects, the gravel used to produce less-weathered oil (LWO) was reused in the second experiment, resulting in more-weathered oil (MWO). The two consecutive experiments each consisted of a graded series of doses plus multiple exposure times at the middle concentration (34 ppb for LWO and 0.7 ppb for MWO). Fertile
herring eggs, attached to glass slides, were incubated in clean or contaminated seawater. We controlled for possible maternal genetic variation within each of these experiments by including eggs from each female in each treatment.

Collection of eggs

Prespawn herring were collected from two locations in southeast Alaska. Herring for the LWO experiment were collected near Cat Island (55.0°N latitude, 131.2°W longitude) on April 11, 1995. Fish for the MWO experiment were collected in Seymour Canal (57.5°N latitude, 133.8°W longitude) on May 13, 1995. Herring were spawned as rapidly as possible after capture in Ketchikan or Juneau. Ovarian membranes were cut longitudinally, and eggs were removed with a stainless steel spatula. From each female, eggs were deposited in a single layer on nine 25 × 75 mm glass slides placed in ambient seawater at the bottom of a shallow glass dish. Deposition was accomplished with a gentle swirling motion; approximately 100 to 150 eggs were deposited per slide. Slides containing eggs from each female were placed in a staining rack and suspended in separate beakers of seawater. Within 30 min of deposition, a few milliliters of milt, pooled from three males, was added to the eggs in each beaker. After 5 min, slides with eggs were removed and gently rinsed in seawater. Eggs from 15 (LWO) or 21 (MWO) females were fertilized in three separate spawning events; a total of 9 males contributed sperm in each experiment. Fertilized eggs (LWO) were transported in ambient seawater from Ketchikan to Juneau. All fertilized eggs were incubated 1 d in clean seawater before experimental treatment.

Eggs were examined for fertilization success within 5 d of spawning. Excess eggs along slide margins and those in areas more than one layer deep were removed. Processing was accomplished in clean seawater with a minimum of emersion. Remaining fertile, infertile, and dead eggs were counted. For all treatments combined, fertility was 79.0% ± 0.8 in LWO experiments and 93.1% ± 0.4 in MWO experiments.

Oil exposure conditions and chemical analysis

Seawater was contaminated by contact with oiled gravel. Water percolated upward through gravel in vertical 30-cm diameter × 122-cm polyvinyl chloride cylinders fitted with a trap to prevent slick overflow [11]. Water flowed at 5 L/min from these oil generators via glass manifold to the bottom of four 40 to 50-L treatment tanks; one cylinder was used per dose. Water flowed through oiled gravel for 5 d before any eggs were exposed. Each cylinder was loaded with 45 kg of oiled gravel (or nonoiled control gravel), except the high-oil treatment generator was loaded with 90 kg gravel. Gravel, approximately 1 to 11 mm in diameter (Md = 5.2 mm, Q1–Q3 = 4.2–6.6 mm), was washed, dried, and then contaminated by spraying measured quantities of artificially weathered Alaska North Slope crude oil on tumbling gravel.

To simulate the composition of oil stranded on PWS beaches in 1989 after the EVO spill, the oil applied to gravel was artificially weathered by heating it to 70°C overnight until its initial mass was reduced by approximately 20% [12]. The resultant PAH composition was dominated by the naphthalenes, followed by phenanthrenes, dibenzothiophenes, fluorenes, and chrysenes. In general, homologs with 2-methyl substitutions (C2) had the highest concentrations followed by C1, C3, and then the parent compounds (Fig. 1).

Eggs from each female were incubated 16 d in each of five treatments (control, trace, low, mid-, and high oil). Additional eggs (also from each female) were exposed in the mid-oil treatment for 1, 2, 4, and 8 d to generate a complete 0 to 16 d time series; controls were reused as zero exposure time these analyses. Eggs were first exposed to oil 1 d after fertilization. After 16 d, all remaining eggs were transferred to clean seawater. Eggs were isolated by female and treatment 1 d later; each slide was individually suspended in a 1-L jar filled with seawater. Temperature was controlled by placing jars in a flowing seawater bath. To facilitate oxygen exchange, slides were suspended from mobile racks. Mean temperature was 5.1°C (4.0–6.7°C) in the LWO experiment and 6.2°C (5.6–7.1°C) in the MWO experiment. Mean salinity was 32 ± 0.3 ppt. At the beginning of treatment, and every fourth day thereafter, 1 to 3 replicate 3.8-L water samples per dose were extracted and analyzed for PAH by gas chromatography-mass spectrometry (GC-MS) [13]. Analysis of PAH samples by GC-MS was performed at the National Marine Fisheries Service, Auke Bay Laboratory [13]. After addition of six internal standards, all samples were ex-
Hatch timing and success, larval viability, and larval abnormalities were observed daily during peak hatch. Observation frequency was reduced to 2- or 3-d intervals during periods of low hatch, and larvae were collected only if five or more were present per jar. Living larvae were assessed for swimming ability and gross morphological deformities, anesthetized with tricaine methanesulfonate, and preserved in 5% buffered formalin. Dead larvae were enumerated and discarded. Without exposing remaining eggs to air, water was exchanged in each jar after completion of these observations. After hatch was complete, the remaining eggs and dead embryos were inspected and enumerated.

The swimming ability of live larvae was categorized as effective, ineffective, or incapable. Effective swimmers were active, frequented the water column, and avoided capture. Ineffective swimmers were generally more lethargic and were more likely to be found on jar bottoms. Incapable larvae were unable to swim in a straight line and were often only capable of spasmodic twitching.

Uptake of PAH by eggs was determined on days 4, 8, and 16, plus days 1 and 2 in the mid-oil treatment of MWO and the high-oil treatment of LWO. Eggs pooled from several females were spawned on nylon plankton netting, fertilized with milt from several males, and attached to mobile racks. (These pooled eggs were used only for hydrocarbon samples.) Egg density was high (often multiple layers). Nets were suspended in incubation tanks separate from but identical to those with eggs on slides. Uptake was measured in all treatments in MWO, but only in control and high-oil treatments in LWO. In LWO, eggs remaining after 16 d were transferred to clean water, and depuration was followed through day 24. For each sample, ≥10 g of eggs were scraped at random from the netting, frozen, and processed using methods of Short et al. [13]. Visual inspection revealed no evidence of oil-coated eggs in any treatment. To estimate variance, three replicate high-oil treatment samples were analyzed on day 16 in each experiment. Reported concentrations of TPAH in tissue are based on wet weight.

For morphological measurement (total body length, yolk volume, and spinal curvature), five preserved larvae were randomly subsampled from each female in each treatment group. Lateral views were digitized with a video camera and a frame grabber. To minimize variance, specimens were rotated to align eyes before image capture and viewed at 6 or 12× for total length measurement and 50× for yolk measurement. Total length was measured from snout to tip of notochord, using as many line segments as necessary to approximate larval posture. Spinal curvature was estimated from the same line segments as the sum of (180° – segment angle). Yolks were generally elliptical; the major axis (yolk length) was measured parallel to the notochord, and the minor axis (yolk height) was perpendicular to the body axis. Yolk volume was estimated from length and height measures [15]. Muscle width, measured for staging purposes, was immediately dorsal to the yolk height measurement.

Edema and jaw size were scored from digital images. Yolk sac edema was indicated if the anterior margin of the yolk membrane was bounded by an area of clear fluid. Pericardial edema was indicated when the pericardium was convex ventrally or had an unusually large clear area. Jaws were classified as small if posterior to the anterior margin of the eye or absent.

Egg and larval measurements

Sensitivity of herring embryos to weathered crude oil

Extracted with dichloromethane. Isolation and purification of calibrated and uncalibrated compounds was completed by silica gel/alumina column chromatography followed by size-exclusion high-pressure liquid chromatography (HPLC) and fractionation; seawater samples were not fractionated by HPLC. Extracts of PAH were separated and analyzed by GC equipped with a mass selective detector. Calibrated PAH were identified by retention time and two mass fragment ions characteristic of each PAH and quantified using a five point calibration curve. Uncalibrated PAH homologs (which included alkyl-substituted isomers of naphthalene, fluorene, dibenzothiophene, phenanthrene, and chrysene) were identified by retention time and the presence of a single characteristic mass fragment ion. Uncalibrated PAHs were quantified by using calibration curves of their respective parent homologs. Experimentally determined method detection limits (MDLs) depended on sample weights and generally were 1 ppb in tissue and 1 to 8 ppb (nanograms per liter) in water. Concentrations below MDL were treated as 0. Tissue concentrations are reported on a wet-weight basis, but wet to dry weight ratios were measured by dehydrating 1 g wet samples for 24 h at 60°C and weighing the remaining mass. The accuracy of the hydrocarbon analyses was about ±15% based on comparison with National Institute of Standards and Technology values, and precision expressed as coefficient of variation was usually less than about 20%, depending on the PAH. Total PAH concentrations were calculated by summing concentrations of individual PAH. Relative PAH concentrations were calculated as the ratio of PAH concentration to the TPAH concentration.

Gravel used in the LWO experiment was reused in the subsequent MWO experiment. Water flow through gravel was restarted 1 d before MWO egg exposures; thus, the oil was more weathered in MWO than in LWO, and TPAH concentrations were lower (Fig. 2). Weathering parameter, w, was estimated from oil gravel using methods of Short and Heintz [14], where w represents the extent of first-order kinetic losses of PAH from petroleum and summarizes the exposure history of the sample (w = 0 in unweathered samples) and increases with weathering. Weathering was dose dependent and ranged from 0.53 to 0.04 in LWO (low- and high-oil treatments, respectively, on day 2), and 1.28 to 0.96 at the end of the MWO experiment (low- and high-oil treatments, respectively).
Genetic and additional morphologic responses in MWO tests were evaluated using blind review. Ten larvae, randomly subsampled from 15 randomly selected females, were examined at 30× magnification. The severity of skeletal, craniofacial, and finfold defects was scored using the graduated severity index (GSI) method [16], where 0 = no effect, 1 = slight defect in structure or size, 2 = moderate defect in structure or size, and 3 = severe defect in structure or size. Types of skeletal defects evaluated were kyphosis or lordosis of the notochord and notching. Craniofacial abnormalities consisted of reduction, malformation or absence of one or both jaws, ocular and otic capsule defects, and microcephaly. Reductions in the width of the dorsal and ventral finfold were recorded. Pectoral fin development was categorized as a bud (no fin rays visible) or containing fin rays. Yolk sac edema was also scored blind as a conformational observation.

During blind review, both pectoral fins were removed, placed onto a glass microscope slide, and stained with acetorcin [16]. All mitotic figures in a fin were enumerated at 1,000× magnification, and anaphase-telophase (AT) configurations were visually assessed for evidence of chromosome/chromatid breakage (bridges and attached fragments), multipolar spindles, and aneuploidy (unequal masses of chromatin in daughter cells). Anaphase aberration rate was calculated for each dose treatment by dividing the total number of aberrant ATs by the total number of ATs present. The number of mitoses per fin was recorded for each larva.

**DATA ANALYSIS**

The percentage of infertile eggs was based on the initial number of eggs. Percentages of eggs that hatched or died were based on the total hatched larvae plus the number of dead eggs determined at the endpoint. Hatched larvae were categorized as live, moribund, and dead, and percentages were based on the total number hatched. Swimming ability of live larvae was categorized as effective, ineffective, or incapable (moribund and dead larvae were not evaluated), and percentages were based on the number of live larvae. The percentage of larvae with spinal aberrations was based on the total of live and moribund larvae. Peak and median hatch were estimated by female parent and averaged.

Scored data (e.g., skeletal defects) were translated into incidence of defective larvae; larvae were either considered normal (score 0) or abnormal (score >0). Additionally, scored data were analyzed directly with the Kruskal-Wallis nonparametric test.

To determine if responses in specific treatments were significant, analysis of variance (ANOVA) techniques were used. The experimental design was a randomized block; where the overall test was significant, treatments were compared to controls with a priori multiple comparisons. Before ANOVA, percentage data were arc-sine transformed and corrected for small sample size where necessary [17]. (The same general conclusions were reached with untransformed data). In cases where multiple larval observations occurred for each female (e.g., larval lengths), larvae were nested by female to avoid pseudoreplication.

Median effective concentrations (EC50s) were estimated from logit fits by female ($x = \ln(\text{initial TPAH})$) and averaged. Analyses were restricted to cases where response in controls and highest treatments spanned 50%, and $r^2 \geq 0.45$. Results of these analyses produced EC50 values comparable to graphical estimates. Comparison of EC50s was accomplished with ANOVA.

Biological responses were regressed against time. In LWO tests, percentage data were analyzed with logistic regression. In MWO tests, linear regression was more appropriate.

To describe changes in PAH composition as a function of time, percentages of homologous families (naphthalenes, fluor- enes, dibenzothiophenes, phenanthrenes, and chrysenes) were regressed against the total number of days water passed through gravel. Models tested were ladder of powers ($x$-transformations from linear through $-1/x^2$ and $y = ae^x$). No single model fit all data adequately.

**RESULTS**

**Oil exposure**

Aqueous TPAH concentrations declined over time during the two consecutive experiments (Fig. 2). Initial aqueous TPAH concentrations in high-, mid-, low-, and trace-LWO treatments were 85.9, 34.3, 9.11, and 1.72 ppb, compared with concentrations of 0.04 to 0.05 ppb in control treatments. Aqueous TPAH concentration in the high-oil treatment declined 75% to 21.4 ppb by the end of the 16-d exposure period. Concentrations of the lower LWO treatments declined more than 90% to final concentrations that ranged from 1.92 to 0.14 ppb. Concentration declines continued in the MWO experiment. Aqueous TPAH concentration in the high-oil MWO treatment declined an additional 84% from 7.6 ppb to 0.84 ppb during the 16-d exposure period. Initial concentrations in the lower MWO treatments ranged from 0.72 to 0.14 ppb TPAH and generally declined during the exposure period. Aqueous concentrations reported throughout the remainder of the article refer to the initial TPAH concentration.

The relative abundances of aqueous PAH changed throughout the two experiments. The predominant PAH initially present were the smaller and less substituted PAH (Fig. 1). Relative PAH abundances progressively shifted to larger and more substituted PAH during the LWO and MWO exposure periods. Thus, less substituted naphthalene homologs were the most abundant PAH initially, but more substituted phenanthrene homologs were most abundant by the end of the MWO exposure as smaller PAH were exhausted from the oil gravel (Fig. 1). The percentage of naphthalenes consistently declined with time ($-0.97 \leq r \leq -0.92, p < 0.001$), whereas percentages of other homologs consistently increased, particularly of phenanthrenes and chrysenes ($0.81 \leq r \leq 0.99, p \leq 0.002$). Smaller PAH were also exhausted from gravel more rapidly at progressively lower oil treatments. Consequently, aqueous concentrations of smaller PAH were greater in LWO tests than in MWO tests, but concentrations of larger PAH were similar. Specifically, concentrations of C3-fluorines, C3-dibenzothiophenes, C3- and C4-phenanthrenes, and chrysenes in the mid- and high-oil treatments of MWO were roughly the same as in corresponding LWO treatments.

Maximum TPAH concentrations in eggs were higher in LWO treatments than in MWO treatments (Fig. 3), and the relative abundance of PAH accumulated by eggs resembled that in contaminated water. In LWO, TPAH concentrations in eggs increased throughout the 16-d exposure and peaked at 13,700 ppb (high-oil treatment). When contaminated eggs were transferred to clean water (days 16–24), PAH depurated exponentially from egg tissue. Naphthalene homologs consistently accounted for more than 80% of the TPAH concentration in eggs throughout LWO exposure and depuration. In contrast,
Sensitivity of herring embryos to weathered crude oil

Fig. 3. Total polynuclear aromatic hydrocarbon (TPAH) concentrations in eggs as functions of exposure time. Treatments are control (C), low (L), mid- (M), and high oil (H). In less weathered oil (LWO), tissue concentrations were only measured in control and high-oil treatments. For clarity, the trace-oil concentration of more weathered oil (MWO) was not displayed. Arrows indicate when eggs were transferred to clean water. Variance was estimated in high-oil treatments on day 16 ($n = 2$); error bars are ±SE.

TPAH concentrations in eggs exposed to the mid- and high-MWO treatments peaked at 226 and 1,020 ppb on day 4 of the exposure period and then declined. Naphthalene homologs consistently accounted for less than 60% of TPAH accumulated by eggs exposed to the high-MWO treatment and for less than 35% of TPAH accumulated during exposure to the mid-MWO treatment. Arithmetic mean TPAH concentration in low-MWO eggs (22 ppb) was twice that in control eggs and was significantly greater than in control eggs ($p = 0.010$). All differences in PAH composition between tissue and water phases were less than 17% and were usually less than 10%.

Biological response to more weathered oil

Exposure of eggs to MWO significantly reduced incubation time ($p < 0.001$). Peak and median hatch time were significantly shorter in the highest three treatments (0.4–7.6 ppb TPAH) than in controls ($p \leq 0.003$), and median hatch time was also significant in the 0.1-ppb MWO treatment ($p = 0.010$). Mean incubation time was reduced 2.6 d in the high-oil treatment.

Egg and larval mortality increased significantly as exposure concentration increased ($p < 0.001$). Egg mortality was significantly elevated at 7.6 ppb TPAH ($p < 0.001$), and larval mortality was significantly elevated at TPAH concentrations $\geq 0.7$ ppb ($p \leq 0.011$) (Fig. 4). Differences between the control and high-oil treatment response were 12% for dead eggs and 2% for moribund plus dead larvae.

Exposure of eggs to low levels of MWO during incubation caused morphological abnormalities in larvae. Percentages of larvae with yolk sac edema were significantly elevated at TPAH concentrations $\geq 0.4$ ppb ($p \leq 0.002$) (Fig. 5). Blind assessment of yolk sac edema produced similar results; percentages of larvae with yolk sac edema were significantly elevated in the 0.4- and 0.7-ppb TPAH treatments ($p \leq 0.012$); the 7.6-ppb treatment was not analyzed. Percentages of larvae with abnormally small jaws were significantly higher than in controls following exposure to TPAH concentrations $\geq 0.7$ ppb ($p < 0.042$). Percentages of larvae with pericardial edema or spinal defects were significantly elevated TPAH concentrations $\geq 0.7$ ppb ($p < 0.001$). Mean differences in response between high-oil treatment and control were 91% (yolk sac edema), 88% (small lower jaws), 43% (pericardial edema), and 58% (spinal defects).

Larval swimming ability was significantly reduced by exposure of eggs to MWO during incubation ($p < 0.001$) (Fig. 5). Percentages of effective swimmers were significantly reduced relative to controls at TPAH concentrations $\geq 0.7$ ppb ($p < 0.001$). The difference in response between the control and the 7.6-ppb TPAH treatment was 67%.

Skeletal and craniofacial defects, finfold defects, and failure to develop pectoral fin rays were semiquantitatively assessed in the control, 0.4-, and 0.7-ppb TPAH treatments of MWO
Fig. 5. Incidence of abnormalities and swimming ability as functions of initial total polynuclear aromatic hydrocarbon (TPAH) concentration. Incidence of yolk sac edema was verified by blind assessment in the more weathered experiment. Curve fits were estimated with logistic regression. Data displayed are means ± SE; r equals estimated correlation coefficients. Solid symbols indicate significant differences from controls.

Fig. 6. Incidences of morphological deformities and genetic aberration as functions of initial total polynuclear aromatic hydrocarbon (TPAH) concentration in a random subsample of larvae from the more weathered of two consecutive experiments. Curve fits were estimated with logistic regression. Data displayed are means ± SE; r equals estimated correlation coefficient. Solid symbols indicate significant differences from controls.

(Fig. 6). Incidences of defects were significantly elevated at 0.7-ppb TPAH in every case (p < 0.001). The most frequently observed skeletal defects were kyphosis or lordosis of the notochord followed by stunting. Craniofacial abnormalities consisted of the reduction or absence of the lower jaw and, less frequently, the upper jaw. Occasionally smaller brain size (microcephaly) or reduced retinal pigmentation was also present in severely affected larvae. Most individuals with yolk sac edema also had reductions in the width of the dorsal and ventral finfolds. Incidences of larvae without fin rays (only pectoral fin buds present) were significantly elevated after exposure to 0.4- and 0.7-ppb TPAH (p ≤ 0.034). Severity of defects, estimated nonparametrically from GSI scores, also increased significantly as oil concentration increased.

Exposure of eggs to TPAH concentrations ≥0.7 ppb in MWO significantly reduced larval length and increased spinal curvature and yolk sac volume (p ≤ 0.006) (Fig. 7). Compared to that of controls, larval length in the 7.6-ppb TPAH treatment decreased by 2.4 mm, spinal curvature increased by 100°, and yolk sac volume increased by 0.12 mm³.

Genetic response to more weathered oil

Chromosomal damage was evaluated in control, 0.4-, and 0.7-ppb treatments of MWO (Fig. 6). The anaphase aberration rate was significantly elevated to 10% at 0.7 ppb TPAH compared to 6% in controls (p = 0.008). The number of mitoses per pectoral fin averaged 11 and was not significantly affected by exposure to TPAH concentrations ≤0.7 ppb.

Comparison of biological response to less and more weathered oil

Biological response to LWO was the same as that to MWO, but higher initial TPAH concentrations were necessary to elicit a significant response. As LWO exposure concentration increased, incubation time decreased, and egg and larval mortality increased (p < 0.001) (Fig. 4). Morphological abnormalities increased as LWO concentration increased, and swimming ability was reduced (p < 0.001) (Fig. 5). Exposure of eggs to LWO significantly reduced larval length, and spinal curvature and yolk sac volume were significantly elevated (p ≤ 0.029) (Fig. 7).

Lowest observed effective concentrations (LOECs) for MWO were generally more than an order of magnitude lower than in LWO. Significant differences in biological responses between the two experiments as a function of concentration is clearly evident in Figures 4, 5, and 7. Responses using LWO were always significant at concentrations ≥34 ppb and occasionally significant at 9 ppb.

The magnitude of biological response to PAH was generally greater in LWO tests than in MWO tests (Figs. 4, 5, and 7), except that the incidence of pericardial edema was about the same or slightly less than after exposure to MWO (Fig. 5). However, pericardial edema induced by LWO tended to be masked by prominent downward rotation of the head. Malformed larvae were less able to swim normally, and spinal condition was the most important predictor of swimming ability (partial r² = 0.90, p < 0.001). Tested with stepwise...
Fig. 7. Larval length and curvature, and yolk volume as functions of initial total polynuclear aromatic hydrocarbon (TPAH) concentration. Data displayed are means ± SE. Solid symbols indicate significant differences from controls.

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*SE, standard error; n, number of calculable observations; NC, not calculable; —, not tested. Lowest observed effective concentrations (LOEC) were determined by analysis of variance (with 15 or 21 observations per treatment in LWO and MWO, respectively).*
MWO, exposures as brief as 4 d elicited significant responses in several cases (spinal defects, reduced jaw size, reduced swimming ability, and yolk sac edema [blind assessment only]) (Fig. 8). For most other responses, an 8-d exposure caused significant differences, except in skeletal deformity, spinal curvature, and yolk volume. The significant 2-d peak hatch response was inconsistent with other measurements. However, consistently significant 2-d responses were observed in LWO.

DISCUSSION

This is the first report of biologically significant, dose-related sublethal effects and mortality in teleost embryos consistently occurring after exposure to aqueous PAHs in the low parts-per-billion range. Exposure of herring eggs to low concentrations of MWO (0.7–7.6 ppb) during incubation caused mortality as well as a number of sublethal effects, including malformations, reduced swimming ability, and genetic damage. Test results were similar with LWO, but LOECs were higher (9–34 ppb).

Laboratory oil exposure

Embryonic herring consistently responded to a suite of aqueous PAH in the low parts-per-billion range that resembled the PAH composition encountered in PWS after the EVOS [14]. Time-to-hatch declined with increasing TPAH concentration; exposed larvae were physiologically more immature and smaller than unexposed larvae. Teratogenic effects included dose-related increases in the incidence and severity of skeletal, craniofacial, fin, cardiovascular, and yolk sac malformations. Most of these adverse responses have been reported elsewhere following exposure to higher oil concentrations [e.g., 6, 7, 16]. However, it is clear from our study that environmental persistence of the larger, more toxic PAHs can be injurious at very low concentrations. In the discussion that follows, we refer to concentrations observed in the MWO experiment unless otherwise stated. Observed skeletal, cardiovascular, and yolk sac malformations appear sufficient to decrease survival [6]. The larval mortality threshold appears to be between mean egg TPAH concentrations of 22 ppb (no observed effect concentration) and 108 ppb of the MWO (LOEC).

There were also significant sublethal effects (yolk sac edema and both physical and temporal evidence of premature hatching) after exposure to only 0.4 ppb TPAH (MWO) that were not accompanied by increased mortality. Mean TPAH concentrations in these eggs were 22 ppb, significantly higher than in controls (11 ppb). The observed reduction in jaw and fin maturity probably reflected precocious hatching [18] and might be reversible with continued development. The concurrent induction of yolk sac edema demonstrates that exposure to 0.4-ppb TPAH causes toxicity, not merely a hormetic or arguably beneficial effect from reduced incubation time [19]. Acites (edema) was also observed in pink salmon (On-
corhynchus gorbuscha) larvae similarly exposed to aqueous PAH [20,21].

Of the observed abnormalities, edema appeared to be responsible for most of the larval mortality. Edema was induced in larvae exposed to initial TPAH concentrations as low as 0.4 ppb (MWO) and was apparent after only a 4-d exposure to 0.7 ppb TPAH. Middaugh [22] reported that cardiovascular malformation (edema or tube hearts) in Menidia beryllina was their most sensitive measured response and was significantly elevated at the lowest dose of water soluble fraction (WSF) of Alaska North Slope crude oil tested, 75 ppb. These conditions resulted in reduced cardiac output and cessation of circulation. The incidence and severity of edema in our study were directly related to exposure duration and dose. In mild cases, slight edema was visible only in the yolk sac; more severe cases were accompanied by increased fluid within the pericardial cavity and the ventricular spaces of the nervous system. Edema in the embryoloral stages apparently results from ionic disturbances and does not require metabolic activation of PAH by the cytochrome P450 system [19].

Two important consequences of the observed sublethal effects would be impairment of swimming and feeding in oilexposed larvae. Although spinal deformation appeared to be the dominant factor, observation of live larvae suggested that edema also adversely affected swimming and would inhibit prey capture. Larvae with enlarged yolk sacs had greater difficulty orienting in the water column than normal larvae and swam more slowly, even with apparent vigorous exertion. Most individuals with yolk sac edema also had reductions in the width of the dorsal and ventral finfolds, which are the respiratory surfaces in pelagic fish larvae [23]. Physical and physiological changes accompanying edema (decreased blood flow to tissues, interference with nervous system function, and increased energy expenditures) reduced finfold surface area, and retarded pectoral fin development undoubtedly contributed to decreased larval swimming ability. As spinal curvature became more pronounced, larvae were less able to swim normally; at the extreme, larvae were only capable of swimming in a circle or spasmodic twitching—movement that resulted in no directed motion. The development of yolk sac edema, spinal deformities, and swimming problems were correlated temporally and in terms of dose, implying that these abnormalities had a causal role in the loss of swimming ability. Oil-exposed eggs hatched early, and embryonic growth was retarded, as indicated by shorter body length, larger yolks, smaller lower jaws, and immature pectoral fins. Because swimming ability improves with maturity, immature larvae are less capable of prey capture and predator avoidance than mature counterparts [23]. However, older larvae with residual edema are less likely to feed successfully [24]. Recent studies with dioxin, a compound that induces malformations in fish larvae identical to those of oil exposure, have demonstrated reduced functioning of the digestive system at levels lower than those that induce edema [25] as well as decreased protein synthesis in both fish larvae and cultured cells [19].

Genetic damage was also induced in oil-exposed larvae following exposure to an initial TPAH concentration of 0.7 ppb (MWO). The genotoxicity endpoint (anaphase aberration rate) measured microscopically visible chromosome/chromatid breaks and bridges during the later stages of mitosis. This method has been previously used to demonstrate genotoxicity in field studies of the Exxon Valdez [1,16] and Argo Merchant oil spills [26], the New York Bight [27], and sediment from the San Francisco Bay [28]. In the first two cases, mitotic aberrations were quantitatively or spatially related to oil. In the sediment toxicity study and in a previous experiment using an oil-water dispersion (OWD) of Prudhoe Bay crude oil [7], the anaphase aberration rate was significantly correlated to TPAH concentration.

The consequences of the low level of genetic damage observed in this study cannot be predicted with any certainty but might include reductions in successful cell division and growth. A previous study found that TPAH concentrations of approximately 130 ppb wet weight (range: 34–844 ppb) were associated with increased defects and mortality of early winter flounder embryos [29]. In that study, the only PAH that yielded strong statistical correlations with mitotic effects and mortality was 1-methylphenanthrene with a mean concentration of 1 ppb wet weight; this PAH is highly toxic in its unmetabolized form [30]. In our study, maximum 1-methylphenanthrene concentrations in egg tissue exceeded 1 ppb in the upper two treatments of MWO and peaked at 32 ppb (wet weight) in the 7.6-ppb treatment of MWO. (In the 86-ppb treatment of LWO, 1-methylphenanthrene concentrations in eggs ranged from 20 to 74 ppb.)

Mechanisms of polynuclear aromatic hydrocarbon toxicity

The induction of deleterious sublethal effects and mortality in herring eggs by exposure to low concentrations of oil appears to conflict with prior studies in our laboratory that indicated far greater concentrations of WSF were required to cause toxicity. However, differences in hydrocarbon composition explain toxicity differences. The LOEC for egg mortality was between 1,000 and 1,400 ppb WSF of Cook Inlet crude oil ([31], concentration reanalyzed with the authors’ permission) with a composition of 82% mono- and 18% diaromatic hydrocarbons. Larval effects were evident at WSF concentrations in the parts-per-million range [31]. In contrast, the oil used in this study had higher proportions of multiring PAH and alkyl-substituted homologs, compounds that are more toxic to fish eggs than the mono- and diaromatics [32]. The composition of PAH accumulated by herring eggs generally mirrored that in water, but concentration in tissue was roughly two orders of magnitude greater than in water.

Comparison of biological responses to MWO and LWO supports the contention that differential toxicity resulted from alteration in PAH composition. For example, comparing the two oil treatments that had very similar TPAH concentrations (9 ppb in LWO and 7.6 ppb in MWO), the MWO, which contained proportionately more high molecular weight PAHs, proved more toxic. It was clear, particularly at higher concentrations, that the higher molecular weight PAHs were more refractory and were present in water passed through the MWO at concentrations roughly equal to those in water passed through LWO. Increased alkyl-substitution also increased compound persistence. Increases in toxicity of PAH with increasing molecular size and alkyl-substitution has been documented in other studies and summarized [33].

Although differences in PAH composition appear to best explain sensitivity differences between LWO and MWO tests, we recognize that differences in parental stock, collection times, temperature, and other uncontrolled factors could potentially confound comparison. Increased temperature, for example, reduced overall incubation time in the MWO test with respect to the LWO test (Fig. 4), but within-test dose responses were consistent, and between-test response patterns were sim-
ilar. The conclusion that MWO is more toxic than LWO remains unchanged if we postulate (based on poorer fertility rate) that the eggs in the LWO experiment were more susceptible to toxic effects than eggs in the MWO experiment. Conversely, there was no evidence to suggest that eggs in the MWO experiment were more vulnerable to oil than eggs in the LWO experiment. In support of the conclusion that differences in PAH composition primarily explain differences between LWO and MWO tests, similar differences in sensitivity between LWO and very weathered oil were observed in pink salmon embryos originating from the same homogeneous stock [21]. Although multiring PAHs may require activation via the cytochrome P450 system to exert toxicity [29,34], alkyl-substituted homologs have received less study and may be directly toxic [30]. The absolute activity of the cytochrome P450 system appears to be negligible until soon after hatching, when a burst of dose-dependent inducible activity occurs [34]. Thus, it is likely that the embryonic effects observed here (premature hatching and defects) instead resulted from cellular oxidative damage and membrane destabilization [25].

Accumulation of TPAH by herring eggs is consistent with first-order kinetics and is determined by the rate of aqueous TPAH concentration decline during exposure. Individual PAH concentrations decline in oil-contaminated seawater according to a first-order kinetic process [14]; thus, the rate of TPAH concentration decline is also approximately first order [21]. Similarly, rates of PAH accumulation and depuration by herring eggs follow first-order kinetics. The solution of the differential equation describing these combined processes indicates that the time of maximum TPAH concentration in eggs depends on the rate of aqueous TPAH concentration decline [21]. Consequently, the more rapid decline of the aqueous TPAH concentrations during the MWO experiment (Fig. 2) explains why maximum TPAH concentration in exposed eggs was earlier in the MWO test than in the LWO test (Fig. 3). This pattern of TPAH accumulation was, therefore, comparable to that observed for similarly exposed pink salmon eggs [21], and both were approximately consistent with the assumed first-order kinetic processes.

Enhanced toxicity of PAHs as a result of photoactivation by UV light has been well documented [e.g., 35], but our apparatus was arranged to minimize the possibility of activation, and the intensity of ambient UV light probably did not activate a significant fraction of the PAH molecules. However, photoactivation in PWS after the EVOS was more likely because of direct exposure of PAH to sunlight. Thus, the LOECs we measured (0.4 ppb, MWO) may actually be conservative compared with conditions existing after the EVOS. Photoactivation definitely did not enhance toxicity in a similar pink salmon study [21] because incubation was accomplished in darkness; yet, LOECs (1 ppb) were similar to those in our study.

In the only other published study demonstrating adverse embryolarval effects at low parts-per-billion oil concentrations [6], similar effects were produced, although the exposure method differed from that reported here. In that study, herring eggs were exposed for 24 h to mechanically dispersed, fresh, Prudhoe Bay crude oil. Aqueous exposure to concentrations ≥4.4 ppb total hydrocarbons caused larval abnormalities, but toxicity was attributed to adherence of microdroplets to the eggs and interference with gas exchange. In contrast, eggs in our experiment were exposed to PAH dissolved in the water (indicated by enrichment of PAH relative to that in parent oil), and eggs were not physically coated by oil, demonstrating that chemical toxicity alone may cause larval abnormalities. Similarly, another researcher concluded that tissue uptake of PAH by pink salmon eggs and alevins from oil-coated gravel was mediated by dissolution of oil in water; significant biological effects were observed at 4.4 ppb [20]. These effects included mortality, spinal deformation, ascites, and opercular hypoplasia. In a follow-up experiment, salmon eggs and alevins were exposed to only aqueous PAH and thus were not in direct contact with the oil-coated gravel [21]. Results of this aqueous exposure were the same as those of simultaneous direct exposures and confirmed that toxicity was mediated by dissolution of PAH [21].

The consistency of response in two dissimilar species, Pacific herring and pink salmon, suggests that these results may be generally applicable to other species of fish. Although both species spawn intertidally, herring eggs are much smaller (1.2–1.5 mm diameter) and adhere to surfaces [36]. Pink salmon eggs are 4.0 to 7.9 mm in diameter and are buried an average of 20 to 30 cm in rocky substrate [37]. Development to hatch in Pacific herring is complete in approximately 27 to 32 d at 5.1°C, and larvae are planktonic. In contrast, development of pink salmon is much slower, 171 d at 5.6°C (mean temperature), and hatched larvae remain buried in gravel for about 142 d; oil exposure continued through emergence in the pink salmon experiment [21]. Despite large differences in development time, habitat, and exposure time, LOECs for sublethal responses to MWO were remarkably similar (0.4–0.7 ppb in herring, 1.0 ppb in pink salmon [21]). Many of the observed abnormalities likely began early in development in both species. The incidence of abnormalities in herring was consistently elevated within 8 exposure days or less, but time to cause abnormalities was not recorded in the pink salmon experiment [21]. As was the case for herring, previously reported sensitivity of pink salmon alevins to WSF (mono- and di aromatics) was in the low parts-per-million range [e.g., 38]. Furthermore, as in herring, PAH from MWO exerted greater effects than PAH from LWO at similar concentrations. For example, 1.3 ppb TPAH from relatively unweathered oil had no discernable effects on pink salmon embryos, whereas water with 1.0 ppb TPAH from weathered oil resulted in reduced survival and growth [21]. It is apparent that the composition of the PAH to which developing eggs is exposed is of prime importance, and it is likely that other species are similarly sensitive to high molecular weight or alkyl-substituted PAH.

We encourage further investigation to explore this hypothesis.

**Relationship to 1989 Exxon Valdez oil spill results**

The intent of this study was to expose herring eggs to oil of similar composition and at concentrations in the range encountered in PWS following the EVOS; thus the oil chosen for study (Alaska North Slope crude oil) and the toxicant delivery method were designed to mimic conditions observed in PWS. After the EVOS, TPAH concentrations in open seawater ranged up to 6.24 ppb, and concentrations up to 1.59 ppb were present 5 weeks after the spill [10]. It is likely that concentrations were even higher in intertidal spawning areas for the herring where oil was stranded on beach substrate and resuspended by each tide or wave turbulence. In our MWO experiment, TPAH concentrations slightly exceeded values reported by Short and Harris [10] only in the highest treatment (7.6 ppb TPAH). In addition, the concentrations we report were based on initial TPAH concentrations. Because laboratory con-
Sensitivity of herring embryos to weathered crude oil

Exposure of herring eggs in PWS probably occurred via two routes, from dissolved PAH and from direct contact with oiled substrates. This experiment, as well as recent studies using Alaska North Slope crude oil [21,22] and soot extracts [19], demonstrate that embryolarval toxicity can result from exposure to dissolved PAH. In 1989, dissolved PAH were bioavailable within the PWS oil trajectory to a depth of 25 m [41]. Early life stages are also sensitive to very low concentrations of dissolved or particulate oil with significant biological effects occurring at 4.4 ppb in both herring [6] and pink salmon [20] and at 0.4 ppb TPAH in this study.

Sublethal effects identical to those described here were consistently observed in herring larvae of different ages from oiled areas within PWS during spring 1989 [1,8,16]. These included temporal and physical evidence of premature hatching, small size, malformations including edema, and genetic damage. In oiled areas of PWS in 1989, herring larvae hatched approximately 3 d earlier, at a more immature stage, than in unoiled areas [1]. Although altered hatching dynamics, stage at hatch, and certain malformations can also be caused by natural stressors such as extreme salinity, temperature, or desiccation [42,43], environmental conditions during 1989 were not unusual and were well within optimal ranges for herring development. Edema could not be evaluated in larvae hatched in the laboratory [16], but residual edema was present in field-collected larvae at 1 to 3 weeks posthatch [24]. In newly hatched larvae, genetic damage was significantly correlated to the EVO–PAH concentration in adjacent mussels in a dose-dependent fashion [16]. Incidences of genetic damage and other malformations were significantly elevated in oiled areas relative to unoiled sites and decreased over time until no measurable differences remained in 1990 [1,8,24].

We expect that most of the abnormal and precocious herring larvae observed in PWS in 1989 did not recover, but rather died prematurely. In a study where similarly deformed pollock larvae were observed for a longer period of time, abnormalities became more pronounced with time, and the majority of abnormal larvae died [44]. Field observations in PWS also suggested premature death of severely affected larvae, because incidence and severity of malformations decreased in larval populations sampled over time. Newly hatched larvae more frequently displayed skeletal bends, absent jaws, and microcephaly than did pelagic larvae [16,24]. Jaws were absent only in the smallest, least mature larvae, and it is assumed that these larvae died after their yolk reserves were utilized. It is also likely that larvae with severe skeletal bends and craniofacial defects would succumb to predators. Manifestations of retarded development (immature pectoral fins and jaws) were generally the only types of morphological defects present in older larvae [8,45]. Larvae with reduced jaws might later recover their ability to feed because jaws continue to grow throughout the larval period [46]. However, Marty et al. [24] found that larvae from oiled areas in PWS, which had higher incidences of ascites (the histological equivalent of edema), also grew less and had significantly less food within their gastrointestinal tract than did larvae from unoiled areas. Growth rates of larval herring from PWS were extremely low (from 0.10 to 0.17 mm/d), bordering on rates found in starvation studies [8,24,47].

Although the existence of toxic effects in PWS herring eggs and larvae has been disputed [9], TPAH concentrations in eggs measured at many locations in 1989 appear sufficient to elicit toxicity, based on the LOECs estimated here (22–108 ppb in egg tissue). Herring eggs collected from some oiled sites in PWS in 1989 and 1990 had TPAH concentrations in excess of 100 ppb [9]. In contrast to Pearson et al. [9], who did not find any consistent, significant correlations between morphological responses and oil exposure, the Natural Resource Damage Assessment studies [1,16] observed morphologic and genetic responses identical to ours among herring larvae of differing ages that decreased with distance and time from the spill. Although it is impossible to directly relate EVO–TPAH concentrations in ambient seawater, mussel tissue, and herring eggs, it is likely that biologically effective oil concentrations were exceeded at many sites within the oil trajectory.

Following the spill, egg to larval mortality was three to six times higher at oiled sites in PWS than at unoiled sites [47]. Although the numbers of eggs deposited were approximately equal at oiled and unoiled sites, estimated larval production was 99.9% less in oiled areas [1]. Because oiled sites tended to be more subject to wave turbulence than unoiled sites, however, oil exposure may not have been the only significant factor that affected mortality rates. Using these differential mortality rates, an overall 52% loss in larval productivity was estimated in PWS following the EVOS [1]. Based on the number of eggs deposited, prediction was that the 1989 year class would be strong, but it was one of the smallest cohorts observed in PWS in over 20 years [1]. However, high natural variability in recruitment precludes a conclusion that the population was affected by oil. Conversely, the same variability also precludes the conclusion that the oil spill did not have an effect on the herring population.

In summary, the findings that very low aqueous TPAH concentrations (0.4 ppb) are detrimental to herring eggs and larvae, that low concentrations of EVO in PWS caused larval mortality, and that another teleost species (pink salmon) is similarly sensitive [21] suggests that current water quality standards are not adequate to protect sensitive early life stages. The State of Alaska standard, 10 ppb, is the lowest state standard in the United States for total aqueous aromatics [48] and was established at 1% of the lowest dose known to kill fish and invertebrates in short-term or chronic exposures [49]. In contrast, the U.S. Environmental Protection Agency acute water quality criterion is 300 ppb TPAH. Results of this study demonstrate that PAH composition is as important as total concentration and should be considered in regulatory guidelines. Composition and weathering of PAH in our experiments was consistent with a major oil spill as shown by Short and Heintz [14]; this suggests general applicability to other spill situations. Mean aqueous TPAH concentrations from 0.9 to
6.2 ppb were present at heavily oiled beaches within 2 weeks of the EVOS [10]; concentrations well above those capable of eliciting both acute and sublethal effects in Pacific herring and pink salmon. Our results underscore the necessity to update nationwide and state standards for dissolved aromatics to reflect realistic exposures to critical life stages.

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Sensitivity of herring embryos to weathered crude oil

Environ. Toxicol. Chem. 18, 1999 493


