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Environmental Toxicology and Chemistry, Vol. 19, No. 6, pp. 1649–1659, 2000
Printed in the USA
0730-7268/00 $9.00 + .00

EXPOSURE OF PACIFIC HERRING TO WEATHERED CRUDE OIL: ASSESSING EFFECTS ON OVA

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(*Received* 2 *June* 1999; *Accepted* 4 *November* 1999)

Abstract—In order to determine if exposure to *Exxon Valdez* oil would adversely affect progeny, reproductively mature Pacific herring were confined in water contaminated with weathered crude oil. Progeny were generally not affected by a 16-d parental exposure to initial aqueous concentrations of ≤58 μg/L total polynuclear aromatic hydrocarbons (PAHs), yielding concentrations of up to 9.7 μg/g in ova. In contrast, previous research indicated that a 16-d direct exposure of herring eggs to similarly weathered oil was detrimental to developing embryos at total initial PAH concentrations of 9 μg/L. Progeny of exposed fish could have been insulated from toxic effects for two reasons. First, as an apparent result of partitioning and metabolism in parental tissues, lower concentrations and less toxic PAHs were preferentially accumulated by ova (primarily naphthalenes; 84–92%). Second, peak exposure concentrations occurred before cell differentiation. The opposite was true for directly exposed eggs; the more toxic multi-ring PAHs (e.g., phenanthrenes and chrysenes) and alkyl-substituted homologues were accumulated, and internal concentrations increased during cell division, differentiation, and organ development. Thus, Pacific herring embryos are more critically sensitive to oil pollution than are gametes.

Keywords—Pacific herring Gametes Larvae Polynuclear aromatic hydrocarbons *Exxon Valdez* oil spill

INTRODUCTION

All life stages of Pacific herring (*Clupea pallasi*) were likely exposed to the crude oil that was spilled by the T/V *Exxon Valdez* in Prince William Sound (PWS) in the spring of 1989, because the spill occurred just prior to the migration of mature herring to nearshore spawning areas [1] and oil persisted for several months in nearshore areas. About one-half of the spawned biomass was exposed to oil [1], and elevated levels of abnormalities and chromosome breakage were observed in newly hatched larvae [2]. Biologically available hydrocarbons from the *Exxon Valdez* oil spill were generally pervasive in the upper water column along the spill trajectory inside PWS during the summer of 1989 [3]. Thus, the potential for exposure of herring eggs and larvae persisted for several months after the spill [1], and there was evidence of adverse effects in planktonic larvae [2,4,5]. The low recruitment of the 1989 year-class to the fishery in 1992 and 1993 [1] suggested, but did not prove, that there was a possible population effect from the spill. Of major concern was the possibility that intergenerational impacts could be caused by genetic damage.

In earlier studies conducted at our laboratory, herring eggs and adults were exposed to water-soluble fractions (WSFs) of crude oil (82% monoaromatic and 18% diaromatic hydrocarbons) [6], but because most or all monoaromatic hydrocarbons likely evaporated within a few days of the *Exxon Valdez* oil spill [7,8], studies that were reliant on monoaromatic hydrocarbons [6,9,10] were inappropriate models for the conditions that were present in PWS. Thus, we adopted an exposure method designed to closely emulate the composition of polynuclear aromatic hydrocarbons (PAHs) observed in PWS after the *Exxon Valdez* oil spill [5,11]. These tests differed significantly from those previously reported by our laboratory [6] and others [9,10,12,13] in that the principal toxicants in our recent study were PAHs.

The general goals of this study were to expose Pacific herring adults to PAHs, to determine biologically effective dosages for embryolarval effects, and to relate the results to direct egg exposures and postspill herring studies in PWS. In preceding companion papers, we reported the results of direct exposure of Pacific herring eggs and adults incubated in water contaminated with Alaska North Slope crude oil [14–16]. The lowest observed effective aqueous PAH concentration causing abnormalities in larvae hatched from directly exposed eggs was 0.4 μg/L [16]; adults were significantly affected at 27 μg/L [15]. In this paper, we report the impacts of indirect exposure of eggs to PAHs, measured through exposure of reproductively mature herring males and females. The effects on the gametes and fertilized eggs of these males and females were judged from fertilization success, embryo mortality, hatch timing and success, and morphological and genetic abnormalities. Most of these parameters were sensitive measures of response in direct egg-exposure tests [16], and results using the two exposure routes are compared.

Hydrocarbon concentrations and composition were determined in treatment water, ova, and in subsequently spawned eggs. For the purposes of this paper, we define ova as eggs that were present in ovaries prior to spawning, and we define eggs as fertile or infertile eggs after spawning. Both aliphatic and aromatic hydrocarbon data were collected, but because it is likely that the aromatic fraction accounts for most toxic

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The research described in this paper was supported by the *Exxon Valdez* Oil Spill Trustee Council. However, the findings and conclusions presented by the authors are their own and do not necessarily reflect the view or position of the Trustee Council.

EX5017-000001-TRB

effects [17], we present only the PAH data in detail. Water in this experiment was contaminated via contact with rock that had been coated with weathered Alaska North Slope crude oil, a method designed to mimic the PAH composition and weathering of *Exxon Valdez* oil that was observed in PWS [18].

MATERIALS AND METHODS

Pre-spawn herring were collected on March 20, 1994, near Shelter Island, Alaska, USA (58.4°N latitude; 134.8°W longitude); pre-spawn herring were maintained as described by Carls et al. [15]. Fish were gravid but not mature when captured. Males began to show evidence of maturation (softening of testes and milt extrusion) by April 11, 1994. Females began to mature (loosening of eggs and yellowing of ovaries) by April 14, 1994. Fish were artificially spawned on May 2 through 4, 1994, and again on May 10 through 12, 1994; these spawnings occurred at about the same time as local natural spawning events (April 28 to May 6, 1994, in Berner's Bay, Alaska [A. McGregor, unpublished data]). Volitional spawning was observed in captive fish as early as April 11, 1994.

There were five treatments (0.03, 3.2, 10, 27, and 58 μg/L initial aqueous total PAH concentration), including control, with three replicates per treatment. Healthy pre-spawn herring were randomly distributed among 15 treatment tanks (50 fish per tank). Water for each replicate 700-L tank percolated upward through gravel or oiled gravel at 6.9 L/min and at 4.2°C in a vertical 30-cm–diameter × 122-cm polyvinyl chloride cylinder that had been fitted with a trap to prevent slick overflow and entered at the bottom of the tank. To simulate the composition of the oil that was stranded on PWS beaches in 1989 after the *Exxon Valdez* oil spill, oil was artificially weathered by heating it to 70°C overnight until its initial mass was reduced by approximately 20%. The resultant PAH composition was dominated by naphthalenes followed by phenanthrenes, dibenzothiophenes, fluorenes, and chrysenes. Gravel (median diameter, 6.8 mm; range, 2–33 mm) was washed on 3-mm screen and thoroughly air-dried. Weathered crude oil, heated to 40°C, was applied with a paint sprayer to 45-kg batches of tumbling gravel (trace-, low-, and mid-oil treatments) or was applied with a Teflon® squirt bottle (high-oil treatment). Batches of gravel were mixed by treatment, and each cylinder was loaded with 45 kg of freshly oiled gravel (or nonoiled control gravel), except for the high-oil treatment cylinders, which were loaded with 90 kg of gravel. Before any fish were exposed, water flowed through oiled gravel for 4 d (control through mid-oil treatments) or for 8 d (high-oil treatment first used for 4 d for an independent study [14]). To characterize the oil and to quantify treatment concentrations, composite water samples (1.27 L from each of three replicate tanks) were collected on exposure days 0, 8, and 16 for hydrocarbon analysis. Mortality and dissolved oxygen were monitored once per day in each tank. Fish ranged in size from 20.3 to 29.9 cm fork length and from 96 to 261 g (wet weight); age ranged from 3 to 10 years.

Tissue hydrocarbons were analyzed according to the methods of Short et al. [19] at the National Marine Fisheries Service (Auke Bay Laboratory, Juneau, AK, USA). Experimentally determined method detection limits depended on sample weights and generally were 1 ng/g in tissue and 1 to 8 ng/L in water. Concentrations below method detection limits were treated as zero. Tissue concentrations are reported on a wet-weight basis so that they can be directly compared to those of previous studies [6,16]. 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 (TPAH) concentrations were calculated by summing concentrations of individual PAHs. Relative PAH concentrations were calculated as the ratio of PAH concentration to the TPAH concentration.

After 8 d of exposure, 21 females per treatment were removed from treatment tanks and artificially spawned; similarly, an additional 9 to 18 females per treatment were collected and spawned after 16 d. The number of fish available for spawning on day 16 was limited by reproductive condition and prior mortality. Fish were weighed and measured as described by Carls et al. [15] and were spawned as described by Johnson et al. [20], except that three males were generally involved in each cross. From each female, approximately 125 eggs were deposited on each of 1,025 × 75–mm glass slides and fertilized within 34 min of deposition. For hydrocarbon analysis, additional eggs from several females were deposited in shallow glass pans, fertilized with milt from several males, and immersed in a 200-L seawater bath (high-oil treatment only, by replicate). Eggs were collected for hydrocarbon analysis 0, 0.5, 1, 2, 4, 8, 16, and 24 d after fertilization and were frozen (10-g minimum per sample). Gonad and muscle samples were also frozen for hydrocarbon analysis (10-g minimum). Dissection tools and glass vials were hydrocarbon-free. Glassware was hydrocarbon-free (as received from the manufacturer); tools were washed, dried, and rinsed with methylene chloride.

Eggs, grouped by replicate treatment, were suspended in 40- to 50-L tanks from a mobile overhead rack designed to cause slow movement through the water (1 rpm). The oil-free seawater input was approximately 1 L/min at 5.3 to 5.4°C. Eggs along the margins of the glass slides were removed to avoid potential egg abrasion by the baskets used to suspend eggs [20]. Numbers of infertile and fertile eggs were recorded for two randomly selected slides from each female, leaving about 250 eggs for further observation. One of these selected slides was isolated in a 1-L glass jar 15 to 18 d after fertilization; isolated eggs were also suspended from the mobile rack. Temperature was controlled by placing jars in a flowing-seawater bath and was monitored daily; salinity (32 ppt, range 30–34 ppt) was monitored weekly. Lighting was natural, supplemented by overhead fluorescent light during daylight hours. Light intensity ranged from 4 to 68 foot candles (ft-c) (mean 18 ± 2.0 ft-c); ultraviolet light represented approximately 13% of the total light energy.

Hatch timing, hatching success, larval viability, and larval abnormalities were observed daily for each fish (except that these variables were observed every other day before hatch began). Hatched larvae were assessed for swimming ability and gross morphological deformities; they were then captured, anesthetized with tricaine methanesulfonate, and preserved in 10% phosphate-buffered formalin. Dead larvae were discarded. Water in incubation jars was exchanged without egg emersion after each observation. After hatch was complete, remaining dead embryos were counted.

Preserved larvae were transferred to physiological saline (two parts distilled water, one part seawater) and measured with an optical micrometer. Total length, yolk length, and yolk height were measured in 50 straight larvae per female. (Curved larvae could not be accurately measured using the micrometer. Observations indicated that curvature in these larvae resulted

from fixation artifacts and was not biased toward any treatment.) Measurements were restricted to the 7- or 8-d period (for 8- and 16-d exposures, respectively) that encompassed the majority of the hatch.

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 noted when the pericardial area was convex ventrally or when it had an unusually large area. Jaws were classified as small if they were absent or posterior to the anterior margin of the eye.

Genetic and additional morphologic responses were evaluated using blind review. A maximum of 15 females were randomly selected from control and high-oil treatments, and 10 stage-1b larvae [21] were randomly selected from each female; 300 larvae were analyzed. Larvae were transferred to 5% phosphate-buffered formalin during this procedure. Pectoral fins were excised and stained with aceto-orcein. The total number of cells undergoing mitosis was counted, and the number of anaphase–telophase aberrations was recorded, including translocation bridges, attached fragments, acentric fragments, stray and lagging chromosomes, and sidearm bridges. The number of micronucleated cells in the pectoral fins was recorded, and interphase cells were categorized as normal or pathologic (swollen, vacuolated, or containing marginated chromatin). Degenerating cells (pycnotic and multinucleated or karyorrhexic) were also counted. The severity of skeletal, craniofacial, and finfold defects was scored using the graduated severity index method described by Hose et al. [2], 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.

Data processing and statistics

To compare PAH composition in water, ova, and eggs, concentrations were first divided by TPAH to yield percentage vector X. A new vector, $y = (y_1, y_2, \ldots y_{n-1})$, where n = number of compounds, consisted of elements y_i, where

$$y_i = \log\left(\frac{x_i}{x_d}\right) \qquad i = 1, 2, \ldots, n - 1$$

Biphenyl, which was present in every sample, was chosen as the denominator (x_d). Vector y was analyzed as though it were a multivariate normal vector [22]; multivariate analysis of variance (MANOVA) was used to test for differences in PAH composition among treatments, tissue, and time. The number of elements in the vector was reduced from 39 to 22 because the MANOVA required that no $x_i = 0$; PAH concentrations not included in this analysis were consistently or frequently zero (Appendix). To eliminate the few remaining zeros, a constant, 0.001, was added to all concentrations before calculation of the y-vector; the resultant axis translation was approximately an order of magnitude smaller than the smallest nonzero values present in the data set. Distributions of transformed compounds did not, in general, appear to be normal, so significance of the MANOVA result was evaluated by randomization [23]. To randomize, tissue type was randomly reassigned to each sample, and the MANOVA statistic (Wilk's Lambda) was recomputed 100 times. The observed statistic was compared with its empirical null distribution to assess its probability under the null hypothesis [23].

The 8- and 16-d exposure groups were not completely independent, because fish were sampled from the same population pool in each tank on two occasions. The statistical design was balanced in the 8-d exposure group, but because of volitional spawning activity and disease, there were insufficient numbers of fish available in the 16-d exposure group to ensure a balanced design. Reported means were based on least-squares estimates.

The denominator used to calculate percentages varied by response type. Percentages of eggs that were fertile, infertile, and initially dead were based on the total number of eggs counted near the beginning of the experiment. Percentages of eggs that hatched or died were based on the total number of hatched larvae plus the number of dead embryos determined at the endpoint. The number of hatched larvae was subdivided into the number of larvae that were alive, the number that were moribund, and the number that were dead. Accordingly, percent alive was the number of living larvae (excluding moribund larvae) divided by the total number hatched. Similarly, number hatched was used as the denominator to calculate percent moribund and percent dead larvae. Swimming ability of alive larvae was categorized as effective, ineffective, or incapable and was expressed as a proportion of alive larvae. 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 swimmers were unable to swim in a straight line and were often capable only of spasmodic twitching. All larvae categorized as ''incapable of swimming'' had spinal defects. Spinal aberrations were assessed only in alive and moribund larvae. Because dead larvae quickly became distorted after death, they were not assessed for spinal condition. Percent spinal aberrations, therefore, was the number of aberrations divided by the number of alive larvae plus the number of moribund larvae.

To estimate hatch timing, the peak hatch day was determined for each fish. In cases in which two peaks of equal magnitude occurred, the first peak was reported. Median hatch day was calculated by two-point linear interpolation of cumulative percent hatch (y) and day (x), from data points that spanned 50% hatch, based on the total number of eggs that hatched.

Percentage data and hatch timing were analyzed with analysis of variance (ANOVA), with replicate nested in treatment and with fish nested in replicate and treatment. Before ANOVA, percentage data were arcsine transformed and corrected for small n where necessary [24]. (The same conclusions were reached with untransformed data.) Scored abnormality data (yolk-sac edema and jaw size) were recoded to yield incidence of abnormality (e.g., percent larvae with small jaws) and were then analyzed with ANOVA. Graduated severity index scores were analyzed with the Kruskal–Wallis nonparametric test.

Because correlation of size (larval length and yolk volume) and time was generally negligible or inconsistent, time was not included as a factor in final size analyses. Yolk-sac volume was estimated as follows: $V = 4/3 \cdot (L/2)^2 \cdot (H/2)$, where V = volume (cubic millimeters), L = length (millimeters) of yolk sac, and H = height (millimeters) of yolk sac [25]. Larval length and yolk volume were analyzed with ANOVA, with replicate nested in treatment and with fish nested in replicate and treatment; size measurements were considered to be random variables.

RESULTS

Reproductive condition of parental stock

The adult herring sampled after 8 and 16 d exposure to oil were reproductively mature, partially spawned, or completely

spawned out. No immature gonads were observed. There were significantly more partially spawned males than females (p = 0.040); gender differences were not significant in the mature and spawned-out categories (p = 0.181 and 0.746, respectively). Significantly more herring in the 16-d group had released some or all gametes than in the 8-d group (p = 0.036). Maturity did not vary significantly among oil treatments ($0.245 \leq p \leq 0.494$).

Hydrocarbon exposure, accumulation, and composition

Aqueous concentrations of TPAH declined during the experiment, and PAH composition changed. Initial TPAH concentrations in water ranged from 0.03 (control) to 58.3 μg/L (high-oil treatment) (Fig. 1). Concentration declines were similar in all treatments; thus, treatment conditions remained clearly separable, which was evident based on TPAH accumulation in ova. Concentrations of individual PAHs generally declined with time; compounds with more rings were lost more slowly, and within homologous families, the more substituted homologues were lost more slowly (Fig. 2a and b). The percentage of naphthalenes declined significantly with time ($p \leq 0.043$); percentages of fluorenes, dibenzothiophenes, phenanthrenes, and chrysenes increased with time ($0.014 \leq p \leq 0.055$). (Monoaromatics were not present in treatment water because the oil was weathered before the tests began.) The composition of PAHs also changed as a function of treatment: weathering was most rapid at the lowest concentrations and slowest in the high-oil treatment. Although relative naphthalene concentrations remained dominant (66–87%) throughout exposures in the high-oil treatment (Fig. 2a and b), they were typically <50% at the end of treatment, and phenanthrenes either became dominant or approached dominance in the lowest two treatments.

Polynuclear aromatic hydrocarbons accumulated in ova of herring exposed to oil and were retained in fertilized eggs. Concentrations of TPAH in ova (y) were correlated with those in water ($0.92 \leq r \leq 0.99$; $p < 0.001$; $y^{0.5}$ transformation), and the maximum mean concentration in ova reached 9,688 ng/g (wet weight) in the high-oil treatment (Fig. 1). Total PAH concentrations in these ova dropped approximately 30%, to 6,719 ng/g, shortly after fertilization. This rapid reduction can be explained by the influx of water as the perivitelline space is formed during fertilization; dry-weight TPAH concentrations were indistinguishable in ova and eggs immediately before and after spawning (p = 0.859). The wet-weight to dry-weight ratio was significantly greater in fertilized eggs (7.4 ± 0.2) than in ova (4.6 ± 0.0) ($p < 0.001$). Concentrations of TPAH declined as eggs incubated in clean water but remained above control concentrations throughout incubation ($p < 0.001$) (Fig. 1c). Initial TPAH concentrations in eggs from adults exposed 16 d were significantly greater than those found in eggs from adults exposed for 8 d ($p \leq 0.002$), but depuration was more rapid in the 16-d eggs, and concentrations were very similar after about 15 d (Fig. 1c).

Composition of PAHs in ova of fish exposed to oil differed significantly from PAH composition in water, particularly on day 16 ($p_{MANOVA} < 0.01$) (Fig. 2). Naphthalenes preferentially accumulated in ova, comprising 92% of the total; C1-naphthalenes were usually dominant, and C2-naphthalenes were usually next in abundance. Relatively few compounds heavier than biphenyl were detected in ova (2–13%); chrysenes and heavier PAHs were never detected. Of the heavy compounds present, unsubstituted fluorene, dibenzothiophene, and phenanthrene homologues were dominant in ova, and highly substituted homologues were underrepresented or not present. (Bias toward naphthalene accumulation in ova was most evident in the lowest oil treatments [not illustrated].) In contrast to the time-dependent composition shifts observed in water, percentages of naphthalenes in ova were roughly similar on days 8 and 16. Not only were percentages of naphthalenes in ova greater than those in water sampled at the same time, but they also consistently exceeded the percentages of naphthalenes in initial water samples.

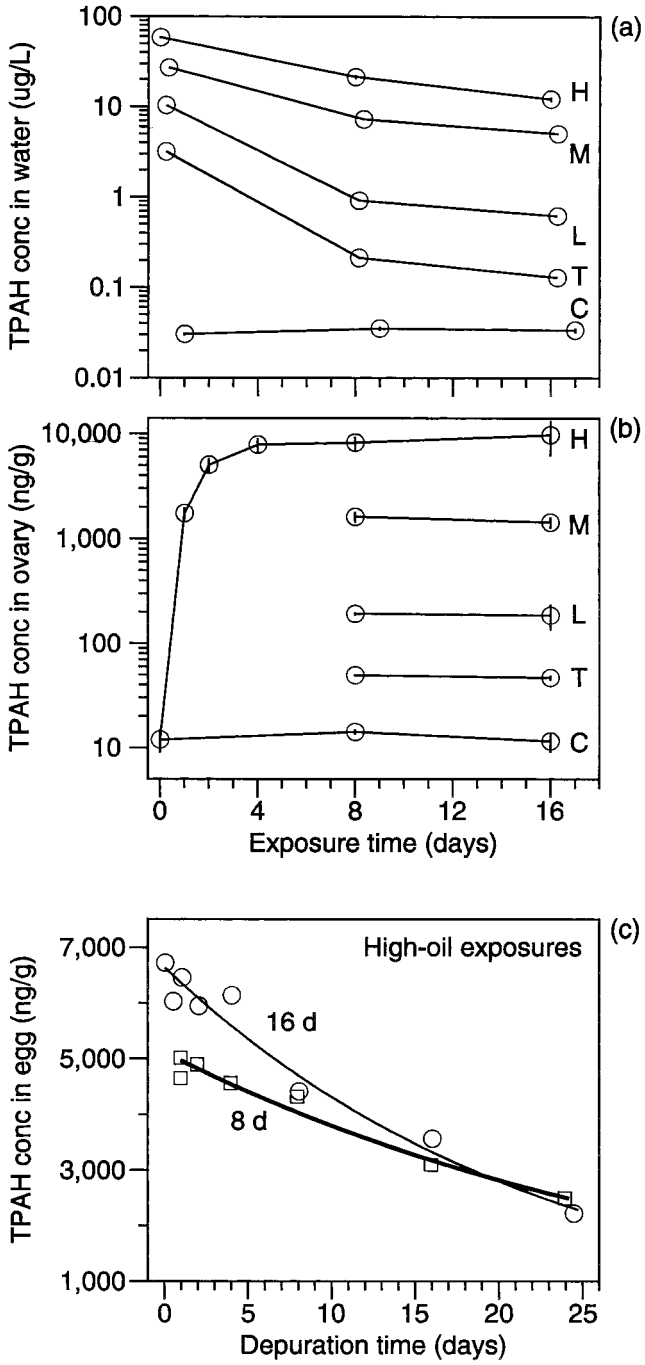

Fig. 1. Mean total polynuclear aromatic hydrocarbon (TPAH) concentrations in water (**a**), ova (**b**), and high-oil treatment eggs (**c**) as functions of time (±SE). Treatments were control (C), trace-oil (T), low-oil (L), mid-oil (M), and high-oil (H). Reproductively mature herring were exposed to oil for 8 or 16 d; thus, gamete exposures were indirect.

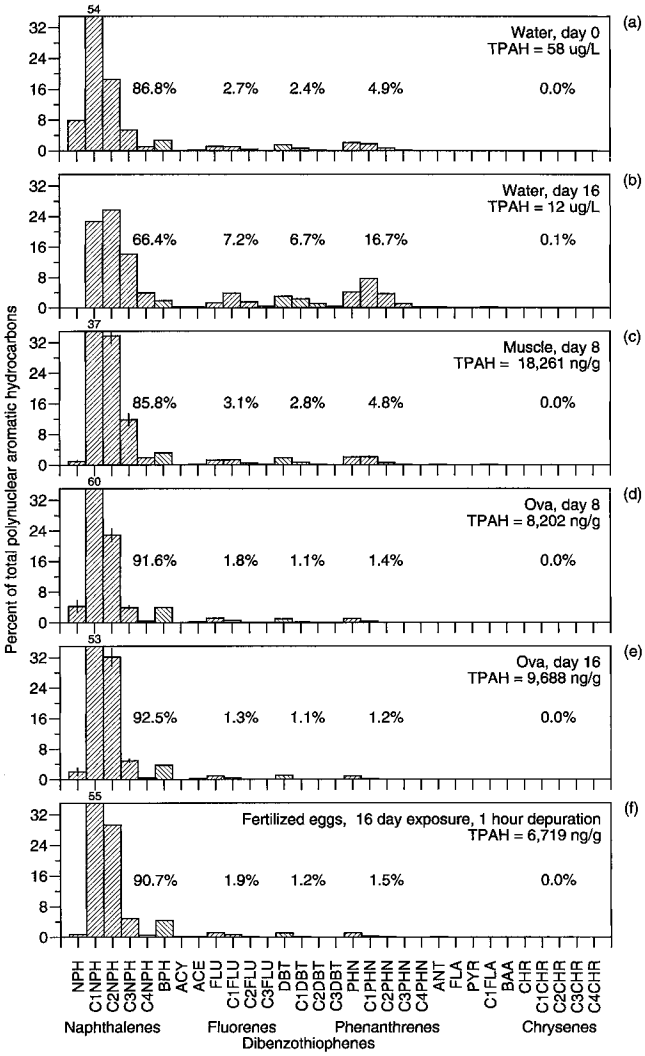

Fig. 2. Composition of polynuclear aromatic hydrocarbons (PAHs) in high-oil treatment water (**a, b**), muscle (**c**), ova (**d, e**), and fertilized eggs (**f**). Percentages printed inside the graphs indicate total percentages of the following homologous chemical groups (left to right): naphthalenes, fluorenes, dibenzothiophenes, phenanthrenes, and chrysenes. Abbreviations within each homologous group indicate the parent compound (e.g., naphthalene, NPH) or the degree of methyl-substitution of the parent compound (e.g., C3-naphthalene, C3NPH, has three methyl substitutions). Total PAH (TPAH) concentrations are reported for each observation.

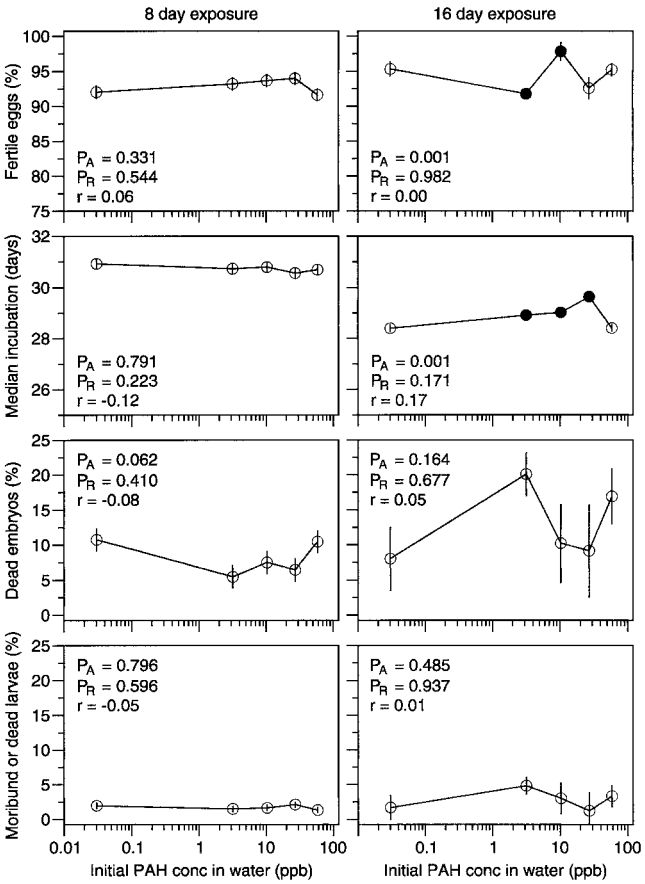

Fig. 3. Egg fertility, median incubation time, embryo death, and larval death as functions of parental exposure to initial total polynuclear aromatic hydrocarbon (TPAH) concentration in water. Reproductively mature herring were exposed to oil for 8 or 16 d; thus, egg exposures were indirect. The p_A value represents the probability of overall treatment effect estimated by analysis of variance, and solid symbols indicate significant differences from controls. The p_R value reports the probability of significant slope, estimated by regression (x = $\log_e$(concentration)), and r = correlation coefficient. Data displayed are least-squares–adjusted means ± SE. Note differences in y-axis scaling.

Compared to muscle tissue, ova accumulated less TPAH, relatively greater amounts of naphthalenes, and fewer substituted PAHs. Total PAH concentrations were consistently higher in muscle tissue than in ova; these differences were significant in the high-oil treatment ($p \leq 0.001$) (e.g., 18,261 ng/g in muscle; 8,202 ng/g in ova on day 8; Fig. 2c and d). The composition of PAHs in ova was consistently enriched in naphthalenes compared to that found in muscle tissue (e.g., 91.6% naphthalenes versus 85.8% in high-oil treatment, respectively, on day 8; Fig. 2c and d). Ova consistently accumulated relatively more unsubstituted PAHs than did muscle tissue (e.g., relatively more phenanthrene than C1- to C4-phenanthrenes) ($0.001 < p \leq 0.034$).

Composition of PAHs in eggs spawned from exposed adults did not differ significantly from composition in ova an hour after fertilization ($p_{MANOVA} = 0.388$) (Fig. 2e and f). The close correspondence between PAH composition in ova and fertilized eggs was verification that hydrocarbons transferred from adults to progeny, that PAHs were present at fertilization, and that naphthalenes accounted for most of the TPAH (91%). As in ova, chrysenes were not detected in eggs.

Rates of PAH-composition change in eggs were significantly lower than the corresponding rates of change in water ($p < 0.001$). Evidence of time-dependent PAH-composition change in tissue was equivocal ($0.36 \leq r^2 \leq 0.99$; $0.001 < p \leq 0.155$). For example, after 16 d in the high-oil treatment, percentages of naphthalenes changed negligibly in eggs but dropped 20% in water.

Adult exposures: Indirect impacts on eggs and larvae

Egg fertility was not correlated with TPAH concentration ($0.00 \leq r \leq 0.06$; $p_{regression} \geq 0.544$) (Fig. 3). Fertility did not differ significantly among treatments in 8-d exposures ($p_{ANOVA} = 0.331$). In 16-d exposures, fertility varied significantly among treatments ($p_{ANOVA} = 0.001$), but it did not vary in a dose-dependent manner; mid- and high-oil treatments did not differ significantly from controls (Fig. 3). Mean fertility was similar in 8- and 16-d exposures (93 and 95%, respectively), but variability was greater in the 16-d exposures.

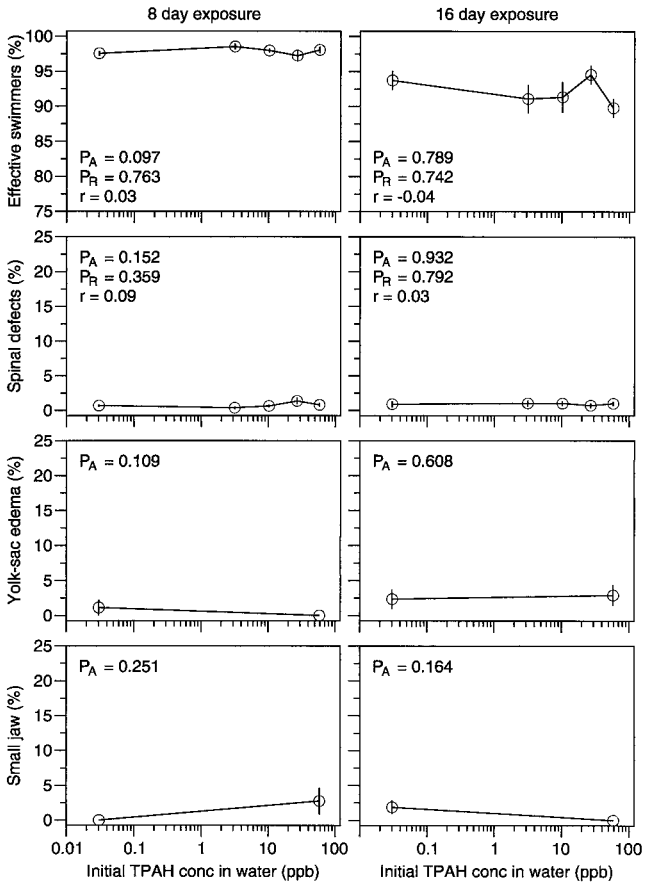

Fig. 4. Swimming ability, spinal defects, yolk-sac edema, and small jaws as functions of parental exposure to initial total polynuclear aromatic hydrocarbon (TPAH) concentration in water. Reproductively mature herring were exposed to oil for 8 or 16 d; thus, egg exposures were indirect. The p_A value is the probability of overall treatment effect estimated by analysis of variance. The p_R value reports the probability of significant slope, estimated by regression (x = $\log_e$(concentration)), and r = correlation coefficient. None of these responses were significantly different from controls. Data displayed are least-squares–adjusted means ± SE.

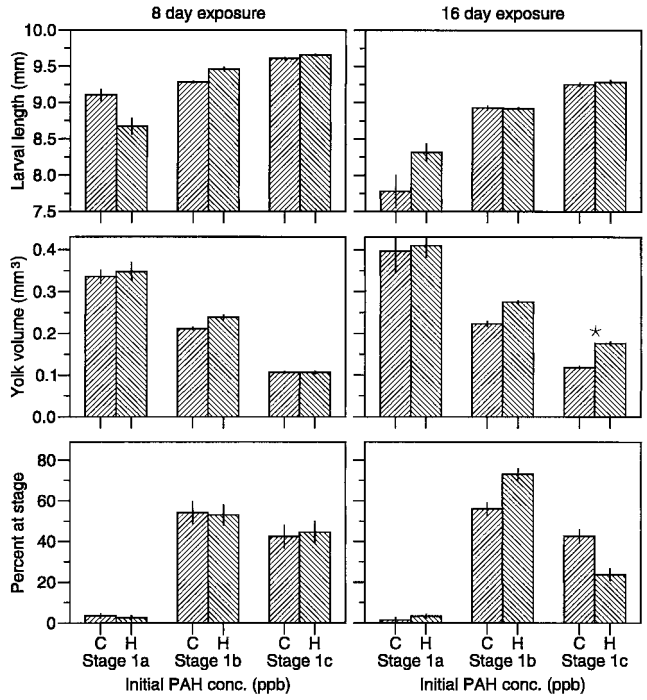

Fig. 5. Length, yolk volume, and maturity of larvae from control (C) and high-oil (H) parental exposures. Adult fish were exposed for either 8 or 16 d. Stage-1a larvae are the least mature; stage-1c larvae are the most mature. Control and high-oil responses were compared for each larval stage: responses were significantly different in only paired comparison (asterisk; p = 0.029). Data displayed are least-squares–adjusted means ± SE.

Incubation time was not affected by parental exposure to oil, and it did not correlate with TPAH concentration ($-0.12 \leq r \leq 0.17$; $p_{\text{regression}} \geq 0.171$) (Fig. 3). In 8-d exposures (5.3°C), mean peak hatch and median hatch were 31 d, and they were not affected by treatment ($p_{\text{ANOVA}} \geq 0.791$). In 16-d exposures (5.4%C), mean peak and median hatch times ranged from 28 to 30 d and varied significantly among treatments ($p < 0.001$). However, these significant differences did not appear to be dose-related; median hatch time was significantly late in trace-, low-, and mid-oil treatments but not in the high-oil treatment. Furthermore, peak hatch was significantly late in the mid-oil treatment but significantly early in the high-oil treatment (not illustrated).

The percentage of dead embryos was not affected by parental exposure to oil and did not correlate with TPAH ($-0.08 \leq r \leq 0.05$; $p_{\text{regression}} \geq 0.410$) (Fig. 3). Embryo death did not differ significantly among treatments ($p_{\text{ANOVA}} \geq 0.062$). Mean percentages of dead embryos were <11% in 8-d exposures and <20% in 16-d exposures (Fig. 3). Significantly more embryos died in 16-d exposures than in 8-d exposures (p = 0.002), but variability was greater in the 16-d test, and there were no apparent dose-related trends.

The percentage of larvae that were moribund or that died within 1 d of hatch was not affected by parental exposure to oil and did not correlate with TPAH concentration ($-0.05 \leq r \leq 0.01$; $p_{\text{regression}} \geq 0.596$) (Fig. 3). Differences among treatments in percentages of moribund plus dead larvae were not significant ($p \geq 0.485$). Fewer larvae were moribund or dead in 8-d exposures than in 16-d exposures (≤2.1 and ≤4.8%, respectively) (p = 0.024).

Larval swimming ability was not affected by parental exposure to oil and did not correlate with TPAH concentration ($-0.04 \leq r \leq 0.03$; $p_{\text{regression}} \geq 0.742$) (Fig. 4). Differences among treatments were not significant ($p_{\text{ANOVA}} \geq 0.097$). Swimming ability was better in 8-d exposures than in 16-d exposures ($p < 0.001$); percentages of larvae classified as effective swimmers exceeded 97 and 90%, respectively.

Larval abnormalities were not caused by exposure of adult herring to oil (Fig. 4). The percentage of larvae with spinal defects was not correlated with TPAH concentration ($0.03 \leq r \leq 0.09$; $p_{\text{regression}} \geq 0.359$). Differences in spinal defects among treatments were not significant ($p_{\text{ANOVA}} \geq 0.152$). The mean percentages of larvae with spinal defects did not exceed 1.3 and 1.1% in 8- and 16-d exposures, respectively. Incidence of larvae with yolk-sac edema (mean ≤ 2.9%) was not elevated in high-oil treatments ($0.109 \leq p_{\text{ANOVA}} \leq 0.608$). Incidence of larvae with undeveloped lower jaws (mean ≤ 2.8%; $0.164 \leq p_{\text{ANOVA}} \leq 0.251$) was not elevated in high-oil treatments. Similarly, graduated severity index scores did not indicate significant morphological response ($p_{\text{Kruskal–Wallis}}$ = 0.516).

Maturity of embryos at hatch was not influenced by parental oil treatment ($0.212 \leq p \leq 0.878$) (Fig. 5). The majority (62%) of newly hatched larvae were classified as stage 1b; only 3% were stage 1a, and the remainder were stage 1c.

Pre-spawning oil exposure generally did not affect progeny

length, but length varied significantly with larval maturity at hatch (Fig. 5). Larval length increased significantly with stage of development ($0.004 \leq p \leq 0.016$), and yolk volumes decreased significantly ($0.001 < p \leq 0.003$). However, larval lengths in high-oil treatments did not differ significantly from the lengths of controls at the same stage of maturity ($0.152 \leq p \leq 0.926$). Similarly, yolk volumes in high-oil treatments did not differ significantly from control volumes in larvae at the same stage of maturity in 8-d exposures ($0.374 \leq p \leq 0.996$). In the 16-d exposures, yolk volumes between oil treatments were not significantly different in stage 1a and 1b larvae ($0.230 \leq p \leq 0.972$), but the volumes of stage-1c high-oil treatment larvae were significantly greater than in stage-1c controls ($p = 0.029$).

Exposure of adult herring to oil for 8 d did not cause a significant elevation in genetic aberrations in the pectoral fins of newly hatched larvae and did not affect the rate of cell division. Percent anaphase–telophase aberration was significantly greater in controls (5 ± 1%) than in the high-oil treatment (4 ± 1%) ($p = 0.032$; $n = 134$ larvae per treatment). Analysis was restricted to control and high-oil treatment larvae. (Based on the 8-d results and on the general lack of response in other 16-d measurements, larvae from 16-d exposures were not analyzed for chromosomal aberrations.) The number of cells undergoing mitosis did not differ significantly between control (18.5 ± 0.4) and high-oil treatment (19.6 ± 0.4) larvae ($p = 0.090$).

Other cell problems were rare and generally were not associated with parental oil treatment. Although statistically significant ($p = 0.036$), micronucleation was observed in only five high-oil treatment larvae (3%). All interphase cells were normal. Pycnotic cells were observed in 10% of the larvae; treatment was not significant ($p = 0.722$). Multinucleated or karyorrhexic cells were detected in 7% of the larvae; treatment was not significant ($p = 0.215$).

DISCUSSION

Effects on progeny of adult herring exposed to weathered crude oil were not discernable when ova contained up to 9.7 μg/g TPAH. Parameters not affected included egg fertility, embryo death, hatch timing, larval death, spinal defects, yolk-sac edema, larval swimming, larval length, stage of larval development at hatch, jaw development, rate of cell mitosis, anaphase–telophase chromosome abnormalities, number of pycnotic cells, and number of multinucleated or karyorrhectic cells. In several cases, significant departures from control responses were observed, but they were not observed in a dose-dependent manner. For example, in the 16-d exposure group, fertility was significantly lower in the trace-oil treatment and significantly higher in the low-oil treatment, but fertility was not significantly different from that observed in the control in mid- and high-oil treatments (Fig. 3). These significant differences were invariably confined to the 16-d exposure group, where variability of response was consistently greater than in the 8-d group.

Lack of egg response to exposure of reproductively mature Pacific herring to oil was not attributable to insufficient data, inadequate test design, or low statistical power. The total number of eggs observed was large in the 8- and 16-d tests (13,499 and 8,035, respectively). Second, in a highly similar experiment, in which eggs were directly exposed to oil during incubation [16], many of the same parameters measured in this study were sensitive measures of response, including embryo death, hatch timing, larval death, spinal defects, yolk-sac edema, larval swimming, larval length, apparent stage of larval development at hatch, jaw size, and anaphase–telophase chromosomal abnormalities.

Gametes apparently were afforded parental protection because chemical partitioning and metabolism within exposed adults limited accumulation of larger PAHs in ova. This partitioning was significant, because PAH toxicity increases with both ring size and alkylation [16,17,26,27]; thus, uptake patterns favored reduced toxicity to ova. Compared to muscle tissue, herring ova contained both a lower concentration and proportion of heavier PAHs (fluorenes, dibenzothiophenes, and phenanthrenes) and had less alkylation within homologous families. We do not, however, fully understand the physiological mechanisms that caused partitioning. Possibly blood-flow patterns caused larger PAHs to bind to other lipophilic tissues and structures before reaching the ovaries. Composition of PAH in tissues may have been partially modified by metabolic action; mixed-function oxidase enzymes were induced in these pre-spawn herring [14]. Metabolites are more soluble than parent PAHs; hence, they are more easily excreted, and the primary site of PAH metabolism was probably the liver. Thus, we suspect metabolite accumulation in ova was relatively small. The net result of differential PAH bioconcentration was that gametes accumulated a larger proportion but lower concentrations of less toxic, lighter PAHs relative to other organs.

Differential accumulation and partitioning was also observed in a previous experiment, in which adult herring were exposed to the WSFs of Cook Inlet crude oil [6]. Following a 48-h exposure to 1.2 mg/L WSF, the total aromatic hydrocarbon concentration in ovary was 80 μg/g versus 150 μg/g in muscle. Monoaromatics predominated in the ovary, whereas in muscle, the proportions of mono- and diaromatics were approximately similar. Despite tissue concentrations that were nearly an order of magnitude above those obtained in the present experiment, herring egg survival was unaffected. Even after a 12-d parental exposure to 1.6 mg/L WSF, hatching success was not reduced by exposure of adults to the WSFs of Cook Inlet crude oil, unless the adults were killed [6].

However, high–molecular weight PAHs such as naphthalene, anthracene, and benzo[*a*]pyrene can reduce the reproductive potential of fishes when introduced during critical phases of reproduction [28–31]. Reproductive stages particularly affected by PAHs appear to be gonadal recrudescence, final oocyte maturation, and spawning. Furthermore, reproductive effects generally occurred following prolonged exposure (≥5 weeks) to high PAH concentrations (high relative to those used in this study). For example, ovarian recrudescence and sexual maturation in female Atlantic croaker exposed to 0.5 mg/L naphthalene or 2.5% WSF of diesel fuel for 8 weeks were impaired [28]. Although PAH concentrations in croaker tissue were not stated, they were likely at least an order of magnitude higher than those reported here for herring. Female fathead minnow exposed to 6 to 12 μg/L aqueous anthracene for 9 weeks laid fewer eggs than did controls [29]. These eggs contained 8.8 μg/g (wet weight) anthracene [29], compared to a measurement of 320 ng/g of three-ring PAHs in herring ova following a 16-d exposure. Reproductive capacity and larval survivorship were reduced in F2 fathead minnows with grandparental exposure to 1 μg/L benzo[*a*]pyrene, where the larval grandparents were exposed for 4 months [30]. Effects on flatfish progeny caused by administration of a high dose of benzo[*a*]pyrene shortly before

spawning [31] probably interfered with steroid-induced final oocyte maturation [28,32]. Because of the lack of knowledge regarding the physiological mechanisms of PAH toxicity on fish reproduction, it is difficult to extend results between different species [33].

Comparison of indirect and direct exposure of eggs to hydrocarbons

The indirect-exposure experiment we present here was carefully matched in a study in which incubating herring eggs were directly exposed to weathered oil. Comparisons between studies were restricted to exposures of the same duration (16 d) and state of weathering; thus, our comparisons are to the less-weathered treatments in the previously published paper [16]. The concentrations and composition of PAHs to which prespawn adult herring (1994) and eggs (1995) were exposed were very similar [34]. Paired concentrations were coincident ($p \geq 0.277$), except that the concentration in the mid-oil treatment in the direct exposures declined faster than it did in indirect exposures (-0.19 ± 0.02 and -0.11 ± 0.03 μg/L per d, respectively) [34]. Most corresponding values for individual PAHs were similar (mean difference = 0.03%; SD = 2.8; n = 337 for nonzero comparisons; range = −11.8 to 16.5%) [34]. Treatment water contained multi-ring compounds in both years, including fluorenes, dibenzothiophenes, phenanthrenes, and chrysenes [34].

The PAH composition in indirectly and directly exposed herring eggs was different even though adult herring and eggs were exposed to PAHs of similar concentration and composition (Fig. 6). Naphthalenes were enriched in eggs spawned from exposed adults, and of the remaining heavier PAHs, unsubstituted homologues dominated (Fig. 6). In contrast, composition of PAHs in directly exposed eggs tended to be more similar to that found in water, and although there was a tendency for naphthalene enrichment, this enrichment was not as pronounced as it was in the indirect exposures (Fig. 6). Consequently, eggs exposed directly to hydrocarbons during incubation contained higher percentages of higher molecular weight PAHs than did indirectly exposed eggs. Furthermore, percentages of higher molecular weight PAHs in directly exposed eggs increased during incubation, and percentages of alkyl-substituted compounds were greater in directly exposed eggs than in indirectly exposed eggs [34].

Patterns of hydrocarbon uptake and depuration were different between eggs exposed indirectly and directly to PAHs, and the peak TPAH concentration in directly exposed eggs was about twice as high as the maximum concentration in indirectly exposed eggs (Fig. 6). In eggs from oil-exposed adults, TPAH concentrations were highest beginning at fertilization, and they depurated slowly thereafter. In contrast, directly exposed eggs continued to accumulate hydrocarbons throughout the 16-d exposure period, thus indicating that there was sufficient lipophilic material present to absorb PAH despite declines in yolk volume. About the time during which organ development began (4 d), TPAH concentrations of directly exposed eggs were approximately equal to maximum concentrations in indirectly exposed eggs. Because of continued bioaccumulation in directly exposed eggs, concentrations quickly exceeded those in indirect exposures as organ development continued. Total PAH concentrations declined rapidly in directly exposed eggs only after they were transferred to clean seawater.

In contrast to the negligible embryonic effects following

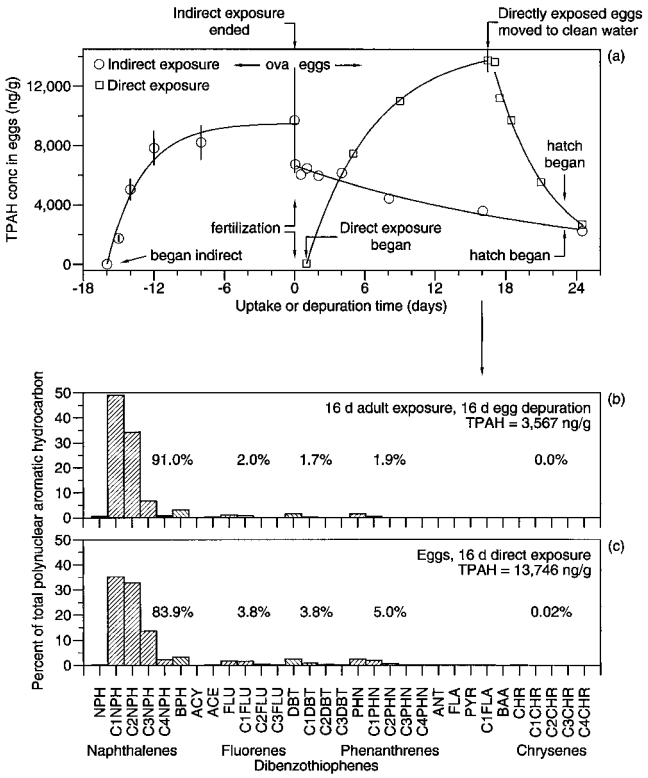

Fig. 6. Comparison of total polynuclear aromatic hydrocarbon (TPAH) uptake and depuration between indirect and direct exposure tests (**a**) and PAH composition in eggs after 16 d of incubation (**b, c**). In indirect tests (**a**), reproductively mature herring (males and females) were exposed to oil; exposure ended at fertilization, and eggs began to lose PAHs. In direct exposure tests (**a**), exposure of eggs to PAHs began 1 d after fertilization and continued for 16 d. All eggs were transferred to clean water before hatching began. Percentages printed inside the graphs (**b, c)** indicate total percentages of the following homologous chemical groups (left to right): naphthalenes, fluorenes, dibenzothiophenes, phenanthrenes, and chrysenes. Abbreviations within each homologous group indicate the parent compound (e.g., naphthalene, NPH) or the degree of methyl-substitution of the parent compound (e.g., C3-naphthalene, C3NPH, has three methyl substitutions).

parental oil exposure (up to 58 μg/L initial aqueous concentration), the oil was highly toxic to directly exposed eggs [16]. Responses of eggs directly exposed to aqueous PAHs were significant in at least the upper two doses (34–86 μg/L) of every measurement presented in this paper, except for fertility. Direct exposure of eggs to oil began after fertilization. The most sensitive measures of response in the direct egg exposure test were increases in the incidence of yolk-sac edema and decreases in larval length, both of which were significant at the 9 μg/L initial aqueous TPAH concentration.

Factors involved in the mitigation of PAH toxicity to progeny by parental exposure include differential partitioning of PAH in parental tissues and concentration changes during embryo development. As a result of differential partitioning and possibly metabolism in parental tissues, embryos were exposed to relatively less-toxic, lower molecular weight PAHs than were those present in water. In contrast, fish embryos have little metabolic potential and accumulate individual PAHs in relation to their abundance in water and their n-octanol–water partition coefficient [26]. Concentrations of PAHs in ova apparently reached equilibrium at 8 to 10 μg/g by 4 d, whereas directly exposed eggs continued to bioaccumulate PAHs throughout the exposure period, with concentrations steadily

increasing to 13.7 μg/g at the termination of the 16-d exposure. Also significant is that peak exposure concentrations in parentally exposed eggs occurred at spawning and were followed by a period of continuous PAH depuration during embryo development. Thus, during the critical state of cell differentiation, TPAH concentrations declined from 6.7 to 2.2 μg/g at hatch. The opposite was true for directly exposed eggs; TPAH concentrations increased to 13.7 μg/g as cell division, differentiation, and organ development proceeded.

Comparison of herring eggs indirectly and directly exposed to hydrocarbons corroborates the observation that toxicity increases as the number of rings increases and with increased alkylation [16,17,26,27]. In this study, aqueous TPAH concentrations of up to 58 μg/L did not evoke biological response in progeny, but when eggs were exposed directly to different proportions of the same compounds during incubation, responses were detectable at TPAH concentrations as low as 9 μg/L [16]. When incubating eggs were exposed to more highly weathered oil, where the proportion of large-ring and alkyl-substituted compounds was even greater, significant biological responses were detected at 0.4 μg/L [16]. (These minimum effective TPAH concentrations represent initial concentrations and do not account for generally rapid declines in exposure concentration.) Previous studies have shown that much larger concentrations of mono- and diaromatic hydrocarbons are required to cause toxicity. For example, in tests in which herring eggs were incubated in the WSFs of Cook Inlet crude oil (82% mono- and 18% diaromatics), significant reduction in hatch was observed at initial aqueous concentrations of 1,400 μg/L but not at 1,000 μg/L [6]. In contrast, we observed a significant reduction in hatch at concentrations as low as 9 μg/L for weathered oil [16]. Similarly, parts-per-million concentrations of WSFs were necessary to cause toxic effects in adult and larval herring [6,35], well above the parts-per-billion concentrations effective in direct exposure of eggs to aqueous PAHs [16].

CONCLUSION

Two factors insulate Pacific herring progeny from toxic oil effects (compared to directly exposed eggs). First, accumulation of hydrocarbons in gametes is biased toward the smaller, less-toxic PAH fractions, probably as a result of chemical partitioning in adult tissues. The second factor is developmental. In the absence of continued exposure, gametes begin to depurate hydrocarbons at spawning, but eggs exposed directly to equivalent aqueous TPAH concentrations are more susceptible to damage, because bioaccumulation progresses during tissue and organ differentiation, a period of maximum developmental vulnerability. Furthermore, PAH composition in directly exposed eggs was more similar to the PAH composition in water, with a higher proportion of the heavier and more-toxic compounds present than in gametes that received parental protection. Combined exposure (before and after spawning) would likely be the worst possibility, because TPAH would continue to bioaccumulate above the concentrations already present in ova, thereby amplifying toxicity in developing eggs.

Because maximal seawater TPAH concentrations were 6 μg/L during the period in which herring spawned in PWS following the *Exxon Valdez* oil spill [36], short-term reproductive effects due to parental exposure were unlikely. Thus, adult herring that were exposed to oil during migration to spawning grounds or as they spawned intertidally probably did not produce impaired progeny, unless their eggs or larvae were directly exposed to oil. Our results do not exclude the possibility of longer term damage to progeny of PAH-contaminated adults, which cannot be entirely discounted, because subtle, behavioral changes were detectable in coho salmon (*Oncorhynchus kisutch*) weeks to months after embryonic exposure to a high dose of benzo[*a*])pyrene [37]. Genetic damage was not detected in somatic tissue; hence, we suspect that it was unlikely in reproductive tissues. Although susceptibilities of these two tissue types in vertebrates may not be equivalent, Brusick [38] suggests a lack of mutation in somatic cells (such as those that we evaluated) in embryos should indicate a corresponding lack of gamete risk. Although environmentally relevant TPAH concentrations did not produce demonstrable toxicity in offspring, adult herring did exhibit other toxic manifestations of oil exposure, such as the activation of viral hemorrhagic septicemia virus, possibly as a result of immunosuppression [15], and significant induction of mixed-function oxidase activity [14].

Of the three life stages of Pacific herring that were potentially exposed to oil after the *Exxon Valdez* spill (those for which we have adequate data), directly exposed eggs were the most sensitive to exposure. In sharp contrast to the lack of response observed in indirectly exposed gametes, eggs directly exposed to PAHs during incubation were adversely affected by initial aqueous TPAH concentrations of as low as 0.4 μg/L in more-weathered oil [16] and of 9 μg/L in less-weathered oil. Mechanisms leading to the abnormal herring larvae observed in PWS following the *Exxon Valdez* oil spill [2,4,5] were, therefore, probably due to exposure of eggs after spawning. Although definitive separation of egg and larval exposure effects was not possible in the field study [5], herring eggs and larvae were similarly sensitive to water-soluble fractions of crude oil [35], and we suspect the same relationship is true for PAH exposure. However, exposed larvae may die without developing the abnormalities characteristic of embryonic exposure [39]. It is therefore crucial for environmental assessments to include laboratory toxicity experiments in which embryolarval development and survival can be more carefully evaluated. To adequately protect aquatic organisms, environmental standards should be based on such critically sensitive periods as egg development.

Acknowledgement—We thank Lori Ewing, Larry Holland, Marie Larsen, Josie Lunasin, Anita Martin, Scott McAllister, Carrita Morris, Jeff Short, and Bob Stone.

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APPENDIX

Polynuclear aromatic hydrocarbons (PAH) identified by chemical analysis, and the abbreviations used in figures. Asterisks indicate which compounds were accepted for multivariate analysis (see text). No compounds heavier than C4-chrysene were detected in water or tissue samples.

Abbreviation	Name
NPH*	naphthalene
C1NPH*	C-1 naphthalenes
C2NPH*	C-2 naphthalenes
C3NPH*	C-3 naphthalenes
C4NPH*	C-4 naphthalenes
BPH*	biphenyl
ACY	acenaphthylene
ACE*	acenaphthene
FLU*	fluorene
C1FLU*	C-1 fluorenes
C2FLU*	C-2 fluorenes
C3FLU*	C-3 fluorenes
DBT*	dibenzothiophene
C1DBT*	C-1 dibenzothiophenes
C2DBT*	C-2 dibenzothiophenes
C3DBT*	C-3 dibenzothiophenes
PHN*	phenanthrene
C1PHN*	C-1 phenanthrenes/anthracenes
C2PHN*	C-2 phenanthrenes/anthracenes
C3PHN*	C-3 phenanthrenes/anthracenes
C4PHN*	C-4 phenanthrenes/anthracenes
ANT*	anthracene
FLA	fluoranthene
PYR	pyrene
C1FLA	C-1 fluoranthenes/pyrenes
BAA	benz[*a*]anthracene
CHR*	chrysene
C1CHR	C-1 chrysenes
C2CHR	C-2 chrysenes
C3CHR	C-3 chrysenes
C4CHR	C-4 chrysenes