Embryonic crude oil exposure causes cardiac hypertrophy and reduced aerobic performance in juvenile pink salmon and Pacific herring

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Abstract

The 1989 Exxon Valdez disaster exposed the embryos of pink salmon and Pacific herring to weathered crude oil in shoreline habitats throughout Prince William Sound. The Pacific herring population collapsed four years later. The role of the oil spill, if any, in the forage fish decline has remained one of the most prominent unanswered questions in modern natural resource injury assessment. Studies subsequent to the spill identified disruption of heart morphogenesis as a major impact of polycyclic aromatic hydrocarbons (PAHs) derived from crude oil. Here we show that pink salmon and herring embryos exposed transiently to trace levels of Alaskan crude oil develop cardiac abnormalities that lead to permanent changes in heart anatomy and physiological performance. When assessed after 7 to 10 months of growth in clean water, both species showed reduced aerobic capacity and common changes in cardiac morphology, including evidence of ventricular hypertrophy. Therefore, the Exxon Valdez oil spill likely impacted pink salmon and herring more than previously appreciated. Moreover, the particular sensitivity of herring embryos to PAHs suggest that the catastrophic collapse of the Prince William Sound population may have been caused in part by delayed mortality due to developmental cardiotoxicity. These findings extend our understanding of the long-term impacts of oil spills, and apply also to more chronic inputs of PAH pollution to aquatic systems from land-based runoff and other sources.

Introduction

The 1989 Exxon Valdez disaster oiled the shoreline spawning habitats for Pacific herring (*Clupea pallasi*) and pink salmon (*Oncorhynchus gorbuscha*), the two most important commercial fish species in Prince William Sound, Alaska. It was subsequently shown that the embryos of both species were sensitive to toxic effects of very low levels of crude oil. Herring larvae sampled in nearshore plankton tows had high rates of morphological abnormalities, including relatively frequent fluid accumulation (edema) around the heart (*1*). Moreover, fertilized eggs collected from pink salmon redds showed elevated rates of embryonic lethality up to four years following the spill (*2*). For both species, subsequent laboratory exposures definitively linked embryolarval deformities and mortality to water-soluble components of crude oil (*3-5*), with defects closely corresponding to the relative abundance of specific classes of polycyclic aromatic hydrocarbons (PAHs).

The most abundant PAHs in the oil spilled from the Exxon Valdez (Alaska North Slope crude oil; ANSCO) are bicyclic (two ring) naphthalenes, tricyclic fluorenes, dibenzothiophenes and phenanthrenes, and tetracyclic chrysenes. In the environment, the composition of dissolved PAHs washing off oiled rocks and other shoreline substrates changes over time in a process known as weathering. The more water-soluble naphthalenes leave the oil first, followed by increasingly hydrophobic tricyclic PAHs and their alkylated derivatives (*6*). In controlled laboratory exposures, the frequency of malformations in salmon and herring embryos increased as the tricyclic compounds became relatively enriched within the larger dissolved PAH mixture (*3, 4*).

In more recent years, embryonic heart failure and the corresponding loss of circulatory function have been shown to underlie most of the familiar defects associated with canonical

crude oil toxicity (*7*). The characteristic accumulation of pericardial edema is secondary to severe cardiac malfunction, including heart malformation. Studies using zebrafish established that individual PAHs act on the developing heart via distinct mechanistic pathways (*8-12*). A major finding was that exposures to fluorene, dibenzothiophene, or phenanthrene as individual compounds can reproduce key aspects of the injury phenotype caused by the more chemically complex PAH mixtures derived from whole oil. Uptake of these PAHs during organogenesis disrupts the form (morphogenesis) and function (pumping blood to other still-forming organs) of the developing heart. Disrupted circulation leads to gross heart malformation, edema, craniofacial defects, and body axis defects (*8, 9*).

The primary cardiotoxic effects of tricyclic PAHs are most likely mediated through a disruption of cardiac excitation-contraction coupling (*8, 9, 13*). In zebrafish embryos, exposure to ANSCO (and other crude oils) caused a specific reduction in ventricular contractility (*8, 14*). The gradual growth and morphogenesis of the heart into a complex, three-dimensional anatomical structure depends critically on normal pumping action and circulation (*15-17*). Larvae with severe cardiac malformation in response to oil exposure typically die at the yolk-sac stage, in part because secondary jaw deformities preclude larval feeding (*18, 19*). However, because cardiac form and function are so tightly linked during morphogenesis, there is potential for milder disruptions of heart rhythm or contractility following transient and sublethal exposure to lower levels of oil. Although subthreshold for overt heart failure and corresponding edema, these more nuanced forms of cardiotoxicity may subsequently alter cardiac shape and reduce performance later in life. Supporting this, adult zebrafish that survived embryonic ANSCO exposure with no externally visible morphological abnormalities grew into adults with rounder hearts and reduced aerobic capacity measured as critical swimming speed (U_{crit}) (*18*).

Extensive mark and recapture studies were also carried out with pink salmon that survived low-level embryonic oil exposure (ANSCO; 20 µg/L total ∑PAH). These fish grew in clean water to become outwardly normal appearing juveniles, and were then released by the tens of thousands to the ocean. Across three distinct brood years, post-release marine survival to adulthood (returning spawners) was reduced by ~ 40% (*20, 21*). This delayed mortality suggests that externally normal fry were in fact physiologically compromised. Although comparable studies for herring were not conducted, the herring population in Prince William Sound collapsed 3-4 years following the spill (*22, 23*) when the cohort spawned in oiled habitats would have recruited into the adult population (*24*). Whether the Exxon Valdez oil spill contributed to this fisheries collapse remains one of the most contentious unanswered questions in modern ecotoxicology.

Swimming performance is important for predator avoidance in larval fishes (*25*), and laboratory measures of swimming speed such as U_{crit} are relevant indicators of physiological condition for migratory salmonids and planktivorous forage fish (*26*). Our previous findings of delayed cardiac effects in zebrafish suggest a potential mechanism underlying the population-scale losses previous documented for pink salmon and herring. For both species, we show here that embryonic oil exposure caused reduced critical swimming speed, abnormal cardiac outflow tract anatomy, and ventricular hypertrophy in juveniles examined after 7-8 months of growth in clean seawater. In addition to improving our retrospective understanding of the long-term impacts of the Exxon Valdez spill, these results show that fisheries are much more vulnerable to future oil spills (and PAHs from land-based runoff and other sources) than previously appreciated.

Results and Discussion

Embryos of pink salmon and Pacific herring were exposed to water-soluble components of ANSCO using oiled gravel generator columns. The exposure window began shortly after fertilization and continued through a critical window of early cardiac development (~ 60% of embryogenesis). Fish were then transferred to clean water to complete embryonic development, larval metamorphosis, and juvenile growth (Fig. S1). Pink salmon were exposed to effluents from a graded series of columns loaded with increasing masses of oil, while herring were exposed to a single mass loading. Initial aqueous ∑PAH concentrations for the pink salmon experiment increased linearly with nominal oil load on gravel ($r = 0.999$, $n = 5$; Fig. S2A), resulting in an exposure range of 0.2 µg/L (control) through 45.4 µg/L (Table 1). Concentrations in all oil treatments dropped exponentially during the 50 d dosing period ($r = 0.984$, $n = 32$, two-phase exponential decay model; Fig. S2B). Pink salmon embryos accumulated PAHs from treatment water with composition consistent with the source of exposure, weathering, and expected changes between compartments (i.e., oil to water to tissue; Fig. 1A, B and Fig. S3). Tissue ∑PAH concentrations ranged from 26 ng/g wet weight (control) to 2474 ng/g (Table 1). Naphthalenes, fluorenes, dibenzothiophenes, and phenanthrenes were present in typical petrogenic patterns for each treatment, with relatively less parent compound and relatively more substituted compounds. Very low levels of chrysenes were detected in embryos, with relatively greater quantities of the parent compound present. Only trace amounts of higher molecular weight compounds (heavier than chrysenes) were measured in all oil treatments. Weathering of sequestered PAHs, estimated by percent chrysenes, was greatest in the lowest oil concentration and least in the highest dose ($r = -1.000$, $n = 4$, two phase exponential decay).

The single gravel column used in the herring embryo exposure had undergone more extensive weathering, and produced a dissolved PAH concentration of 0.230 ± 0.010 μg/L at the beginning of the exposure, relative to a background PAH concentration of 0.039 ± 0.003 μg/L in effluent from the clean gravel control column (Table 1). Greater weathering relative to the pink salmon exposure was reflected in the larger depletion of parent and C1-naphthalenes, fluorenes, dibenzothiophenes, and phenanthrenes, and higher proportions of the C2-alkyl homologs (Fig. 1). While control herring embryos had very low background levels of PAHs (∑PAHs 9.3 ± 3.7 ng/g wet weight), embryos exposed to oiled gravel effluent accumulated a ∑PAH concentration of 28.6 ± 10.2 ng/g wet weight, with a pattern that closely matched the composition of dissolved PAHs in water (Fig. 1D). This very low level PAH exposure was still sufficient to cause induction of the PAH-metabolizing enzyme cytochrome P4501A, as was evident from a 5-fold induction of *cyp1a* mRNA relative to controls (Fig. 1E).

Relative to herring embryos, pink salmon embryos are much larger, with a higher lipid content. As expected, PAH concentrations were correspondingly much higher in salmon than herring on the basis of wet weight (Table 1). Lipid correction more closely aligned tissue ∑PAH concentrations in both species. The lowest dose in pink salmon (2066 ng/g lipid) was roughly equivalent to the dose in herring embryos (1787 ± 256 ng/g lipid), and control levels were 240 and 582 ± 178 ng/g lipid in salmon and herring, respectively. Therefore, although herring were exposed to dissolved PAHs at a concentration roughly 40-times lower than the lowest dose for salmon, the two treatments produced tissue concentrations in the same range, and hence could be expected to produce overlapping biological effects.

Morphological defects were assessed in hatched pink salmon alevins and dechorionated herring embryos, the latter at exposure day 8 (Table S1). Only low levels of overt cardiotoxicity

were evident as pericardial edema in 11% of pink salmon embryos (high dose) and 11.8 ± 3.2% of oil-exposed herring embryos. For pink salmon, minor edema at the posterior end of the yolk sac was more frequent than pericardial edema, albeit not dose-dependent (Table S1). Minor hemorrhages were also observed in a small percentage of oil-exposed alevins. Herring embryos were evaluated at a stage that precedes blood cell formation, and thus corresponding effects of oil on vascular integrity were not assessed. Overall, the targeted exposure concentrations yielded minimal acutely lethal toxicity (e.g., overt heart failure), as intended.

Pink salmon were grown out in a single tank for each treatment. Herring embryos were hatched and raised in replicate tanks (3 each) for the control and single oil dose exposures. Juvenile growth rate was assessed for pink salmon, and was slowed by exposure to oil. At emergence (the end of yolk absorption), juvenile mass among treatments was approximately the same (0.2 g), and the size of controls and fish from the highest dose were indistinguishable ($P_{ANOVA} = 0.914$). Subsequent growth was exponential in each treatment but slower with increasing dose. Specific growth rates (Fig. S4) as measured by both mass and length, declined significantly with dose (mass r = -0.954, $P_{linear\ regression} = 0.012$; length r = -0.912, $P_{linear\ regression} = 0.031$).

Critical swimming speed (U_{crit}) was measured in juvenile pink salmon and Pacific herring eight and seven months after hatch, respectively. For both species, U_{crit} was significantly reduced in juvenile fish that were exposed to oil during embryonic development ($P < 0.001$; Fig. 2). For pink salmon, U_{crit} was determined for three treatments (control, ∑PAH 15.4 µg/L and 45.4 µg/L exposures). Absolute U_{crit} was greatest in control fish (43.5 cm/sec) and lowest in fish from the ∑PAH 45.4 µg/L exposure (34.6 cm/sec, Fig. 2A). Although there was a weakly positive correlation between fish length and U_{crit} in juvenile pink salmon (r = 0.394; Fig. S5A), the slope

of the linear regression was significant ($P < 0.001$). For swim tunnel experiments, fish from the high oil treatment were significantly shorter ($P = 0.002$) and lighter ($P = 0.006$) relative to controls (Fig. S5B, C). Fish also grew during the month-long experimental window (Fig. S5D; $P_{ANCOVA} < 0.001$). Although the slopes for the two oil treatments were equal ($P_{ANCOVA} = 0.915$), the controls were bigger on a given date ($P_{ANCOVA} = 0.001$). However, dose-dependent reduction in swimming speed was not entirely explained by differences in fish size, in particular because the smaller oil-exposed fish had lower relative U_{crit}. Critical swimming speed normalized by length (relative U_{crit}) produced the same dose-dependent pattern and significance (Fig. 2B), and relative U_{crit} in the ∑PAH 45.4 µg/L treatment group (5.7 ± 0.3 BL/sec) was significantly less than in controls (6.2 ± 0.2 BL/sec, $P = 0.02$). Reduction in relative U_{crit} was also significant after accounting for time and growth using 2-factor ANOVA models that included either length and U_{crit} or time and $U_{crit.}$

Absolute U_{crit} in juvenile herring transiently exposed to oil as embryos (∑PAH 0.23 µg/L) was significantly reduced to 20.7 ± 1.6 cm/sec relative to 26.5 ± 2.1 cm/sec for controls ($P = 0.04$, ANOVA with tank nested in treatment; Fig. 2C). Oil-exposed herring were larger than controls (length 5.6 ± 0.1 cm vs. 5.3 ± 0.1, respectively; weight 1.6 ± 0.1 g vs. 1.3 ± 0.1 g), most likely as a consequence of enhanced juvenile growth at lower densities due to higher in-tank early life stage mortality. This suggests further that the smaller size of oil-exposed pink salmon was not responsible for reduced absolute U_{crit}. The relative U_{crit} of exposed juvenile herring was significantly reduced from 5.0 ± 0.4 body lengths per second (BL/sec; controls) to 3.7 ± 0.3 BL/sec ($P = 0.03$, ANOVA with tank nested in treatment; Fig. 2D). There was no tank effect for either measure ($P = 0.15$ and 0.18, respectively). Oxygen consumption was measured during herring swim trials and used to calculate active metabolic rates (Fig. 2E). Oil exposure

significantly reduced maximum metabolic rate as fish approached U_{crit}, from 1824 ± 96 mg O_2/kg/hr in controls to 1642 ± 100 mg O_2/kg/hr for those exposed to crude oil ($P = 0.04$; tank effect $P = 0.15$). The standard metabolic rate was not determined, thus preventing a quantification of aerobic scope. However, this finding is consistent with reduced aerobic capacity and cardiac output during sustained swimming (*27*).

In addition to reducing swimming performance (and by proxy aerobic capacity) in both species, embryonic oil exposure caused similar changes in juvenile cardiac morphology, affecting the heart ventricle and outflow tract (Fig. S6). The morphology of the juvenile salmon ventricle was measured following eight and ten months of growth in clean water. At eight months, in correspondence to ∑PAH dose, there was a significant increase ventricle length ($R^2 = 0.8$, $P = 0.03$; Fig. 3A) but not width ($R^2 = 0.3$, $P = 0.4$). This yielded a highly significant dose-dependent increase in the ventricular aspect ratio (Fig. 3B; $R^2 = 0.96$, $P = 0.003$). This persisted to ten months post-exposure (Fig. 3B), albeit with weaker significance ($R^2 = 0.76$, $P = 0.055$). The length of the bulbus arteriosus (normalized to ventricular length) was reduced by oil exposure (Fig. 3C). Although not clearly dose-dependent, the effect of oil exposure was highly significant (ANOVA $P = 0.0012$) with salmon from all oil treatments having shorter outflow tracts relative to controls (all P values < 0.005) save the lowest ∑PAH exposure (9.8 µg/L). Although measurements of the outflow tract angle were more highly variable, there was a clear trend that mirrored the effects on the length of the bulbus arteriosus (Fig. 3D).

For juvenile herring a broader suite of cardiac measurements were taken from the same individuals used in U_{crit} assays (Table 2). As noted above, juveniles exposed to oil embryonically (∑PAH 0.23 ± 0.01 µg/L) were larger and had a higher condition factor than controls (Table 2). Oil exposure resulted in a specific increase in the lateral aspect ratio of the ventricle at 1.52 ±

0.02 relative to controls at 1.45 ± 0.02 (Table 2), but no change in the normalized lateral length or width, nor the ventral dimensions or aspect ratio. Ventricular volumes were estimated with an elliptical cone formula using the lateral length, ventral length and ventral width measures. The ventricular volume was significantly higher after oil exposure (0.050 ± 0.002 mm^3) compared to controls (0.042 ± 0.003 mm^3). Although there also was no change in the length of the bulbus arteriosus, there was a highly significant reduction of the outflow tract angle from 21.9 ± 1.8º in controls to 15.4 ± 1.4º in oil-exposed fish (Table 2). As indicated in Table 2, many of the anatomical measures in herring showed significant tank effects, both in the presence and absence of a significant effect of oil exposure. However, the results of post-hoc statistical tests indicated that tank effects were unlikely to confound any of the significant oil exposure effects, because there was no single tank consistently associated with a tank effect. For example, although higher condition factor was associated with different ventricular shape in adult rainbow trout (*27*), the tank with the most different condition factor (C11) was not the same as the tanks with the most different aspect ratio (O8) or ventricular volume (C6).

Changes in ventricular shape could be indicative of cardiac hypertrophy. Extensively characterized in mammals (*28*) and also occurring through similar pathways in salmon (*29*), cardiac hypertrophy is initially a compensatory response to cardiac stress or injury. At the cellular level, hypertrophy in mammals is indicated primarily by changes in cardiomyocytes that occur concentrically (increased cross-sectional area of individual cardiomyocytes), or eccentrically (lengthening of individual cardiomyocytes). However, fish hearts have the capacity to regenerate cardiomyocytes (*30*), and hypertrophy in fish also involves hyperplasia (*28, 29*). We therefore examined hearts dissected from pink salmon eight months post-exposure for histopathological evidence of hypertrophy. Although oil-exposed salmon had elongated

ventricles, we were unable to obtain measures of individual cardiomyocyte lengths (eccentric hypertrophy) from sections of the highly trabeculated and sponge-like heart. Rather, cardiomyocyte diameters were measured in cells randomly selected from three consistent areas of the ventricle (Fig. 4A). Analysis in fixed sections from 5 – 6 individuals for each treatment group showed a trend of increased cardiomyocyte width with increasing oil exposure that was not statistically significant (Fig. 4B). This may be due in part to the relative insensitivity of the technique (*31*). Oil exposure was associated significantly with hyperplasia in the ventricle (ANOVA $P < 0.0001$), with an increase in the density of cardiomyocyte nuclei in the highest exposure group (123% of control, $P = 0.0002$; Fig. 4C). The thickness of the epicardium was also reduced significantly by oil exposure (ANOVA $P = 0.02$; Fig. 4D). We examined the mRNA expression levels of atrial and B-type natriuretic peptides (ANP/*nppa* and BNP/*nppb*) in both salmon and herring hearts, as these genes are strongly linked to cardiac hypertrophy and heart failure in humans (*32*). Although there was a trend of increasing *nppa* and *nppb* mRNA levels with exposure level in pink salmon (Fig. 4E), there was no statistically significant dose-response relationship, with only the lowest exposure group (tissue ∑PAH 222 ng/g) showing significantly higher expression of *nppa* at 2.5-times above control ($P = 0.04$, Dunnett's method). Although the etiology of the pathological remodeling that underpins heart failure in humans and fish is clearly different, our combined results strongly suggest that a transient and sublethal oil exposure during embryogenesis triggers a subsequent hypertrophic response in juvenile fish.

Studies involving numerous fish species have shown that maximum cardiac output is linked to an optimal ventricular shape. In fast swimmers such as salmon and herring, a pyramidal ventricle confers a high cardiac output in support of prolonged swimming (*27, 33-36*). Adult rainbow trout (*O. mykiss;* ~1 kg) with atypical rounder hearts (i.e., reduced aspect ratio) have a

lower cardiac output and, by extension, a reduced U_{crit} (*27*). Similarly, zebrafish exposed transiently to ANSCO as embryos and then assessed one year later as reproductively mature adults also had rounder hearts and reduced U_{crit} relative to unexposed controls (*18*). Although our current findings for pink salmon and herring are dissimilar (elongated hearts following oil exposure), the relationship between cardiac output and cardiac anatomy has not been characterized for relatively young fish in the size range tested here (1 - 3 g). In zebrafish the most dramatic changes in ventricular shape occur between the larval and juvenile stages, with juveniles having a more elongated ventricle than the final adult form (*37*). The young salmon studied here would have grown considerably to adulthood (~ 2 kg), and an extended hypertrophic response during this interval may have eventually transformed elongated hearts into a more rounded adult morphology. On the other hand, the precise nature of cardiac dysfunction during embryonic exposure is likely to influence the final shape of the ventricle. In zebrafish, mutant embryos lacking atrial myosin heavy chain have reduced atrial contractility and yet survive to adulthood under laboratory rearing conditions. Unlike the rounder ventricles of adult oil-exposed zebrafish, these mutants as adults had elongated ventricles with a reduced outflow tract angle (*37*). While the precise effects of ANSCO exposure on cardiac function have not been determined for pink salmon, the crude oil reduces ventricular contractility without affecting the atrium in zebrafish embryos (*14*) and causes a different functional defect (atrial fibrillation) in Pacific herring embryos (*13*). Thus the anatomical changes we observed in juvenile salmon and herring may reflect an initiating pathophysiology (predominantly atrial dysfunction) that progresses to an altered ventricular shape and reduced ventricular performance later in life.

Lastly, our findings have important implications for understanding the long-term impacts of episodic disasters (e.g., oil spills) as well as more chronic inputs of PAH pollution from land-

based runoff and other sources. For herring in particular, our results shed new light on a possible connection between the Exxon Valdez spill and the ecosystem-scale crash of the Prince William Sound herring population several years later. We have established that cardiac injury occurs in a significant portion of herring that survive a transient and very low level PAH exposure without showing outward signs of acute heart failure. The injury threshold for aqueous ∑PAHs is as low as 0.23 μg/L. Accordingly, forage fish losses in Prince William Sound due to the Exxon Valdez disaster may have been grossly underestimated. For example, a recent analysis of all the water samples collected up to 1.4 years after the spill concluded that only 7% (89/1288) were potentially hazardous to herring, based on a toxicity threshold in the range of 1 – 10 μg/L (*31*). Notably, however, the arithmetic mean of all 1288 samples was 0.4 μg/L ∑PAHs, a concentration higher than that which caused significant delayed cardiotoxic injury in this study (0.23 μg/L). It is therefore likely that herring spawned in oiled nearshore habitats were affected in larger numbers over a more extensive geographic range than previously appreciated. Additional studies are needed to establish a precise threshold for cardiac dysregulation in herring embyros at exposure concentrations between 0.2 – 1 μg/L, a range that is near background environmental levels in urban spawning habitats known to receive PAH inputs from stormwater runoff, marine vessel traffic, and other sources.

Experimental Procedures

Embryo production. Eggs were collected from nineteen pink salmon females at Auke Creek, Alaska, on August 23, 2010. Eggs from each female were partitioned into six dry plastic cups and fertilized with milt pooled from multiple males (three males contributed to each cross,

fourteen males total). Eggs with milt were gently decanted three times between two cups to mix gametes. Water was added and all eggs and milt remained in contact 1 min. Eggs were added to incubators for each oiled gravel dose at random so that each female contributed approximately equal numbers of eggs to each treatment (mean 5854 ± 89 eggs per incubator, range 5595-6062).

Ripe Pacific herring were captured by cast net on March 24, 2010 from a spawning aggregation of the Holmes Harbor stock (northern Puget Sound), over eelgrass at 48º 6' N - 122º 31' W. Gonads were dissected from 45 females (length 17. ± 0.4 cm, weight 58.4 ± 2.5 g) and 12 males (length 18.9 ± 0.3 cm, weight 61.3 ± 2.2 g). A total of 527 g ovaries (mean 11.7 ± 0.7 g each) were dissociated and the eggs pooled. Testes (104 g total, mean 8.7 ± 0.9 g each) were pooled and macerated in herring Ringer's solution (206 mM NaCl, 7.2 mM KCl, 2.1 mM $CaCl_2$, 3.1 mM $MgCl_2 \cdot 6H2O$, pH 7.6). For fertilization and controlled adherence onto nylon mesh, eggs were first dispersed in calcium/magnesium-free artificial seawater containing 0.25% polyvinyl alcohol, and then evenly distributed onto six 18 cm X 40 cm nylon sheets in normal seawater containing diluted milt from macerated testes. After 30 min incubation with milt, fertilized eggs were rinsed three times in seawater, and then transferred to gravel column effluents. Fertilization rates were 95-98% based on counts of cleavage stage embryos 24 hours after fertilization.

Oil exposure and post-exposure culture. Embryos of both species were exposed to effluent water from oiled gravel columns. ANSCO was partially weathered to remove monoaromatic compounds by heating at 70 °C for 12 h until approximately 20% evaporated (*5*). Oiled and clean gravel columns were prepared and operated as previously described (*4, 5, 38*). For pink salmon exposure, dried 6 mm gravel was coated with weathered oil to yield four oil treatment levels (0.5, 1, 2, and 3 g oil/kg rock). Each treatment comprised one polyvinylchloride generator column (30 cm diameter × 122 cm tall) with 65 kg rock per treatment with an effluent hose

directly connected to similar cylindrical egg incubators (30 × 61 cm). Fresh water entered each column at the bottom (3.8 L min^{-1} approximate flow rate) and exited at the top. Exposure to oiled column effluent flow began 1 h after fertilization and extended 50 days, with all eggs remaining on top of perforated aluminum plates during this phase. Salinity was increased for 1 h every other day through January 2011 with seawater to 31 psu to limit microbial growth. Temperature, monitored daily, declined from about 9 °C to a minimum of 2.8 ºC then increased to 3.4 ºC. Embryos were at the eyed stage at the end of dosing, and were then removed from incubators and decanted into water. The incubators were cleaned and refilled with clean gravel, and live embryos replaced in perforated mesh bags. Two week later embryos were transferred to a vertical Heath tray stack for hatching (treatment positions were random). Hatching commenced the second week of November, and alevins were sampled for live morphological assessment (~ 100 per treatment). After yolk absorption (February 22-25), juveniles (about 3300 per treatment) were transferred from Heath trays to 300-L tanks containing seawater. Juvenile fish were fed pelletized food ad libitum beginning immediately after transfer. Food pellet size was increased as the fish grew, from #0 crumble (Bio-Oregon) in February to 1.2 mm in July. Fish subsamples (typically about 50 fish per treatment) were weighed and measured several times (1 to 4 week intervals) without replacement until August 30, 2011 (372 dpf).

For Pacific herring, gravel oiled at 6 gm/kg was re-used (after storage at -20ºC) from a previous study in which the column ran for 10 days (*13*). Herring embryo treatments (control and oil-exposed) comprised a polyvinylchloride column (9.6 cm diameter × 57.6 cm height) containing 2.6 kg gravel with the effluent draining into a 90-L aquarium. A steady-state effluent reservoir (~ 80 L) was maintained in each aquarium with a standpipe drain. For temperature control, effluent reservoir aquaria were submerged in a fiberglass hatchery raceway tank with

continuous flow (~ 20 gal/min) of ambient seawater. Column flow was initiated March 3, 2010 and embryo exposure began March 24. For each treatment three sheets of nylon mesh with adherent fertilized eggs (~ 50,000 total) were suspended in mid-water within the effluent reservoir. Temperature was maintained at 9.1 ± 0.1ºC in both treatments. For the first six days of exposure, dissolved oxygen was 93.7 ± 0.7% and 94.3 ± 0.1% in in the control and oiled reservoirs, respectively. Dissolved oxygen dropped to 64% in both treatments on day 7, and was raised to 86% using air stones for the last two days of exposure. At 8 d post-fertilization, embryos were subsampled for live morphological analysis and preservation for RNA extraction (flash frozen on liquid nitrogen) or PAH analysis (stored at -20 ºC) by cutting sections from each of the three mesh sheets in each treatment. After sample collection, mesh sheets were cut in half and suspended in 600-L tanks with filtered seawater flowing at 1 L/min. Tanks were held outdoors under an awning with light intensity at the surface of 360 lux. Hatching commenced on April 7, 2010 (14 dpf) and was complete by April 13, with 4.7 ± 0.9% and 2.7 ± 0.4% of eyed embryos unhatched in control and oiled tanks, respectively. From days 1-20 larvae were fed rotifers enriched with phytoplankton (*Nannochloropsis* and *Pavlova* algae paste, live *Tetraselmis* and *Nichia*; Brine Shrimp Direct, Ogden. UT). Newly hatched *Artemia* brine shrimp were introduced at day 5, and larvae were fed phytoplankton-enriched *Artemia* until day 20, after which frozen copepods (Cyclop-eeze, Argent Laboratories, Redmond, WA) and minced frozen krill were added to the diet. Fish were fed three times daily on weekdays, and five times daily on weekends via autofeeder.

Swimming performance. Juvenile fish were individually challenged in a modified Blazka-style 1.5-L swim tunnel respirometer (model SW10040, Loligo Systems, Tjele, Denmark) in seawater at 8 – 9ºC. For pink salmon assays were limited to three treatment levels (control,

∑PAH 15 µg/L, and ∑PAH 45 µg/L) due to the time required to assay enough individuals while minimizing the influence of continued growth. For both species, fish were randomly captured from each treatment group, and the swim tunnel operator was blind to treatment. Fish were allowed to acclimate in the tunnel for 30 minutes at water flow rate of 5 to 10 cm/sec; rates were then increased by 5 cm/sec every 5 minutes for salmon and every 10 minutes for herring. Failure was defined as impingement on the downstream baffle for > 1 s; each impinged fish was given a chance to resume swimming at a lower water velocity and velocity was quickly returned to the failure speed. The second failure time was recorded for fish that resumed swimming; the first failure time was recorded for those that did not resume. Times were rounded up or down to the nearest minute. U_{crit} was recorded as the failure velocity plus the minutes the fish swam at that speed. This calculation assumes that the time addition approximates a water velocity increase of 1 cm/sec per minute. The maximum tunnel flow rate was 50 cm/sec, and fish that reached or exceeded this velocity were allowed to swim without time restriction until failure (8% of juvenile pink salmon achieved this performance level). Swim tunnel tests extended from October 21 to November 17, 2010 for Pacific herring and from July 8 to Aug 3, 2011 for pink salmon. Oxygen consumption was measured for herring by recording the dissolved oxygen level (mg/L) at the end of acclimation and at each velocity increment up to the U_{crit}. Linear regressions between time and dissolved oxygen concentration were calculated with Microsoft Excel, and resultant slopes with $r^2 \geq 0.9$ ($N = 32$ controls, 33 oil-exposed) were used to calculate oxygen consumption based on the volume of the swim tunnel (1.5 L) and fish mass.

Cardiac anatomy and histology. For pink salmon, heart shape was assessed in animals randomly selected from each treatment group at a point at the beginning of swim assays (July 6-8, 2011) and after completion (September 8-9, 2011). For Pacific herring, heart shape was

assessed in the same individuals for which U_{crit} measures were made. For herring and salmon assessed in July 2011, fish were sacrificed with an overdose of MS-222 and hearts excised at the ventral aorta and sinus venosus, positioned in a petri dish flooded with either herring Ringer's solution or phosphate-buffered saline, and imaged on Nikon SMZ800 or Wild M5A stereomicroscopes fitted with FireI-400 (Unibrain, San Ramona, CA) or QImaging Micropublisher 5.0 RTV (Surrey, BC, Canada) digital cameras, respectively. For salmon measured in September 2011, hearts were too large to be imaged on the stereomicroscope. Instead, after MS-222 overdose, fish were partially dissected to expose the lateral aspect of the ventricle, and hearts were imaged in situ with a hand-held digital camera with a macro lens setting. Images of dissected hearts were calibrated with a stage micrometer, allowing absolute measurements of cardiac dimensions (ventricular length and width, bulbus arteriosus length). Relative measurements only (aspect ratio) were obtained from hearts imaged in situ.

Hearts dissected from pink salmon in July 2011 were fixed in neutral buffered formalin, transferred to 70% ethanol, and processed for paraffin embedding and microtome sectioning as described in detail elsewhere (*18*). Hearts were embedded in the same dorsal-ventral orientation and cellular measurements taken in sections from the same mid-line plane in which the ventricle lumen, bulbus arteriosus and ventriculobulbar valve were all in view. Sections were imaged on Nikon Eclipse 50i or Nikon E600 compound microscopes. Cardiomyocyte diameters were measured in 10 cells each in three areas of the ventricular wall in three sections of each heart (90 cells total from each heart). Epicardial thickness was measured in three areas of the ventricle in three sections from each heart. Cardiomyocyte nuclei were counted in four areas of the ventricle in each of three sections; total nuclei were counted in images that covered 225 μm^2 by 300 μm^2.

cDNA cloning. Clones for *nppa* and *nppb* were isolated from adult herring and juvenile pink salmon hearts, while herring *cyp1a* was isolated from ANSCO-exposed embryos (*13*). Tissue was either frozen in liquid nitrogen, or placed in RNA*later*, (Ambion, Life Technologies, Grand Island, NY, USA) before storage at -80 ^{o}C. Tissue total RNA was purified using either TRIzol reagent (Ambion) or the RNeasy Mini Kit (Qiagen, Valencia, CA, USA) according to the manufacturer's instructions. Reverse transcription polymerase chain reactions (RT-PCR) and rapid amplification of cDNA ends (RACE) were performed using previously published primers (*39, 40*) (Table S2). DNA sequencing of amplicons was performed on a 3100 Genetic Analyzer (Applied Biosystems, Life Technologies).

SMARTer RACE cDNA Amplification Kit and Advantage 2 PCR Enzyme System (Clontech, Mountain View, CA, USA) were used according to the manufacturer's instructions to amplify a 3' (*nppa*) or 5' (*nppb*) herring cDNA fragment, followed by amplicon sequencing and design of herring *nppa* and *nppb* gene-specific primers for use in additional RACE steps to complete cloning of each cDNA in the opposite direction. Salmon *nppa* and *nppb* were amplified via 3' and 5' RACE reactions that generated overlapping amplicons. For herring *cyp1a*, cDNA synthesis was performed using SuperScript III First-Strand Synthesis SuperMix, followed by RT-PCR amplification of an internal fragment using Platinum *Taq* DNA polymerase (Invitrogen, Life Technologies), amplicon sequencing, and design of a gene-specific primer for use in a 5' RACE reaction, using reagents described above. GenBank accession numbers are provided in Table S3.

Total RNA extraction and QPCR Analysis. Before storage at -80 ºC, salmon embryos and heart tissue were placed in RNA*later* (Ambion) for several weeks at 4 ºC and herring embryos were snap frozen in liquid nitrogen. Manufacturer's instructions were used to extract total RNA

with TRIzol, followed by treatment with DNA-free DNase (Ambion) using the rigorous DNase treatment option. RNA was then ethanol precipitated, and quantitated using a NanoDrop ND-1000 spectrophotometer (Thermo Scientific, Wilmington, DE, USA). RNA purity was assessed by measuring the A_{260}/A_{280} ratio, which was ~ 2.0 for all samples.

All DNased RNA samples were determined to be free of real-time reverse transcription quantitative polymerase chain reaction (RT-qPCR) inhibitors by testing 100 ng in a SPUD assay (*41*), using protocols and cycling parameters as described below. RNA integrity was assessed by visualizing samples via ethidium bromide agarose gel electrophoresis, and by confirming that all samples had a 3':5' ratio of < 4. Briefly: for salmon samples, two primer pairs were designed – one to amplify a 3' region and one to amplify a 5' region of *nppa*. Per RT-qPCR plate, two identical standard curves were included, each amplified by either the 3' or 5' primer pair, and a C_T for each primer pair was determined for each sample. The same threshold and baseline settings were applied to all wells, and the two primer pairs were determined to have approximately equal efficiencies by confirming that the absolute value of the slope of the standard curve log input amount versus ΔC_T was < 0.1, where $\Delta C_T = C_T$ 5' primers - C_T 3' primers for each standard curve point. For each sample, the 3':5' ratio = $2^{\Delta C}{}_T$. A purified PCR product that spanned the target areas for the 3' and 5' primers was used as a positive control to verify a 3':5' ratio of ~1.0. For herring samples, the same approach was used except that the target gene was *nppb*. These assays were performed using the same protocols and cycling parameters as described below.

Synthesis of cDNA was performed in 10 μl reactions using SuperScript III First-Strand Synthesis SuperMix (Invitrogen) and was primed with oligo(dT) primers, using the

manufacturer's instructions. Synthesis of embryonic and cardiac cDNA was performed using 1.875 μg and 0.1 μg of total RNA, respectively.

Gene-specific RT-qPCR primers (Table S3) were designed using Primer3 (http://www-genome.wi.mit.edu/genome_software/other/primer2.html), and synthesized by Sigma-Aldrich, St. Louis, MO, USA. To confirm primer specificity, the DNA sequence of all amplicons was determined as described above. RT-qPCR experiments were done in white 96 well optical plates, sealed with optical adhesive film, and analyzed on an ABI 7700 Sequence Detector (Applied Biosystems). Power SYBR Green Master Mix in a 25 μL reaction volume was used for salmon samples, and SYBR Select Master Mix (Applied Biosystems) in a 20 μL reaction volume was used for herring samples, with 4 μL of cDNA and primer concentrations of 0.15 μM each. Per reaction, 4 ng of embryonic or 1 ng of cardiac cDNA was used. Thermal cycling conditions were 2 min at 50 ºC, 10 min at 95 ºC for salmon samples or 2 min at 95 ºC for herring samples, followed by 41 amplification cycles at 95 ºC for 15 s plus 60 ºC for 30 s, and ending with a melting curve protocol for amplicon specificity assessment of 95 ºC for 15 s, 60 ºC for 20 s, 20 s continuous increase from 60 ºC to 95 ºC, then 95 ºC for 15 s.

For each primer pair, every plate included a standard curve of five 5-fold serial dilutions of pooled sample cDNA. All standard curves had a slope of –3.2 to –3.5, and $R^2 > 0.98$. Plate controls, plate calibrator samples, and reverse transcription calibrator samples were included and evaluated (*42*). The absence of genomic DNA contamination was confirmed by including a no-reverse transcription control for each gene from all samples – these control C_Ts were determined to be >7 cycles above the corresponding reverse transcribed sample. Each target gene was normalized to a reference gene, *ef1a (43)*, subsequent to confirming the approximately equal

efficiencies of the target and reference gene assays (described above), and the stable expression of *ef1a* across control and treatment samples.

Statistical analysis. Analysis of variance (ANOVA; one- or two-factor) was used to determine statistical significance for pink salmon growth and swimming performance data. Regression was used to explore relationships between dependent and independent variables; Pearson's correlation coefficient and F-tests (mean square regression / mean square residual) were used to assess significance. Analysis of covariance (ANCOVA) was used to explore the relationship between responses such as critical swimming speed and fish length and treatment. Statistical software applications used for the above analyses were SAS (SAS Institute, Cary, NC) and Minitab (Minitab, Inc., State College, PA). Dose-response data from pink salmon hearts (morphology and histology) were analyzed using linear and non-linear regression models using Prism 6.0b for Macintosh (Software McKiev, Boston, MA). Non-linear models were compared to linear models statistically, and most data fit straight-line models. Irrespective of whether data could be fit to regression models, ANOVA and post-hoc analyses (Dunnett's method) were applied using JMP 8.0.1 for Macintosh (SAS Institute, Cary, NC) to determine thresholds or treatment groups that were different than controls. Standard one-way ANOVA was used for single measurements from individuals (e.g., aspect ratio), while histological measurements taken from multiple sections were analyzed by one-way ANOVA with replicate nested within treatment (PAH dose), with individual fish representing replicates. One-way ANOVA with heart region nested within individual showed no difference between heart regions. Data for all herring measures were analyzed by one-way ANOVA with tank (replicate) nested within treatment (control or oil-exposed), with Student's t and Tukey Kramer Honestly Significant Differences tests for post-hoc means comparisons for treatment effect and tank effect, respectively.

Acknowledgements: This work was funded in part by a grant from the Prince William Sound Regional Citizens Advisory Council to G.S.R, M.G.C., and J.P.I. The authors thank Heather Day, Richard Edmunds, Jana Labenia, Cathy Laetz, Paul Olson, Frank Sommers, Julann Spromberg, and Maryjean Willis for assistance with various aspects of the study.

References

1. G. D. Marty, J. E. Hose, M. D. McGurk, E. D. Brown, D. E. Hinton, Histopathology and cytogenetic evaluation of Pacific herring larvae exposed to petroleum hydrocarbons in the laboratory or in Prince William Sound, Alaska, after the Exxon Valdez oil spill. *Can. J. Fish. Aquat. Sci.* **54**, 1846 (Aug, 1997).
2. S. D. Rice *et al.*, Impacts to pink salmon following the Exxon Valdez oil spill: Persistence, toxicity, sensitivity, and controversy. *Rev. Fish. Sci.* **9**, 165 (2001).
3. M. G. Carls, S. D. Rice, J. E. Hose, 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*). *Environ. Toxicol. Chem.* **18**, 481 (1999).
4. R. A. Heintz, J. W. Short, S. D. Rice, Sensitivity of fish embryos to weathered crude oil: Part II. Increased mortality of pink salmon (*Oncorhynchus gorbuscha*) embryos incubating downstream from weathered Exxon Valdez crude oil. *Environ. Toxicol. Chem.* **18**, 494 (1999).
5. G. D. Marty *et al.*, Ascites, premature emergence, increased gonadal cell apoptosis, and cytochrome P4501A induction in pink salmon larvae continuously exposed to oil-contaminated gravel during development. *Can. J. Zool.-Rev. Can. Zool.* **75**, 989 (1997).
6. J. W. Short, R. A. Heintz, Identification of *Exxon Valdez* oil in sediments and tissues from Prince William Sound and the Northwestern Gulf of Alaska based on a PAH weathering model. *Environ. Sci. Technol.* **31**, 2375 (1997, 1997).
7. J. P. Incardona, T. K. Collier, N. L. Scholz, Oil spills and fish health: exposing the heart of the matter. *J. Expo. Sci. Environ. Epidemiol.* **21**, 3 (2011).
8. J. P. Incardona *et al.*, Aryl hydrocarbon receptor-independent toxicity of weathered crude oil during fish development. *Environ. Health Perspect.* **113**, 1755 (2005).
9. J. P. Incardona, T. K. Collier, N. L. Scholz, Defects in cardiac function precede morphological abnormalities in fish embryos exposed to polycyclic aromatic hydrocarbons. *Toxicol. Appl. Pharmacol.* **196**, 191 (2004).

10. J. P. Incardona, H. L. Day, T. K. Collier, N. L. Scholz, Developmental toxicity of 4-ring polycyclic aromatic hydrocarbons in zebrafish is differentially dependent on AH receptor isoforms and hepatic cytochrome P450 1A metabolism. *Toxicol. Appl. Pharmacol.* **217**, 308 (2006).
11. J. P. Incardona, T. L. Linbo, N. L. Scholz, Cardiac toxicity of 5-ring polycyclic aromatic hydrocarbons is differentially dependent on the aryl hydrocarbon receptor 2 isoform during zebrafish development. *Toxicol. Appl. Pharmacol.* **257**, 242 (Dec, 2011).
12. J. A. Scott, J. P. Incardona, K. Pelkki, S. Shepardson, P. V. Hodson, AhR2-mediated; CYP1A-independent cardiovascular toxicity in zebrafish (Danio rerio) embryos exposed to retene. *Aquat. Toxicol.* **101**, 165 (2011).
13. J. P. Incardona *et al.*, Cardiac arrhythmia is the primary response of embryonic Pacific herring (Clupea pallasi) exposed to crude oil during weathering. *Environ. Sci. Technol.* **43**, 201 (2009).
14. J.-H. Jung *et al.*, Geologically distinct crude oils cause a common cardiotoxicity syndrome in developing zebrafish. *Chemosphere* **91**, 1146 (2013).
15. N. S. Glickman, D. Yelon, Cardiac development in zebrafish: coordination of form and function. *Semin. Cell Dev. Biol.* **13**, 507 (Dec, 2002).
16. J. J. Schoenebeck, D. Yelon, Illuminating cardiac development: Advances in imaging add new dimensions to the utility of zebrafish genetics. *Semin. Cell Dev. Biol.* **18**, 27 (Feb, 2007).
17. O. B. C. S. a. E. T. The Federal Interagency Solutions Group, "Oil Budget Calculator - Deepwater Horizon" (2010).
18. C. E. Hicken *et al.*, Sub-lethal exposure to crude oil during embryonic development alters cardiac morphology and reduces aerobic capacity in adult fish. *Proc. Natl. Acad. Sci. U. S. A.* **108**, 7086 (2011).
19. J. P. Incardona *et al.*, Exxon Valdez to Deepwater Horizon: comparable toxicity of both crude oils to fish early life stages. *Aquat. Toxicol.* **in press**, (2013).
20. R. A. Heintz, Chronic Exposure to Polynuclear Aromatic Hydrocarbons in Natal Habitats Leads to Decreased Equilibrium Size, Growth, and Stability of Pink Salmon Populations. *Integr. Environ. Assess. Manag.* **3**, 351 (2007).
21. R. A. Heintz *et al.*, Delayed effects on growth and marine survival of pink salmon *Oncorhynchus gorbuscha* after exposure to crude oil during embryonic development. *Mar. Ecol. Prog. Ser.* **208**, 205 (2000).
22. P. J. F. Hulson *et al.*, Data conflicts in fishery models: incorporating hydroacoustic data into the Prince William Sound Pacific herring assessment model. *ICES J. Mar. Sci.* **65**, 25 (Jan, 2008).
23. R. E. Thorne, G. L. Thomas, Herring and the "Exxon Valdez" oil spill: an investigation into historical data conflicts. *ICES J. Mar. Sci.* **65**, 44 (January 1, 2008, 2008).
24. J. F. Schweigert, D. E. Hay, T. W. Therriault, M. Thompson, C. W. Haegele, Recruitment forecasting using indices of young-of-the-year Pacific herring (Clupea pallasi) abundance in the Strait of Georgia (BC). *ICES J. Mar. Sci.* **66**, 1681 (September 1, 2009, 2009).
25. L. A. Fuiman, K. A. Rose, J. H. Cowan, E. P. Smith, Survival skills required for predator evasion by fish larvae and their relation to laboratory measures of performance. *Anim. Behav.* **71**, 1389 (Jun, 2006).
26. I. Plaut, Critical swimming speed: its ecological relevance. *Comp. Biochem. and Physiol. A-Mol. Integ. Physiol.* **131**, 41 (Dec, 2001).

27. G. Claireaux *et al.*, Linking swimming performance, cardiac pumping ability and cardiac anatomy in rainbow trout. *J. Exp. Biol.* **208**, 1775 (May, 2005).
28. I. B. Johansen *et al.*, Cortisol response to stress is associated with myocardial remodeling in salmonid fishes. *The Journal of Experimental Biology* **214**, 1313 (April 15, 2011, 2011).
29. V. Castro *et al.*, Cardiac Molecular-Acclimation Mechanisms in Response to Swimming-Induced Exercise in Atlantic Salmon. *PLoS One* **8**, e55056 (2013).
30. K. D. Poss, L. G. Wilson, M. T. Keating, Heart regeneration in zebrafish. *Science* **298**, 2188 (Dec 13, 2002).
31. A. M. Gerdes, Cardiac myocyte remodeling in hypertrophy and progression to failure. *J. Card. Fail.* **8**, S264 (2002).
32. E. Braunwald, Biomarkers in Heart Failure. *N. Engl. J. Med.* **358**, 2148 (May 15, 2008, 2008).
33. A. P. Farrell, Cardiorespiratory performance in salmonids during exercise at high temperature: insights into cardiovascular design limitations in fishes. *Comp. Biochem. and Physiol. A-Mol. Integ. Physiol.* **132**, 797 (Aug, 2002).
34. A. P. Farrell, Cardiorespiratory performance during prolonged swimming tests with salmonids: a perspective on temperature effects and potential analytical pitfalls. *Philos. Trans. R. Soc. B Biol. Sci.* **362**, 2017 (Nov 29, 2007).
35. D. Sanchez-Quintana, V. Garcia-Martinez, V. Climent, J. M. Hurle, Morphological analysis of the fish heart ventricle: myocardial and connective tissue architecture in teleost species. *Ann. Anat.* **177**, 267 (May, 1995).
36. R. M. Santer, M. Greer Walker, L. Emerson, P. R. Witthames, On the morphology of the heart ventricle in marine teleost fish (teleostei). *Comp. Biochem. Physiol. A-Physiol.* **76**, 453 (1983).
37. N. R. Draper, H. Smith, *Applied regression analysis*. (John Wiley & Sons, Inc, New York, NY, 1981).
38. M. G. Carls, R. A. Heintz, G. D. Marty, S. D. Rice, Cytochrome P4501A induction in oil-exposed pink salmon Oncorhynchus gorbuscha embryos predicts reduced survival potential. *Mar. Ecol. Prog. Ser.* **301**, 253 (2005).
39. K. Inoue *et al.*, Structural and functional evolution of three cardiac natriuretic peptides. *Molecular Biology and Evolution* **22**, 2428 (Dec, 2005).
40. K. Yamazaki, H. Teraoka, W. Dong, J. J. Stegeman, T. Hiraga, cDNA cloning and expressions of cytochrome P450 1A in zebrafish embryos. *J. Vet. Med. Sci.* **64**, 829 (Sep, 2002).
41. T. Nolan, R. E. Hands, W. Ogunkolade, S. A. Bustin, SPUD: a quantitative PCR assay for the detection of inhibitors in nucleic acid preparations. *Anal. Biochem.* **351**, 308 (Apr 15, 2006).
42. T. Nolan, R. E. Hands, S. A. Bustin, Quantification of mRNA using real-time RT-PCR. *Nat. Protoc.* **1**, 1559 (2006).
43. J. A. Luckenbach, J. T. Dickey, P. Swanson, Regulation of pituitary GnRH receptor and gonadotropin subunits by IGF1 and GnRH in prepubertal male coho salmon. *Gen Comp Endocrinol* **167**, 387 (Jul 1, 2010).

Table 1: PAH concentrations in exposure water and embryonic tissues

Species	Treatment	∑PAH water (µg/L)	∑PAH in embryos (ng/g wet weight)	∑PAH in embryos (ng/g lipid)
Pink salmon	clean gravel	0.2	26	240
	0.5 g/kg oiled gravel	9.8	222	2066
	1 g/kg oiled gravel	15.4	634	5895
	2 g/kg oiled gravel	30.0	1279	11,900
	3 g/kg oiled gravel	45.4	2474	23,012
Pacific herring	clean gravel	0.039 ± 0.003	9.3 ± 3.7	582 ± 178
	oiled gravel	0.230 ± 0.010	28.6 ± 10.2	1787 ± 256

Table 2: Anatomical measures in juvenile Pacific herring

measure	control	oil exposed	*P* oil effect[b]	*P* tank effect[c]
Fork length (cm)	5.3 ± 0.1	5.6 ± 0.1	0.0005	0.0007 (C6)
Mass (g)	1.26 ± 0.07	1.65 ± 0.10	0.0003	0.0004 (O2)
Condition factor	0.82 ± 0.01	0.87 ± 0.01	0.01	0.02 (C11)
Ventricle length - lateral[a]	69.5 ± 1.0	69.8 ± 1.2	0.2	0.001 (O8)
Ventricle width - lateral[a]	51.3 ± 0.9	49.8 ± 0.7	0.7	0.3
Aspect ratio - lateral	1.45 ± 0.02	1.52 ± 0.02	0.04	0.03 (O8)
Ventricle length - ventral[a]	64.0 ± 1.0	63.6 ± 1.5	0.6	0.004 (O8)
Ventricle width - ventral[a]	63.5 ± 0.9	64.3 ± 1.0	0.07	0.01 (O8)
Aspect ratio - ventral	1.14 ± 0.01	1.13 ± 0.01	0.2	0.003 (C11)
Ventricle volume (mm^3)	0.042 ± 0.003	0.050 ± 0.002	0.007	0.002 (C6)
BA length (relative to ventricle)	0.45 ± 0.01	0.47 ± 0.01	0.6	0.5
OFT angle (degrees)	21.9 ± 1.8	15.4 ± 1.4	0.004	0.5

[a]Normalized to fork length

[b]Data analyzed by one-way ANOVA with tank nested under treatment (oil exposure)

[c]Parentheses indicate identity of statistically different tanks, e.g., C6 = control tank 6

Figure legends

Figure 1. Exposure to oiled gravel effluent resulted in uptake of PAHs into embryos and induction of *cyp1a*. (A) Waterborne PAHs from the highest exposure concentration (3 g oil/kg gravel; 45 µg/L ∑PAH) for pink salmon embryos, measured in a single water sample. (B) Tissue PAHs in pink salmon embryos at exposure day 21 (∑PAH 2474 ng/g wet weight), measured in a single pooled sample (7 g tissue wet weight). (C) Waterborne PAHs in column effluent for Pacific herring embryo exposure (∑PAH 0.23 µg/L) measured in triplicate water samples (mean ± s.e.m.). (D) Tissue PAHs in herring embryos at exposure day 8, measured in triplicate samples of pooled embryos (mean ± s.e.m.; 1 -3 g wet weight each). (E) Levels of *cyp1a* mRNA in herring embryos exposed to gravel effluents. RNA was extracted from embryos at exposure day 8 and levels of *cyp1a* and *ef1α* determined by QPCR as described in Materials and Methods. Data are mean ± s.e.m. of *cyp1a* levels normalized to *ef1α* levels measured in two replicates from each treatment with ~ 150 embryos each. N, naphthalenes; AY, acenaphthylene; AE, acenaphthene; F, fluorene; D, dibenzothiophene; P, phenanthrene; ANT, anthracene; FL, fluoranthene; PY, pyrene; FP, fluoranthenes/pyrenes; BAA, benz[*a*]anthracene; C, chrysene; BBF, benzo[*b*]fluoranthene; BKF, benzo[*j*]fluoranthene/benzo[*k*]fluoranthene; BEP, benzo[e]pyrene; BAP, benzo[a]pyrene; PER, perylene; IDY, indeno[1,2,3-*cd*]pyrene, DBA, dibenz[*a*,*h*]anthracene/dibenz[*a*,*c*]anthracene; BZP, benzo[*ghi*]perylene. Parent compound is indicated by a 0 (e.g., N0), while numbers of additional carbons (e.g. methyl groups) for alkylated homologs are indicated as N1, N2, etc.

Figure 2. Critical swimming speed of juveniles was reduced by embryonic oil exposure. U_{crit} was measured as described in the Materials and Methods. Mean U_{crit} (± s.e.m.) is given as an absolute speed (A and C, cm/s) or relative to body length (B and D, BL/s) for (A, B) salmon exposed to clean effluent (∑PAH 0.2 µg/L, $N = 52$) or oiled gravel effluent with ∑PAH 15 µg/L ($N = 45$) and 45 µg/L ($N = 52$); and (C, D) herring exposed to clean gravel effluent (∑PAH 0.04 µg/L, $N = 32$) and oiled gravel effluent with ∑PAH 0.23 µg/L ($N = 33$). Statistical analysis is discussed in the text. For salmon data, P values are shown for effect of oil exposure from ANOVA while P values over oil-exposed groups represent comparison to controls in post-hoc analysis. For herring data, P values shown are for effect of treatment (oil exposure) and tank effect, respectively from a nested ANOVA (replicate nested under treatment).

Figure 3. Dose-dependent changes in juvenile cardiac morphology following embryonic oil exposure. Anatomical locations of measurements are shown in Fig. S6. (A) Ventricular length and width normalized to fish fork length measured in juveniles 8 months after exposure (mean ± s.e.m.). Data were fit to a linear regression model, *P* value indicates significance of the slope. (B) Ventricular aspect ratio measured in juveniles 8 months (triangles) and 10 months (diamonds) after exposure, and both age groups pooled (circles) fit a linear regression model (mean ± s.e.m). *P* values indicate significance of slope. (C) Length of the bulbus arteriosus normalized to ventricular length in juveniles 8 months after exposure (mean ± s.e.m.). Data did not fit linear or non-linear regression models, but were highly significant by ANOVA (P = 0.0012); three highest doses were significantly different from control by post-hoc Dunnett's test. (D) Outflow tract angle in juveniles 8 months following exposure (mean ± s.e.m.). For 8 month fish, $N = 12$ for all

oil exposed groups, 13 for control; for 10 month fish, $N = 20$-23 for all groups except lowest oil exposure dose, $N = 11$.

Figure 4. Evidence of ventricular hypertrophy in juvenile pink salmon following embryonic oil exposure. A subset of hearts from fish 8 months post-exposure for which dimensions were measured in Fig. 3 were sectioned and stained with hematoxylin/eosin. (A) Sections were selected that showed the atrium and ventricle, with a clear midline plane through the bulbus arteriosus and ventricular-bulbar valve. (B) Cardiomyocyte diameters, (C) thickness of the epicardium, and (D) numbers of cardiomyocyte nuclei were measured in the indicated areas (A, red and blue squares, black circles, respectively). (E) Levels of mRNA for atrial (*nppa*) and B-type natriuretic peptide (*nppb*) in pink salmon hearts. RNA was isolated from juveniles at 10 months post-exposure following dissection and preservation in RNALater, and ANP and BNP mRNA levels measured by quantitative real-time RT-PCR using gene-specific primers and a SYBR green assay. ANP/BNP levels were normalized relative of the levels of a reference gene (pink salmon *ef1α*). All data are mean ± s.e.m. ANOVA showed significance for effect of oil exposure for epicardial thickness ($P = 0.02$) density of nuclei ($P < 0.0001$). Treatments that differed significantly from controls are indicated by asterisks (Dunnett's post-hoc test, $\alpha = 0.05$).

Supplemental Material

Table S1: Morphological signs of toxicity in exposed embryos

Species	∑PAH water (µg/L)	N	Pericardial edema (%)	Yolk sac edema (%)	Hemorrhage (%)
Pink salmon	0.2	96	0	1	0
	9.8	86	0	22	3
	15.4	120	9	38	7
	30.0	89	2	24	3
	45.4	88	11	33	9
Pacific herring	0.039 ± 0.003	68	1.2 ± 1.2	0	NA
	0.230 ± 0.010	81	11.8 ± 3.2	0	NA

Table S2. Genes and primers used in cDNA cloning, 5' to 3'

Gene		Primer
pink salmon		
nppa –	5' RACE[a]	GACTGCAGCCTAAACCGCTCGAGGTC
	3' RACE[a]	AGGACTGCCGTCTCATGGGGACTTCTG
nppb	5' RACE[b]	GTATTTGCCAACYGTGKTGCATCC
	3' RACE[b]	CGTATCCTGTCTACAAYGGGYTACTGAC
Pacific herring		
nppa –	5' RACE[c]	TCATTCGTGGGCCATGTTCTCAACC
	3' RACE[d]	TGCTTTGGNGCCAGRATGGAYCGNATHGG
nppb	5' RACE[b]	CANCCCAGAGARCTCATGGANCCDAT
	3' RACE[c]	GGCGGTCCGGAACGACTCGAAGAG
cyp1a	internal[e]	F:ATHGAYCAYTGYGARGAYMG
	internal[e]	R:TCYTTNGGDATRAARTANCC
	5' RACE[c]	TGAAGGCTTCCAAAAGAGGGAGGCTGG

[a]designed based on alignment of available teleost *nppa* sequences
[b]designed based on alignment of available teleost *nppb* sequences
[c]gene-specific primer
[d]Inoue et al. 2005, fishANPS-1 primer
[e]Yamazaki et al. 2002, F = forward primer, R = reverse primer

Table S3. Genes and primers used for RT-qPCR, 5' to 3'

Gene	Primer Pair	GenBank accession number or reference
pink salmon		
nppa – 5'	F: ACCAGGAGTAAAGCTGTGTCTG	KF271796
	R: GACTAGCTACGTCTTTTAGGACTGC	
nppa – 3'	F: ATCATAGAGGCACATTGACTGG	KF271796
	R: TGTGTGTCACTACACCCTCTCTC	
nppb	F: AATGAGCTCTCTTGGATGCAC	KF271797
	R: AGTAATGACGTTGCTGTTCCAG	
*ef1*α	F: CCCCTGGACACAGAGATTTCATC	Lukenbach et al., 2010
	R: AGAGTCACACCGTTGGCGTTAC	
Pacific herring		
nppa	F: GCTGCTGGACCTCATTATGG	KF271794
	R: AGCAAAATCCAGAGTCATCGTG	
nppb – 5'	F: CCAACACCACAAGCAATACATC	KF271795
	R: GTTGTAGGTTCACGAATGCAAG	
nppb – 3'	F: CATTACGCCATTACGCCATAAG	KF271795
	R: GGGCATGGACGTATATCAGAAG	
cyp1a	F: AGGAGCACATCAGCAAGGAG	KF271793
	R: ACCACCTGTCCGAACTCATC	
*ef1*a	F: CTGGTATGGTTGTGACCTTCG	DQ334851.1
	R: ACGGATATCCTTGACTGACACG	

F = forward primer; R = reverse primer

Figure S1: Experimental design for embryonic exposure and post-exposure growth. The top bar demarcates the embryonic (blue), larval (amber) and juvenile (brown) life stages relative to exposure and growth. Relative length of each stage is arbitrary and does not represent actual time for a given stage. The middle and lower bars indicate the relative exposure and rearing periods for herring (middle) and pink salmon (lower), and points where measurements were made (arrows). Red indicates the proportion (%) of embryonic development during which each species was exposed to oiled-gravel effluent, with absolute exposure time given for each species in days (d); green indicates the period spent in clean water. Gray bars provide the time spent in different culture environments. Exposure and rearing paradigms were tailored to the different life history strategies of each species, e.g., herring as nearshore demersal spawners that produce pelagic larvae and salmon as anadromous spawners that deposit eggs in streambed nests, within which embryos complete larval development. Herring embryos were exposed in gravel effluent reservoirs, then transferred to rearing tanks to complete embryonic development, hatch, complete larval development and juvenile growth. Salmon embryos were maintained in gravel effluent reservoirs with switching of oiled effluent to clean effluent, followed by transfer to a vertical stack incubator for hatching and completion of larval development (yolk absorption), followed by transfer to rearing tanks for juvenile growth.

Figure S2. Total aqueous PAH concentrations in column effluents during pink salmon exposure. (A) Total sum (∑) PAHs in column effluent (µg/L) at the beginning of exposure (day 0) as a function of oil mass applied to gravel. Linear regression results are provided above the line. (B) Relationship between ∑PAH aqueous concentration and time. All data from the five exposure levels were combined in this analysis, each normalized to initial aqueous concentration.

Figure S3. Individual PAH concentrations in column effluent at the start of exposure and in embryos at exposure day 21 for pink salmon at all treatment levels. Left panels show aqueous PAHs measured in single water samples, right columns show PAHs measured in embryos from single pooled samples (each ≥7 g tissue wet weight). (A) 3 g/kg oil load, (B) 2 g/kg oil load, (C) 1 g/kg oil load, (D) 0.5 g/kg oil load. (E) control clean gravel. N, naphthalenes; AY, acenaphthylene; AE, acenaphthene; F, fluorene; D, dibenzothiophene; P, phenanthrene; ANT, anthracene; FL, fluoranthene; PY, pyrene; FP, fluoranthenes/pyrenes; BAA, benz[*a*]anthracene; C, chrysene; BBF, benzo[*b*]fluoranthene; BKF, benzo[*j*]fluoranthene/benzo[*k*]fluoranthene; BEP, benzo[e]pyrene; BAP, benzo[a]pyrene; PER, perylene; IDY, indeno[1,2,3-*cd*]pyrene, DBA, dibenz[*a*,*h*]anthracene/dibenz[*a*,*c*]anthracene; BZP, benzo[*ghi*]perylene. Parent compound is indicated by a 0 (e.g., N0), while numbers of additional carbons (e.g. methyl groups) for alkylated homologs are indicated as N1, N2, etc.

Figure S4. Reduced growth rates in juvenile pink salmon following embryonic oil exposure. Specific growth rates as a function of embryonic aqueous ∑PAH concentration for mass (A) and length (B) were determined during growth up to the point of swim trials. Values are mean ± s.e.m. for ~ 250 fish at each dosing level, derived from weight and length measures on a minimum of 10 and up to 55 fish at each time point.

Figure S5. Fish size in swimming performance assays. (A) Relationship of absolute U_{crit} (cm/sec) to fish length for all fish assayed. Data represent individual U_{crit} measure and fork length of all fish from each of the three treatment groups assayed. (B) Fork length and (C) weight

of individual assayed for each of three treatment groups. Data are mean ± s.e.m. (control, $N = 45$; 15 µg/L, $N = 52$; 45 µg/L, $N = 52$). Asterisks indicate statistically different groups determined by one-way ANOVA and post-hoc means comparison. (D) Length of fish as a function of swim trial date (July 3 through August 12).

Figure S6. Locations and orientation for measure of cardiac dimensions, shown for a representative pink salmon heart. Hearts dissected from both pink salmon and Pacific herring were oriented and imaged as shown. (A) Ventricular lengths were measured from the center of the bulbus arteriosus to the apex (black line, *VL*), while ventricular widths were measured from the dorsal apex perpendicular to the length line (green line, *VW*), Aspect ratio was measured by tracing the perimeter of the ventricle with the freehand line tool in ImageJ (yellow dashed line, *AR*). Length of the bulbus arteriosus was measured from the midline of where it joins the ventricle to the midline of the distal lumen at the dissection point (blue line, *BAL*). (B) The outflow tract was measured by drawing a line with one segment running from the apex to the midline of the ventricular – bulbar junction, and a segment running along the midline of the bulbus (*OFT* ∠).