Sublethal exposure to crude oil during embryonic development alters cardiac morphology and reduces aerobic capacity in adult fish

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Exposure to high concentrations of crude oil produces a lethal syndrome of heart failure in fish embryos. Mortality is caused by cardiotoxic polycyclic aromatic hydrocarbons (PAHs), ubiquitous components of petroleum. Here, we show that transient embryonic exposure to very low concentrations of oil causes toxicity that is sublethal, delayed, and not counteracted by the protective effects of cytochrome P450 induction. Nearly a year after embryonic oil exposure, adult zebrafish showed subtle changes in heart shape and a significant reduction in swimming performance, indicative of reduced cardiac output. These delayed physiological impacts on cardiovascular performance at later life stages provide a potential mechanism linking reduced individual survival to population-level ecosystem responses of fish species to chronic, low-level oil pollution.

cardiac toxicity | fish populations | heart development | oil spills

Oil spills such as the 1989 Exxon Valdez spill and the 2010 Deepwater Horizon disaster pose major threats to fish health and population vitality. Both spills have catalyzed research efforts to discern how to detect fish injury (1). The population effects on pink salmon (*Oncorhynchus gorbuscha*) and Pacific herring (*Clupea pallasi*) after Exxon Valdez are cause for concern regarding the effects of any major spill, including the Deepwater Horizon. Evidence of population effects is strongest for pink salmon, for which studies of embryos in spawning gravels in the intertidal zone of streams crossing oiled beaches demonstrated elevated mortality for at least 4 y after the spill (2–4). Laboratory studies subsequently showed that water contaminated with PAHs in the low parts per billion (ppb or μg/L) dissolved from oiled gravel produced a characteristic syndrome of edema in both species (5–8). Moreover, a series of mark and recapture studies with pink salmon found that morphologically normal juveniles that survived embryonic exposure to water containing <20 μg/L total PAHs had elevated rates of postrelease mortality in the marine environment, with an average reduction in adult survival by 36% (6, 9, 10). Growth rate depression was also measured in juveniles several months after exposure ceased, but cellular or tissue mechanisms were not investigated (10). There is evidence for similar effects in Pacific herring after the spill (11, 12), but comparable studies for this species were not conducted.

Because of the logistical difficulties of identifying mechanisms of toxicity in wild species, we use the zebrafish (*Danio rerio*) model to explore the long-term impacts of sublethal oil exposure. Oil exposure studies using zebrafish embryos demonstrated a heart failure syndrome that is lethal to larvae (13, 14), findings that were validated in Pacific herring (15). As oil weathers, the proportional composition of dissolved-phase PAHs becomes dominated by the tricyclic fluorenes, dibenzothiophenes, and phenanthrenes (5, 6, 16), which were shown to be directly cardiotoxic (13, 14). In herring embryos, cardiotoxicity occurred at tricyclic PAH concentrations in the tissue as low as 0.8 μmol/kg (150 ppb) wet weight, indicating a specific, high-affinity cellular target (15). Individual nonalkylated tricyclic PAHs caused atrioventricular conduction arrhythmias indistinguishable from those caused by drugs known to block potassium channels required for the repolarization phase of cardiac action potentials (13, 14). PAH mixtures from weathered crude oil caused more complex cardiac dysfunction, suggestive of additional targets, including pacemaker currents and plasma membrane or sarcoplasmic calcium channels (14, 15). Consistent with genetic analyses of cardiac form and function in zebrafish (17, 18), this oil-induced cardiac dysfunction affects later morphogenetic steps, such as looping of the atrial and ventricular chambers into their normal side-by-side positioning (13, 14). Although these morphological defects are lethal, these aggregate findings raise the question of whether milder, transient cardiac dysfunction caused by low doses of PAHs can have subtle impacts on cardiac form that could ultimately influence physiological performance later in life and, in turn, reduce survival.

We hypothesized that low levels of embryonic oil exposure might influence ventricular shape and, ultimately, cardiac output in adult animals because previous studies showed that intermediate PAH concentrations caused a compensatory dilation of the cardiac chambers in larvae (13). Ventricular shape is linked to maximum cardiac output as demonstrated by critical swimming speed (U_{crit}) studies (19, 20). Continuously swimming species such as salmon or herring have pyramidal ventricles (21, 22), and fish with rounded ventricles (reduced length/width ratio) are slower swimmers with reduced cardiac output (23). Zebrafish are an appropriate model because they have pyramidal ventricles (24) and are among the highest measured critical swimming speeds (13 body lengths per s at 28 °C) (25).

Results

Zebrafish embryos were exposed to low PAH concentrations from a few hours after fertilization to just before the hatching stage (48 h) in the effluent of a continuously flowing oiled or clean (control) gravel generator column (14), which mimics the

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natural weathering of an oiled shoreline (16) (*Materials and Methods*). We assessed the critical swimming speed and cardiac anatomy of adult survivors after rearing them in clean water for 10–11 mo. Contaminated effluent initially contained 60 ppb ∑PAH causing lethal pericardial edema in 100% of exposed embryos (e.g., at day 0; Fig. 1*A*). Clutches of embryos from common parents were divided in half and added to either control or oiled effluent every several days, and after 3 wk of weathering, ∑PAH dropped in oiled gravel effluent to a point where most embryos appeared normal. Approximately 100 adult fish were reared from each half-clutch incubated in either oiled or clean gravel effluent during three independent replicate exposures. The oiled-gravel exposures had an overlapping range of ∑PAH concentrations (24–36 ppb), and total tricyclic PAHs (10–14 ppb; Fig. 1*A*). The composition of PAHs was similar among the doses (Fig. S1), although the first exposure had slightly higher levels of noncardiotoxic alkyl-naphthalenes (13).

Oil exposure led to reduced larval survival (ANOVA, $P < 0.01$), and larval survival for each clutch was not significantly different within treatments (ANOVA, $P > 0.05$; Fig. 1*B*) despite slightly different ∑PAH in the exposed group. On average, 95% of control embryos survived the larval-juvenile transition, whereas 80–87% of the exposed embryos survived (Fig. 1*B*). Embryos with edema generally failed to feed as larvae and did not survive metamorphosis. The increased mortality observed here was higher than that reported for pink salmon embryos exposed to somewhat lower ∑PAH (18–20 ppb) (6). Although pink salmon had a mortality rate 1.2 times higher than unexposed controls (from 29.6 to 35%), here the average mortality for zebrafish embryos was 3.4 times higher (from 5 to 17%) after exposure to ∑PAH in the range of 24–36 ppb.

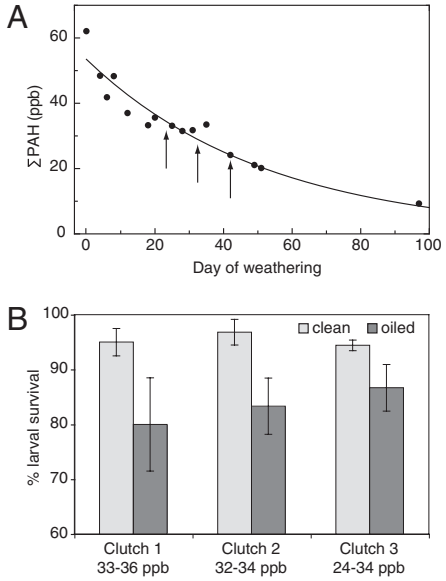

Fig. 1. (*A*) Experimental PAH exposure concentrations over time. Values represent the sum of 44 PAH and alkyl-PAH analytes (ppb) as described in *Materials and Methods*. Each point represents a single water sample taken on the indicated day after initiation of water flow to the column (day of weathering). The curve was fitted by using an exponential function using pro Fit 6.1.9 for Macintosh. Arrows indicate the three points (day 23, day 33, day 42) where exposure was initiated for the three clutches of embryos raised to adulthood. (*B*) Cumulative survival of embryos through larval-juvenile metamorphosis. Dead larvae were counted daily through 12 d after fertilization, the period of peak mortality. Values represent the total mean percent mortality (±SE) for five replicate tanks of 30 fish for each group exposed to clean gravel (light gray bars) or oiled gravel (dark gray bars) effluent. Survival of oiled fish was significantly reduced ($P = 0.004$) but exposure day and interaction between oil exposure and exposure day were not significant (two-way ANOVA). Because water samples were collected in some cases before and after the initiation of exposure, ranges of ∑PAH concentrations are given for each oil-exposed group.

Subsamples of embryos were assayed for induction of cytochrome P4501A (CYP1A) immediately after exposure. CYP1A is the primary detoxification enzyme for PAHs (14) and a biomarker of PAH exposure. Whereas control embryos showed only background immunofluorescence, oil-exposed embryos showed a similar pattern of immunofluorescence consistent with previous oiled gravel column studies (14) (Fig. S2).

We exposed another clutch of embryos to 9 ppb ∑PAH at day 97, (Fig. 1*A*) to examine the effectiveness of CYP1A in protecting embryos. This exposure included a subset of embryos injected with *cyp1a* antisense morpholino to knock down expression of CYP1A (14). Few oil-exposed uninjected (3% or 1/31) or control-injected embryos (0/17) exhibited pericardial edema and failed cardiac looping compared with 92% (33/36) of CYP1A-blocked morphants (Fig. S3). No edema was observed in control embryos exposed to clean gravel effluent (0/29 for uninjected, 0/17 for standard control morpholino, and 0/33 for *cyp1a* morphants). Therefore, CYP1A induction clearly plays a protective role rather than contributing to toxicity of petrogenic PAHs in early life history stages of fish. The sensitivity of zebrafish to PAH is similar to that of salmonids and other marine species (5, 6), in contrast to other classes of contaminants, such as the dioxins, to which zebrafish are orders of magnitude more resistant (26). The metabolic capacity of CYP1A enzymes for petrogenic PAH substrates in different fish species may account for variation in oil toxicity.

Although we did not measure growth rates during the juvenile period, sublethal oil exposure did not result in dramatically different final growth trajectories. There were some significant differences in final size measures for some groups of female fish, but there was not a consistent relationship with exposure (Table S1). Females from both oil-exposed day 33 and day 42 clutches were slightly longer than their paired control groups. Females from the oil-exposed day 33 clutch were significantly heavier than all other groups except the oil-exposed day 42 females, whereas the latter had a condition factor significantly higher than all other groups. There were no significant differences for male fish between treatment groups within or across all clutches for length and weight (Table S1). Two-way ANOVA indicated an effect of clutch on male condition factor, with clutch 3 fish (oil-exposed and clean) having a slightly larger K value. However, there was no interaction between oil exposure and clutch.

After growing to adulthood in clean water (>10 mo), exposed zebrafish had reduced U_{crit}, indicative of reduced cardiac output (19, 23, 27). For each clutch, U_{crit} was measured by using a standard swim tunnel for equally sized fish from each of five replicate rearing tanks (Table 1; two-way ANOVA, $P > 0.2$). All oil-exposed groups showed reduced U_{crit} compared with controls from the same clutch, whether determined by absolute speed (cm/s; Fig. 2*A*) or relative to body length (BL per s; Fig. 2*B*). For oil-exposed groups, swimming speed was reduced by 17%, 22%, and 15%, respectively. Oil exposure affected both absolute ($P < 0.01$, two-way ANOVA) and relative U_{crit} ($P < 0.001$), but there was no effect of clutch, nor an interaction between oil exposure and clutch. Hence, the three clutches can be treated as replicates, with oil exposure producing a reduction in mean relative U_{crit} by 18 ± 4%.

Changes in ventricular shape correlated with the reduced swimming performance from oil-treated fish. We measured ventricular shape as the length-to-width ratio of the heart by using digital images. We determined length as the distance between the apex of the heart and the center of the ventriculobulbar valve, and width as the widest distance perpendicular to the length (Fig. 3*A*, arrows). Oil-exposed fish as a group had rounder hearts, indicated by a length-to-width ratio of 1.38 ±

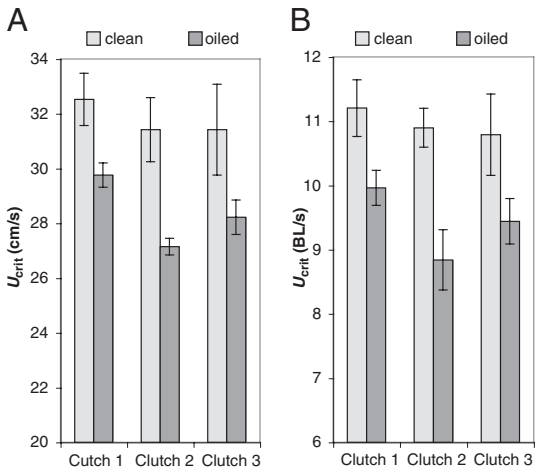

Fig. 2. Reduced swimming performance in adult fish exposed to oiled gravel effluent as embryos. U_{crit} was measured as described in *Materials and Methods*. Mean U_{crit} (±SE) for fish embryonically exposed to effluent from clean gravel (light gray bars, n = 5) or oiled gravel (dark gray bars, n = 9) is given as an absolute speed (*A*; cm/s) or relative to body length (*B*; BL/s). Statistical analysis is discussed in the text.

0.03 compared with a ratio of 1.53 ± 0.05 (Student's t test, P = 0.007) for control fish exposed to clean gravel effluent (Fig. 3*B*).

Discussion

Observations of reduced swimming performance and change in ventricular shape are consistent with our predictions based on the cardiac toxicity of crude oil. The developing heart is one of the first organs to become functional during organogenesis. In zebrafish (and other species), a regular heart rate is established during the tubular stage when both chambers have walls that are a single cell layer. Subsequently, looping brings the chambers into an adjacent arrangement, an atrioventricular conduction pathway is established, valves are formed, and the ventricular myocardium proliferates to become multilayered (24). The genetics of heart development in zebrafish has established the inseparable relationship between cardiac form and function during early stages of cardiac morphogenesis (17): Mutations affecting heart structure impact its function, whereas mutants with impaired function concomitantly experience impacts to the form of the chambers or valve structure. At sufficient concentrations, the tricyclic PAH compounds in petroleum produce severe arrhythmias mimicking those cardiac function mutants. These arrhythmias are lethal owing to circulatory failure. Moreover, sublethal exposure to PAHs induces subtle changes in heart shape (e.g., a 9% decrease in length-to-width ratio) that translate into larger impacts on aerobic performance (a reduction of U_{crit} by 18%).

Sustained swimming (U_{crit}) is a relevant indicator of individual fitness for pelagic planktivores (e.g., herring) and migratory salmonids (28). Even for larval fishes, swimming performance has ecologically relevant implications for predator avoidance (29). Moreover, a rounded ventricle is associated with increased stress-induced mortality (30). Survivorship depends on optimizing physiological performance. We show a direct link between oil exposure during embryonic development and delayed effects on physical capacity of adults.

Our finding that transient embryonic oil exposure affects the performance of adult zebrafish, together with the previously documented population-scale effects of pink salmon exposed to Exxon Valdez oil during early life stages, strongly suggests a physiological mechanism linking individual-based toxicity and population-level response. A similar mechanism could be the basis for the population impacts of other cardiotoxic pollutants such as the dioxins and planar polychlorinated biphenyls (31). With the high degree of evolutionary conservation among vertebrate hearts, these findings in zebrafish also have implications beyond both fish populations and contaminants, relating to environmental impacts on heart shape and cardiac output in humans. Similar relationships exist in the human heart (32), and physical factors that influence intracardiac forces during fetal development can result in rounder hearts with reduced output (33). Without being overtly teratogenic, chemicals with the ability to affect embryonic or fetal cardiac function could potentially produce morphological changes that could underlie some of the variation in human cardiac performance.

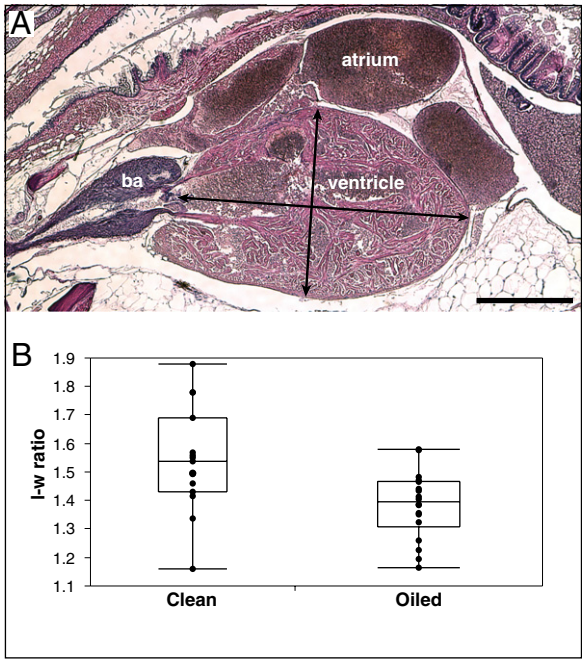

Fig. 3. Embryonic oil exposure changes the cross-sectional dimensions of the adult cardiac ventricle. (*A*) Representative transverse section of an adult zebrafish heart showing the two main chambers (atrium and ventricle) and the bulbus arteriosus (ba). Arrows indicate locations of length and width measurements. (*B*) Box plots with individual data points showing length/width ratios for fish embryonically exposed to clean gravel effluent (n = 16) or oiled gravel effluent (n = 17). (Scale bar, 0.5 mm.)

Materials and Methods

Zebrafish Culture and Exposures. Zebrafish (*D. rerio*) wild-type AB broodstock were maintained in a modular system on a 14-h light/10-h dark cycle at 28.5 °C, using methods detailed (34). Fish spawning, embryo exposures, and adult zebrafish maintenance were all carried out with system water (reverse-osmosis water with Instant Ocean sea salt added to adjust it to a conductivity of ≈1,500 μS/cm and a pH between 7 and 8). Spawning and egg collection

Table 1. Length and condition factor of adult fish used in swim test

Treatment group	n	Length, cm	K
Clutch 1 clean	25	2.91 ± 0.03	2.01 ± 0.07
Clutch 1 oiled	45	2.99 ± 0.03	2.12 ± 0.05
Clutch 2 clean	25	2.88 ± 0.05	1.97 ± 0.06
Clutch 2 oiled	45	3.07 ± 0.03	2.10 ± 0.06
Clutch 3 clean	25	2.92 ± 0.05	2.36 ± 0.17
Clutch 3 oiled	45	2.99 ± 0.03	2.22 ± 0.06

K, condition factor.

was carried out as described in detail (34). Embryos used in the exposures were all between the four- and eight-cell stage at the start of the exposure.

Embryos were exposed for 48 h to partially weathered Alaska North Slope (ANS) crude oil by using oiled gravel columns, as described (14). System water was passed through columns (2-L glass beakers) containing oiled (6 g/kg) and control (unoiled) gravel, using centrifugal pumps to maintain a constant flow of 10 mL/min. The resulting effluent was collected in rectangular 23 × 33 cm glass baking dishes, which were set at a slight angle to allow the water to overflow out the far end. Embryos were placed in open 60 × 15 mm diameter glass Petri dish replicates (n = 5, 30 embryos per dish) set in the baking dishes. The temperature in each baking dish was maintained between 27 and 28.5 °C with submersible heaters, and recorded up to three times daily. System water was pumped continuously through both the oiled and control gravel columns 24 h a day, 7 d a week, for 97 d. During this time, new embryos were exposed every 3 to 4 d. After each 48-h exposure, embryos were examined for pericardial edema with a Nikon SMZ800 stereomicroscope, subsampled, and preserved in paraformaldehyde for CYP1A analysis. Embryos from clutches whose exposure started on column days 23, 33, and 44 were transferred to clean water in 150-mm Petri dishes and placed in an incubator at 28 °C for an additional 48 h. At this point, they were transferred to a temperature-controlled zebrafish facility and reared to 12 d after fertilization (dpf) in 1-L larval tanks on a diet of paramecia, newly hatched *Artemia*, and finely powdered dry food (14). Mortality was assessed daily through 12 dpf, and surviving larvae from each clutch were reared to adulthood in five subgroups in the modular colony, with each subgroup treated as a replicate to control for potential tank effects.

CYP1A Immunofluorescence and Confocal Microscopy. CPY1A immunofluorescence processing was carried out as described (35). In general, embryos were fixed overnight in 4% phosphate-buffered paraformaldehyde (pH 7.4) and then transferred to methanol plus 10% DMSO for storage. Primary antibodies used were monoclonal anti-CYP1A C10-7 (Cayman Chemical) and anti-myosin heavy chain monoclonal MF20 (Developmental Studies Hybridoma Bank) (36). Secondary antibodies used were Alexa Fluor 488-conjugated goat anti-mouse IgG_3 (S46) and Alexa Fluor 568-conjugated goat anti-mouse IgG_{2b} (MF20), both from Invitrogen/Molecular Probes. Mounted immunolabeled embryos were imaged by using a Zeiss LSM 5 Pascal confocal system with Ar and He/Ne lasers (Carl Zeiss Advanced Imaging Microscopy).

PAH Analysis. Water samples (200 mL) were taken at the beginning and end of each exposure from both the control and oiled gravel columns. Each sample was stored in a brown glass bottle with the addition of 20 mL of dichloromethane in a 4 °C refrigerator until extraction. After deuterated internal standards were added, each sample was extracted twice with 25 mL of dichloromethane. The extractions were stored in a −20 °C freezer until further processing to remove any residual water. The solvent dichloromethane was replaced with hexane via boiling evaporation. Boiling stones were added to each sample extract, and the extracts were placed on a 40 °C hot plate and concentrated to ≈10 mL, and then 1 mL of hexane was added. The extract was further boiled down to 2 mL At this point, the extract was transferred to an amber sample vial, to which more boiling stones and 0.5 mL of hexane was added, and the vial was placed on a 70 °C hot plate. The extract was boiled down to 1 mL and removed from the heat in preparation for instrument analysis. The samples were processed for and analyzed by GC/MSD and GC/FID as described (37).

Swimming Performance Assay. Sustained critical swimming speed (U_{crit}) of adult zebrafish (treated as embryos to control or oiled effluent ≥10 mo earlier) was determined with a rectangle-shaped swim tunnel, using a modification of a previous design (38). A water current was generated by a magnetic drive pump, and the flow was controlled by a knife gate valve located directly downstream from the pump. The swimming chamber was located in a section of clear pipe to observe and measure fatigue. Other knife gates, screened and solid, prevented the fish from moving out of the testing chamber. Plastic straws and varying diameters of pipe helped to linearize the flow through the testing chamber. A flow meter installed in the swim tunnel measured flow in liters per minute. An electric barrier was installed immediately downstream from the testing chamber to discourage fish from resting against the downstream screen.

Five fish (mixed males and females ages 10–11 mo) were used in each swim trial. Fish were selected to be as close in size as possible. They were placed in the swim tunnel and allowed to swim to the testing chamber, after which the screened knife gate was lowered to prevent escape. The fish were allowed to acclimate for 10 min before a low flow (6.2 cm/s) was introduced. The flow was maintained at 6.2 cm/s for 30 min to allow the fish to orient themselves to the flow direction. After 30 min, the flow was increased to 10.3 cm/s and maintained there for 20 min. The flow was increased in 10.3 cm/s increments every 10 min until the first fish was fatigued. Fatigue was defined as when a fish fell against the downstream screen and could not recover despite a shock from the electric barrier. Once fatigue was determined, the trial was ended and the fish removed from the swim tunnel. All fish were anesthetized after each trial, and their lengths and weights were recorded.

Histology. Live adult zebrafish (formerly exposed as embryos to control or oiled effluent) selected for assessment of histopathological changes were killed by being placed on ice. Any external abnormalities (e.g., frayed fins, cloudy eyes, ulcers, skin discolorations, parasites) were assessed visually. The gills were similarly examined after cutting away the operculum, and internal organs were also examined after making a midline incision on the belly from the anus to the pectoral girdle, and assessed for ascites, hemorrhage, or other abnormalities. The visceral cavity was then further exposed by removing a small section of the abdominal wall, and the tail was excised posterior to the anus. Fish for histology were preserved whole in Dietrich's fixative solutions for histology, at ≈1:20 (vol/vol) tissue to fixative, typically requiring 15 mL of fixative for 1–2 zebrafish. To ensure uniform and complete fixation, tissues were fixed for 3 d on a rotor or other agitating device. Fixed fish were then rinsed in two or three changes of water, and placed in 70% ethanol. The fish were then bisected along their length by using a fresh razor blade, making the cut parallel to and on the left side of the spinal cord, before they were processed. The bisected fish were then loaded into cassettes for processing, using a VIP Tissue-Tek enclosed processor (Sakura Finetek). The tissue processing protocol followed that specified by the Zebrafish International Research Center, University of Oregon (http://zebrafish.org/zirc/health/diseaseManual.php). Processed tissues were infiltrated with Surgipath Formula 'R' infiltrating and embedding paraffin (Surgipath Medical Industries) and a 50:50 ratio of Fisher Paraplast PLUS tissue embedding medium (Fisher Scientific) and Surgipath Formula 'R' in the final paraffin bath. Tissues were then embedded in Surgipath Formula 'R' paraffin and cut, either in step or serial sagittal sections, as needed, at 5- to 7-μm thickness with a high-profile disposable blade. If the fish were difficult to cut, the block face was soaked in ice-cold 0.05% Tween 20. To focus on the morphology of the heart, serial sections of each fish were cut until it was possible to view a full section of the ventricle, atrium, and bulbus arteriosus, with the key feature being a full longitudinal section of the bulbus arteriosus. This approach standardized the orientation and sectioning plane of the three heart chambers as much as possible for imaging and measurements.

Sections of gills, spinal cord, and all internal organs were taken to ensure proper pathological evaluation. Because the fish were cut to one side of the spinal cord, sections were cut toward the midline on one half of the fish, and away from the midline on the other. This procedure produced a ribbon of sections containing gills (one bisected half) and spinal cord (the other bisected half), along with a complete sampling of internal organs, while continuing to focus on the plane of section with the heart.

Sections were routinely stained by hematoxylin and eosin, using protocols described (39). Sections were then screened by using light microscopy for proper heart visualization and orientation and imaged on a Nikon E600 compound microscope by using a Spot RT camera and Spot 4.5.9.9 software (Diagnostic Instruments). Images of the hearts were subsequently examined blind to consistently select sections that rigorously met the standard criteria for measuring cardiac dimensions (plane of section through the midline of the bulbus arteriosus and showing both atrium and ventricle). A total of 16 controls and 19 oil-exposed fish were successfully sectioned from the day 23 and day 33 clutches. From these samples, images passing criteria for measurements included 8/16 controls and 9/19 oil-exposed from clutch 1, and 8/16 controls and 8/19 oil-exposed from the clutch 2. Measurements were made blind to treatment group by using ImageJ 1.43 (http://rsbweb.nih.gov/ij). Because statistically significant differences due to oil exposure were observed in the pooled clutch 1 and 2 fish, animals from clutch 3 were not sectioned. There were no significant differences in condition factor between oil-exposed and control fish among the clutch 1 and clutch 2 treatment groups as a whole (Table S1).

Statistical Analysis. Statistical analyses were performed with JMP 6.0.2 for Mac (SAS Institute). Common measurements made on all three clutches (mortality, length, weight, condition factor, U_{crit}) were analyzed by two-way ANOVA (α = 0.05) with treatment and clutch as independent variables. In cases where effects of or interactions between variables were detected, post hoc means comparisons were performed by using either Tukey–Kramer Honestly Significant Differences test or Student's t test, depending on the number of groups.

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1. Incardona JP, Collier TK, Scholz NL (2011) Oil spills and fish health: Exposing the heart of the matter. *J Expo Sci Environ Epidemiol* 21:3–4.
2. Bue BG, Sharr S, Moffitt SD, Craig AK (1996) Effects of the Exxon Valdez oil spill on pink salmon embryos and preemergent fry. *Proceedings of the Exxon Valdez Oil Spill Symposium*, eds Rice SD, Spies RB, Wolfe DA, Wright BA (Am Fisheries Soc, Bethesda, MD), pp 619–627.
3. Geiger HJ, Bue BG, Sharr S, Wertheimer AC, Willette TM (1996) A life history approach to estimating damage to Prince William Sound pink salmon caused ty the Exxon Valdez oil spill. *Proceedings of the Exxon Valdez Oil Spill Symposium*, eds Rice SD, Spies RB, Wolfe DA, Wright BA (Am Fisheries Soc, Bethesda, MD), pp 487–498.
4. Rice SD, et al. (2001) Impacts to pink salmon following the Exxon Valdez oil spill: Persistence, toxicity, sensitivity, and controversy. *Rev Fish Sci* 9:165–211.
5. Carls MG, Rice SD, Hose JE (1999) 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: 481–493.
6. Heintz RA, Short JW, Rice SD (1999) 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* 18:494–503.
7. Marty GD, Hose JE, McGurk MD, Brown ED, Hinton DE (1997) 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* 54:1846–1857.
8. Marty GD, et al. (1997) 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.* 75:989–1007.
9. Heintz RA (2007) 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* 3:351–363.
10. Heintz RA, et al. (2000) Delayed effects on growth and marine survival of pink salmon Oncorhynchus gorbuscha after exposure to crude oil during embryonic development. *Mar Ecol Prog Ser* 208:205–216.
11. Hulson PJF, et al. (2008) Data conflicts in fishery models: Incorporating hydroacoustic data into the Prince William Sound Pacific herring assessment model. *ICES J Mar Sci* 65:25–43.
12. Thorne RE, Thomas GL (2008) Herring and the "Exxon Valdez" oil spill: An investigation into historical data conflicts. *ICES J Mar Sci* 65:44–50.
13. Incardona JP, Collier TK, Scholz NL (2004) Defects in cardiac function precede morphological abnormalities in fish embryos exposed to polycyclic aromatic hydrocarbons. *Toxicol Appl Pharmacol* 196:191–205.
14. Incardona JP, et al. (2005) Aryl hydrocarbon receptor-independent toxicity of weathered crude oil during fish development. *Environ Health Perspect* 113: 1755–1762.
15. Incardona JP, et al. (2009) Cardiac arrhythmia is the primary response of embryonic Pacific herring (Clupea pallasi) exposed to crude oil during weathering. *Environ Sci Technol* 43:201–207.
16. Short JW, Heintz RA (1997) 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* 31:2375–2384.
17. Glickman NS, Yelon D (2002) Cardiac development in zebrafish: Coordination of form and function. *Semin Cell Dev Biol* 13:507–513.
18. Schoenebeck JJ, Yelon D (2007) Illuminating cardiac development: Advances in imaging add new dimensions to the utility of zebrafish genetics. *Semin Cell Dev Biol* 18:27–35.
19. Farrell AP (2002) Cardiorespiratory performance in salmonids during exercise at high temperature: Insights into cardiovascular design limitations in fishes. *Comp Biochem Physiol A Mol Integr Physiol* 132:797–810.
20. Farrell AP (2007) Cardiorespiratory performance during prolonged swimming tests with salmonids: A perspective on temperature effects and potential analytical pitfalls. *Philos Trans R Soc B Biol Sci* 362:2017–2030.
21. Sanchez-Quintana D, Garcia-Martinez V, Climent V, Hurle JM (1995) Morphological analysis of the fish heart ventricle: Myocardial and connective tissue architecture in teleost species. *Ann Anat* 177:267–274.
22. Santer RM, Greer Walker M, Emerson L, Witthames PR (1983) On the morphology of the heart ventricle in marine teleost fish (teleostei). *Comp Biochem Physiol A-Physiol* 76:453–457.
23. Claireaux G, et al. (2005) Linking swimming performance, cardiac pumping ability and cardiac anatomy in rainbow trout. *J Exp Biol* 208:1775–1784.
24. Hu N, Sedmera D, Yost HJ, Clark EB (2000) Structure and function of the developing zebrafish heart. *Anat Rec* 260:148–157.
25. Plaut I, I, Gordon MS (1994) Swimming metabolism of wild-type and cloned zebrafish Brachydanio rerio. *J Exp Biol* 194:209–223.
26. Henry TR, Spitsbergen JM, Hornung MW, Abnet CC, Peterson RE (1997) Early life stage toxicity of 2,3,7,8-tetrachlorodibenzo-p-dioxin in zebrafish (Danio rerio). *Toxicol Appl Pharmacol* 142:56–68.
27. Gallaugher P, Thorarensen H, Farrell AP (1995) Hematocrit in oxygen transport and swimming in rainbow trout (Oncorhynchus mykiss). *Respir Physiol* 102:279–292.
28. Plaut I (2001) Critical swimming speed: Its ecological relevance. *Comp Biochem Physiol A Mol Integr Physiol* 131:41–50.
29. Fuiman LA, Rose KA, Cowan JH, Smith EP (2006) Survival skills required for predator evasion by fish larvae and their relation to laboratory measures of performance. *Anim Behav* 71:1389–1399.
30. Poppe TT, Johansen R, Gunnes G, Tørud B (2003) Heart morphology in wild and farmed Atlantic salmon Salmo salar and rainbow trout Oncorhynchus mykiss. *Dis Aquat Organ* 57:103–108.
31. Cook PM, et al. (2003) Effects of aryl hydrocarbon receptor-mediated early life stage toxicity on lake trout populations in Lake Ontario during the 20th century. *Environ Sci Technol* 37:3864–3877.
32. Adhyapak SM, Parachuri VR (2010) Architecture of the left ventricle: Insights for optimal surgical ventricular restoration. *Heart Fail Rev* 15:73–83.
33. Crispi F, et al. (2010) Fetal growth restriction results in remodeled and less efficient hearts in children. *Circulation* 121:2427–2436.
34. Linbo TL (2009) Zebrafish (Danio rerio) husbandry and colony maintenance at the Northwest Fisheries Science Center. *NOAA Technical Memorandum NMFS-NWFSC-100* (US Depart of Commerce, Seattle, WA).
35. Incardona JP, Day HL, Collier TK, Scholz NL (2006) Developmental toxicity of 4-ring polycyclic aromatic hydrocarbons in zebrafish is differentially dependent on AH receptor isoforms and hepatic cytochrome P4501A metabolism. *Toxicol Appl Pharmacol* 217:308–321.
36. Bader D, Masaki T, Fischman DA (1982) Immunochemical analysis of myosin heavy chain during avian myogenesis in vivo and in vitro. *J Cell Biol* 95:763–770.
37. Short JW, Jackson TJ, Larsen ML, Wade TL (1996) Analytical methods used for the analysis of hydrocarbons in crude oil, tissues, sediments, and seawater collected for the natural resources damage assessment of the Exxon Valdez oil spill. *Am Fish Soc Symp* 18:140–148.
38. Ward DL, Maughan OE, Bonar SA (2002) A variable-speed swim tunnel for testing the swimming ability of age-0 fish. *N Am J Aquaculture* 64:228–231.
39. Stehr CM, Myers MS, Willis MJ (1993) Collection of fish tissues for the National Benthic Surveillance Project: Necropsy procedures, tissues processing, and diagnostic procedure for Histopathology. *Sampling and analytical methods of the National Status and Trends program, National Benthic Surveillance and Mussel Watch Projects, 1984-1992, NOAA Technical Memorandum NOS ORCA 71* (US Dept of Commerce, Silver Spring, MD).