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 million (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 weathered, 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 cardiotoxicity 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 (Ucrit) 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
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 \(\Sigma PAH\) causing lethal pericardial edema in 100% of exposed embryos (e.g., at day 0; Fig. L4). 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, \(\Sigma PAH\) 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 \(\Sigma PAH\) concentrations (24–36 ppb), and total tricyclic PAHs (10–14 ppb; Fig. L4). 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. L1B) despite slightly different \(\Sigma PAH\) in the exposed group. On average, 95% of control embryos survived the larval-juvenile transition, whereas 88–87% of the exposed embryos survived (Fig. L1B). 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 \(\Sigma PAH\) (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 \(\Sigma PAH\) in the range of 24–36 ppb.

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 \(\Sigma PAH\) at day 97 (Fig. L4) to examine the effectiveness of CYP1A in protecting embryos. This exposure included a subset of embryos injected with cypl 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 cypl 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 (226). 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 \(U_{\text{crit}}\) indicative of reduced cardiac output (19, 23, 27). For each clutch, \(U_{\text{crit}}\) 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 \(U_{\text{crit}}\) compared with controls from the same clutch, whether determined by absolute speed (cm/s; Fig. 2A) or relative to body length (BL per s; Fig. 2B). 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 \(U_{\text{crit}}\) (\(P < 0.001\)), 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 \(U_{\text{crit}}\) 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, arrows). Oil-exposed fish as a group had rounder hearts, indicated by a length-to-width ratio of 1.38 ±
Cardiac toxicity of crude oil. The developing heart is one of the 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 $U_{\text{crit}}$ by 18%).

Sustained swimming ($U_{\text{crit}}$) 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.

**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 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>
<thead>
<tr>
<th>Treatment group</th>
<th>Length, cm</th>
<th>$K$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Clutch 1 clean</td>
<td>2.91 ± 0.03</td>
<td>2.01 ± 0.07</td>
</tr>
<tr>
<td>Clutch 1 oil</td>
<td>2.99 ± 0.03</td>
<td>2.12 ± 0.05</td>
</tr>
<tr>
<td>Clutch 2 clean</td>
<td>2.88 ± 0.05</td>
<td>1.97 ± 0.06</td>
</tr>
<tr>
<td>Clutch 2 oil</td>
<td>3.07 ± 0.03</td>
<td>2.10 ± 0.06</td>
</tr>
<tr>
<td>Clutch 3 clean</td>
<td>2.92 ± 0.05</td>
<td>2.36 ± 0.17</td>
</tr>
<tr>
<td>Clutch 3 oil</td>
<td>2.99 ± 0.03</td>
<td>2.22 ± 0.02</td>
</tr>
</tbody>
</table>

$K$, condition factor.
Swimming Performance Assay. Floating adult zebrafish (formerly exposed as embryos to control or oil treatments) selected for assessment of histopathological changes were killed by being placed on ice. Any external abnormalities (e.g., frayed fins, cloudy eyes, ulcers, skin discolourations, 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 asciites, 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 solutes for histology, at \(\approx 1:20\) (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 and lining the midline of the fish to the sectioning plane of the testing chamber, 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/2010HealthAndDiseaseManual.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-width 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 anatomy 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 section 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 and screened 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 clutch 2 treatment groups as a whole (Table S1), 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, \(U_{v0}\)) were analyzed by two-way ANOVA (\(\alpha = 0.05\)) with treatment and clutch as independent variables. In cases where significant differences in condition factor between oil-exposed and control fish 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.

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, \(U_{v0}\)) were analyzed by two-way ANOVA (\(\alpha = 0.05\)) with treatment and clutch as independent variables. In cases where significant differences in condition factor between oil-exposed and control fish 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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