Cardiac Arrhythmia Is the Primary Response of Embryonic Pacific Herring (Clupea pallasii) Exposed to Crude Oil during Weathering

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Teleost embryos develop a syndrome characterized by edema when exposed to water that weathers substrates contaminated with crude oil. Previous studies using zebrafish demonstrated that crude oil exposure causes cardiogenic edema, and that the most abundant polycyclic aromatic hydrocarbons (PAHs) in weathered crude oils (tricyclic fluorenes, dibenzothiophenes, and phenanthrenes) are cardiotoxic, causing arrhythmia through a pathway that does not require activation of the aryl hydrocarbon receptor (AHR). We demonstrate here for Pacific herring, a species impacted by the Exxon Valdez oil spill, that the developing heart is the primary target of crude oil exposure. Herring embryos exposed to the effluent of oiled gravel columns developed dose-dependent edema and irregular cardiac arrhythmia soon after the heartbeat was established. At a dose that produced cardiac dysfunction in 100% of exposed embryos, tissue levels of tricyclic PAHs were below 1 µmol/kg, suggesting a specific, high affinity target in the heart. These findings have implications for understanding the mechanism of tricyclic PAH cardiotoxicity, the development of biomarkers for the effects of PAH exposure in fish, and understanding the long-term impacts of oil spills and other sources of PAH pollution in aquatic environments.

Introduction

The Exxon Valdez oil spill occurred in the spring of 1989, coincident with the spawning of Pacific herring stocks in Prince William Sound. Herring deposit adhesive, demersal eggs on shallow nearshore vegetation and other substrates, and spawning along oil-contaminated shorelines resulted in oil exposure of developing embryos and larvae. Larvae hatched from contaminated sites had high rates of abnormalities and mortality (1–4). Although they did not spawn until months after the spill, similar effects were observed with pink salmon (Oncorhynchus gorbuscha), which deposited their eggs in intertidal stretches of stream deltas. Oiled gravel persisting in some streams was a continuous source for exposure of pink salmon embryos, resulting in elevated mortality (5).

These observations prompted a host of laboratory studies that examined the effects of Alaska North Slope crude oil (ANSCO) on fish development. Two broad conclusions can be drawn from these studies. First, a common syndrome of developmental abnormalities was induced in both herring and salmon embryos by exposure to crude oil constituents dissolved in water (6–10). These findings, coupled with recent studies on other species (11–15), demonstrate what appears to be a conserved response of teleost embryos to petroleum exposure. Gross features of this syndrome included pericardial and yolk sac edema, small jaws, and body axis defects. Indeed, pericardial or yolk sac edema appears to be the most sensitive indicator of exposure to crude oil in fish embryos (7, 9, 16).

Second, the frequency and severity of oil-associated developmental defects was correlated with the composition of polycyclic aromatic hydrocarbons (PAHs) dissolved from oiled substrate. Neat crude oils generally contain PAH fractions that consist of roughly 50–60% naphthalenes, 40–50% tricyclic compounds (fluorenes, dibenzothiophenes, and phenanthrenes), and 1–3% chrysenes (17). Higher molecular weight PAHs such as benzo[a]pyrene usually constitute <1% of the total PAHs in crude oils. During the weathering of oiled substrates such as beach gravel by water, PAHs (and other constituents) move into water from the substrate over time. This timed release is in essence the definition of weathering, described by first-order loss-rate kinetics (18), and results in a “water-washed” pattern of dissolved PAHs. Lower molecular weight compounds with fewer alkyl substitutions are dissolved most readily, and dissolution rates are proportional to hydrophobicity. Effluent from substrates with relatively fresh oil is initially dominated by the relative proportions of naphthalenes. Over time, the concentrations of tricyclic PAHs and alkylated isomers become proportionately greater. As the pattern of dissolved PAHs shifts to these tricyclic compounds, both mortality and defects such as pericardial edema occur at much lower total PAH concentrations (9, 10). Thus, toxicity of ANSCO to fish embryos is predominantly associated with fluorenes, dibenzothiophenes, and phenanthrenes.

To better understand the mechanisms of petroleum toxicity to fish embryos, we previously carried out a systematic analysis of the effects of individual PAHs and weathered crude oil in zebrafish embryos (15, 19, 20). Exposure of zebrafish embryos to a series of nonalkylated PAHs containing 2 to 4 rings demonstrated that different PAH compounds act through unique toxic pathways. The tricyclic compounds fluorene, dibenzothiophene, and phenanthrene all caused cardiac dysfunction relatively early in the organogenesis phase of development (30–36 h postfertilization [hpf]), which resulted in downstream effects including edema, craniofacial defects, and body axis defects (19). These individual PAHs essentially reproduce the most pronounced effects of exposure to crude oil, which do not require activation of the aryl hydrocarbon receptor (AHR) pathway (15). Thus, the edema that occurs in oil-exposed embryos is cardiogenic, and crude oil contains a directly cardiotoxic fraction that is consistent with the activities of the most abundant tricyclic compounds. In contrast, exposure to the tetracyclic compound chrysenne has no discernible effects on embryonic
development, while the tetracyclic compounds pyrene and benz[a]anthracene, which are not abundant in crude oil, produce syndromes that are distinct from oil exposure and that require AHR activation (20).

The easily visible beating heart of most fish embryos, and zebrafish in particular, allows for detailed functional observations via simple digital videomicroscopy. Moreover, the integration of these types of observations with genetic analysis of cardiac function in zebrafish provides the capability to infer likely physiological targets of cardiotoxic compounds such as the tricyclic PAHs (21). Exposure to relatively high concentrations of nonalkylated tricyclic PAHs produces a dose-dependent reduction of heart rate (bradycardia), followed by more complex arrhythmias consistent with atrioventricular block (conduction block) (19). Somewhat more complex effects, including reduced contractility, were observed in zebrafish embryos exposed to weathering crude oil that produced total tricyclic alky PAH aqueous concentrations in the range of 20–30 ppb (15). Comparison to the phenotypes of known zebrafish cardiac mutants suggests several potential myocardial targets for oil toxicity, including cardiac potassium channels (22–24), sarcoplasmic or plasma membrane calcium channels (25), or gap junctions (26).

This identification of direct cardiovascular pathophysiology as a key process underlying petrogenic PAH toxicity provides a new conceptual framework for identifying improved biomarkers for the effects of oil spills and other PAH sources in native fish species. Although the effects of weathered ANSCO on Pacific herring embryos are well-documented (9), those studies analyzed primarily late end points of toxicity in hatching stage larvae. We therefore sought to determine whether effects on cardiac physiology similar to those observed in zebrafish occur in herring embryos during early phases of crude oil exposure. Due to the optical clarity of the embryo, transparency of the chorion, and the relative ease of mechanical dechorionation, Pacific herring is an ideal marine forage fish species for developmental studies.

Materials and Methods

Materials and methods are described here in brief. Complete details are available in the Supporting Information.

Production of Herring Embryos. Herring embryos were produced from ripe spawner-stage adult fish captured by gill net March 2006 in central Puget Sound, WA. Ripe eggs from dissected ovaries were distributed on glass microscope slides targeting ~100–200 adherent eggs and fertilized as described previously (9). Eggs were not pooled, and each ovary contributed to a separate batch of 10 slides. Six fertilizations with viabilities ranging from 63 to 82% were selected for use in oiled gravel column exposures, providing 60 slides that were distributed among the six dosing columns so that each fertilization was equally represented.

Production of Oiled Gravel Effluent. The basic approach to producing water contaminated with weathered crude oil was to percolate continuously flowing (0.5–0.7 L/hr) filtered seawater through columns of oiled gravel (8, 9, 15, 18). Embryos were incubated in a reservoir that collected a steady-state volume of effluent continuously, maintained at ambient sea temperature (9 ± 0.5 °C). ANSCO was partially weathered by heating at 70 °C until reduced to 80% volume, and crushed rock (1-cm diameter) was coated with oil at 6.0 g/kg by shaking in 1 gallon unlined steel paint cans. A graded series of dosing columns was assembled by combining different ratios of the 6.0 g/kg oiled gravel and clean gravel by weight in 1 L beakers: 100% oiled gravel, 50%, 25%, 12.5%, 6.25%, and 0% (effluent from columns are referred to below as OGE100, OGE50, etc., and 0% control is referred to as CGE). Column flows were initiated the morning of adult herring collection and run 24 h to clear any particulates and provide initial weathering before embryo exposure. Embryos were placed in column effluent (10 slides, >1000 embryos per dose) at ~24 hpf (late blastula stage). Flow rates and temperatures were monitored and embryos were observed daily.

Live Observation of Embryos, Videomicroscopy, and Immunofluorescence. Embryos were manually dechoronated if necessary and maintained at 9–10 °C during observation on a Nikon SMZ800 stereomicroscope or Zeiss Axioplan 2 compound microscope, and imaged with an iFire400 digital video camera (Unibrain) and Apple iBook G4 laptop with VT-B Carbon Pro software (www.bensoftware.com) or Zeiss AxioCam. Dechoronated embryos were fixed in 4% phosphate-buffered paraformaldehyde and processed for CYP1A and myosin heavy chain confocal immunofluorescence as described elsewhere (15).

Quantification of Heart Rate and Rhythm. Heart rates were determined from 20-s video segments collected from individual embryos. Statistical analysis of heart rate data was carried out with JMP 6 for Macintosh (SAS, Cary, NC). A quantitative assessment of cardiac arrhythmia was obtained by determining the interbeat variability from the same video segments (collected at 30 frames/sec). The initiation of cardiac contraction was noted for each beat, and the corresponding video frame number was recorded. Using Microsoft Excel, the number of frames between beat initiations was calculated and the mean and standard deviation were obtained for each embryo. This standard deviation is a measure of heart rate irregularity. By comparison, a regular rhythm would have essentially the same number of frames between beats and therefore a low standard deviation. The standard deviations for individual embryos were then averaged to obtain a mean interbeat variability for each exposure group (N = 5 embryos per group).

PAH Analysis. At 7 and 8 dpf (exposure day 6 and 7) pooled samples of embryos with intact chorions (~1.5 g) were collected, rinsed in clean filtered seawater, and frozen at −80 °C until analysis. These time points were chosen because the primary biological effects were most pronounced by 7 dpf. The samples were extracted and analyzed using a previously described method (27). Quality assurance guidelines for measuring PAHs were followed as described elsewhere (28). A Standard Reference Material (SRM) from the National Institute of Standards and Technology (SRM 1974b) and a method blank sample were analyzed concurrently with the samples. The lower limits of quantification for PAHs ranged from 0.19 to 0.37 ng/g wet weight.

Results

Accumulation of PAHs in Embryos. TPAH concentrations in embryos were correlated with dose (e.g., r = 0.993 after 7 days exposure, TPAH = 11375*(1 – e−0.0128*treat), where “treat” is equal to the percent of oiled gravel in a given treatment level; Table 1). Composition was dominated by naphthalenes (69 ± 0.4% by weight (mean ± SE), Figure 1a). Phenanthrenes were the next most represented homologous family, at about 12% of TPAH. The total concentration of phenanthrenes reached 78 ng/g wet weight in the lowest treatment and 980 ng/g wet weight in the highest, while the total concentration of tricyclic compounds (fluorenes, dibenzothiophenes, and phenanthrenes) respectively reached 190 and 2300 ng/g. Few chrysene were accumulated (<0.1% of TPAH, about 10 ng/g in the highest treatment). Using molecular weights of parent and alkylated naphthalenes, fluorenes, dibenzothiophenes, and phenanthrenes, these levels represent 3.6 µmol/kg (wet weight) TPAH and 1 µmol/kg total tricyclics at the lowest dose, and 47 µmol/kg TPAH and 12 µmol/kg tricyclics at the
microscopic evidence of visible oil droplets in contact with embryos (Figure S1). Hence, these herring eggs were exposed primarily (or entirely) to dissolved PAHs, consistent with other oil-gravel column assays (7, 9, 10, 30, 31).

Early Cardiac Dysfunction Associated with Weathered Crude Oil Exposure. A published atlas of Atlantic herring development described the first heartbeat at 6 dpf at 8°C (48 accumulated thermal units, atu) (32). By 4.5 dpf (40.5 atu, 40+ somites) we observed heart contractions with an irregular rate of 22–30 beats per minute (bpm) in embryos exposed to either CGE or OGE100. However, at 5 dpf (i.e., at 4 days of exposure) dose-dependent effects on heart rate were apparent (Figure 2a, white bars). Embryos exposed to OGE6.25 had mean heart rates indistinguishable from control (37 ± 2 vs 35 ± 4 bpm, respectively), but significant bradycardia was observed at higher doses (22 ± 4 bpm in OGE12.5, 15 ± 1 bpm in OGE100). There was also a dose-dependent effect on cardiac rhythm (Figure 2b). By 5 dpf, control embryos had established a regular rhythm, with a very low interbeat variability (55 ± 14 msec). Although the overall rate in OGE6.25-exposed embryos was indistinguishable from control, they showed an irregular rhythm with a mean interbeat variability of 120 ± 85 msec. There was a trend of increasing irregularity of rhythm with increasing oil dose. By 7 dpf (exposure day 6), the impacts of OGE on cardiac function were more pronounced, with all oiled gravel doses displaying marked bradycardia (Figure 2a, black bars). Embryos exposed to OGE6.25 showed a significantly reduced rate of 37 ± 5 bpm relative to controls at 75 ± 8 bpm (p = 0.001). Although a dose-dependent trend was observed at 7 dpf, there were no statistically significant differences among heart rates in the OGE-exposed groups. However, there were distinct dose-dependent differences in function. Compared to controls, embryos exposed to OGE6.25 displayed a fairly regular bradycardia with concomitant slowing of ventricular contraction (Movies S1 and S2 in the Supporting Information). In contrast, OGE100-exposed embryos showed bradycardia with a markedly irregular atrial rate. In this case ventricular contraction always followed an atrial contraction, but there was a notable shift in the relaxation time of the atrium (Movies S3). Instead of atrial contraction being followed by rapid dilation, the atrium remained contracted for a longer portion of the cardiac cycle. Finally, pericardial edema was apparent in some OGE-exposed embryos by 6 dpf, and was present in all OGE doses by 7 dpf (Figure 3a–f).

To determine if oil-induced bradycardia represented a true developmental defect (e.g., disruption of heart patterning) or a physiological effect, control embryos were moved into OGE100 at 7 dpf (a point where they had well-established, regular heart rates) and exposed overnight. Compared to controls at 8 dpf, embryos exposed to OGE100 for only a 20-h window showed a significantly reduced mean heart rate (93 ± 3 vs 64 ± 2 bpm, t test p = 0.0001; Figure 2a, gray bars). At later stages, mortality was high at doses ≤OGE50 (data not shown). By 12 dpf (exposure day 11) only the OGE6.25 and OGE12.5 doses had large numbers of viable embryos.

### TABLE 1. TPAH Concentrations in Embryos

<table>
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<th>Oiled gravel dose</th>
<th>Day 6 TPAH</th>
<th>Day 6 P0–P4</th>
<th>Day 6 total tricyclics</th>
<th>Day 7 TPAH</th>
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<tr>
<td></td>
<td>(ng/g wet weight)</td>
<td>(ng/g wet weight)</td>
<td>(ng/g wet weight)</td>
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<td>3</td>
<td>10</td>
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<tr>
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<td>2300</td>
<td>297</td>
<td>700</td>
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*CGE, control gravel effluent (0% oiled gravel); OGE, oiled gravel effluent (6.25% oiled gravel, etc.). * Due to limited tissue availability, all values are from single determinations.

### FIGURE 1. PAH composition in herring eggs after 6 days exposure (7 dpf) (a) compared to typical PAH composition in weathered Alaska North Slope crude oil (ANSCO) (b), PAH composition in water (c), and PAH composition in corresponding herring eggs (d) exposed to oil-gravel effluent in a previous experiment. Values in (a) represent mean levels across all doses. N, naphthalene; BP, biphenyl; AC, acenaphthylene; AE, acenaphthene; F, fluorene; D, dibenzothiophene; P, phenanthrene; AN, anthracene; FL, fluoranthene; PY pyrene; C, chrysene. Numbers of additional carbons (e.g., methyl groups) for alkylated homologues are indicated as N1, N2, etc.
However, 100% of viable OGE-exposed embryos at all doses displayed morphological and functional abnormalities, including marked pericardial edema, reduced cardiac chambers, and bradycardia or other arrhythmias. Nevertheless, there were few other anatomical abnormalities associated with oil exposure (Figure S2). Most structures appeared similar in embryos exposed to CGE or OGE6.25, including the eye, brain and neural tube, otic vesicle, and notochord. Development of the liver bud was similar (not shown), as was the pectoral fin bud (Figure S2c and d, arrows). Due to the severity of the cardiovascular defects observed in even the lowest dose of oiled gravel, exposure was terminated at this point, an estimated 4–5 days prior to the expected hatching period.

**Distribution of CYP1A Immunoreactivity in Oil-Exposed Herring Embryos.** The distribution of CYP1A at exposure day 6 (7 dpf), assessed by immunofluorescence and confocal microscopy, was essentially identical for embryos exposed to each OGE dose (Figure S3). Similar to what we previously observed in OGE-exposed zebrafish embryos, CYP1A immunofluorescence was strong in the endothelium of head (Figure S3a, c) and trunk vessels (Figure S3e, g), the endocardium (Figure S2a–c), the epidermis (Figure S2e), the liver (Figure S3e, g), and urinary pore (not shown). However, in contrast to our results with zebrafish, the myocardial cells of OGE-exposed herring embryos were strongly CYP1A+ (Figure S3a, b). CGE-exposed embryos showed weak CYP1A immunofluorescence in the epidermis and in scantily distributed endothelial cells of the sinus venosus and caudal vein (Figure S4).

**Discussion**

Previous crude oil exposure studies with Pacific herring embryos focused on relatively late end points in hatched larvae (4, 6, 7, 9). As a consequence, the events leading to mortality or morphological abnormalities were unknown. In the current study, exposure to ANSCO during weathering resulted in dose-dependent defects in heart rate and rhythm at a very early stage, as soon as a regular heartbeat was established (5 dpf), and well before lethality was observed (12 dpf). The observed bradycardia and irregular arrhythmia are consistent with direct effects on cardiac function predicted from findings in zebrafish, and occurred at roughly the equivalent developmental stage [i.e., pharyngula (33)].
The effects of short-term exposure at a later developmental stage (i.e., between 7 and 8 dpf) is consistent with a physiological target in the heart, rather than cardiac dysfunction resulting as a consequence of a true developmental defect caused by exposure starting shortly after fertilization.

Although the phenotypic effects on cardiac function largely validate a common response in zebrafish and herring, the pattern of CYP1A induction in herring was unexpected. After similar exposures, zebrafish embryos showed robust endocardial CYP1A induction, but no induction in the myocardium (15). In herring embryos exposed to even the lowest OGE dose, strong CYP1A immunofluorescence was observed in the myocardium at 7 dpf. Therefore, despite the consistency of the cardiac functional effects between species, we cannot unequivocally rule out AHR-dependent effects of crude oil exposure in herring. Nevertheless, cardiac rhythm disturbances are not the primary response associated with exposure to potent AHR ligands such as 2,3,7,8-tetrachlorodibenzo-p-dioxin or the pyrogenic PAH benz(a)anthracene, both of which cause AHR-dependent cardiac malformations at later developmental stages (20, 34). Thus, the cardiac phenotype is most consistent with direct effects on a target involved in the electrophysiological properties of the myocardium (e.g., a channel protein).

Tissue PAH levels in this experiment were within the range observed in previous studies (9). Although water PAH concentrations were not measured here, similar columns typically generate initial water TPAH levels in the range of 60–100 µg/L (9, 15, and our unpublished observations). In the current experiment, TPAH tissue concentrations reached about 600 ng/g wet weight in the lowest and 8000 ng/g in the highest exposure after 7 days at 9 °C. The patterns of PAHs observed here are consistent with the “less weathered oil” exposure in the previous experiment (9), in which the highest exposure dose resulted in about 10,000 ng/g wet weight after 8 days at 5 °C. Although we expected the graded column series to produce exposures ranging from lethal to no effects concentrations, it is clear that the lowest dose was above a no effects concentration, with 100% of exposed embryos showing signs of toxicity. In the previous study, pericardial edema was observed in ~40% of hatched larvae exposed to initial water TPAH < 1 µg/L from gravel with “more weathered oil” (i.e., higher tricyclic fraction), producing tissue TPAH levels at ~150 ng/g wet weight (9).

The profound effects on cardiac function observed here were associated with relatively low tissue burdens of PAHs. Embryos exposed to OGE6.25 showed pericardial edema and a 50% reduction in heart rate at 7 dpf with a tissue level of 0.8 µmol/kg (150 ng/g) total tricyclics (TPAH 2.8 µmol/kg, 480 ng/g). This level is 3 orders of magnitude lower than the critical body residue range of 2–8 mmol/kg in canine myocardium (38, 39). MS-222 acts by binding neuronal voltage-sensitive sodium channels with a relatively low affinity [dissociation constant in the high micromolar range (40, 41)], while ouabain acts by binding to Na⁺-K⁺ ATPase with high affinity [dissociation constant in the nanomolar range (42)]. The levels of tricyclic PAHs producing cardiotoxicity in herring embryos are consistent with a specific, high affinity target involved in the electrophysiological properties of the myocardium, specialized conduction cells, or pacemaker. The irregular arrhythmia observed at higher doses could be characterized as atrial fibrillation. However, in the adult human heart with atrial fibrillation, the rate is usually increased, and the ventricle responds irregularly to chaotic atrial stimulation (43). Moreover, unlike some zebrafish mutants with fibrillation (25, 44, 45), multiple foci of disorganized contraction were not observed in the atria of OGE-exposed herring.

These findings confirm that toxicity from crude oil to a temperate marine species is consistent with the cardiotoxic properties of individual tricyclic PAHs observed in zebrafish embryos (15, 19). While the precise target and mechanism remain elusive, altered cardiovascular physiology is likely to play a role in both the acute sublethal effects and long-term impacts of oil spills and other chronic sources of aquatic PAHs. There are clear links between cardiac function and form during fish development (46), and a well-established relationship between the shape of the fish heart, maximum cardiac output, and swimming performance (47–49), a behavior that is highly relevant for fitness and survival. This suggests a hypothetical basis for the development of biomarkers relating to cardiac performance, such as cardiac natriuretic peptides secreted in response to hemodynamic stress (50, 51), that in conjunction with biomarkers of exposure such as CYP1A or levels of fluorescent aromatic compounds in bile (52), may provide a more sensitive and efficient means for assessing oil spill impacts on fisheries resources.

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**Supporting Information Available**

Detailed Materials and Methods, Figure S1 showing the absence of oil droplets adhering to herring eggs, Figure S2 showing the gross morphology of oil-exposed and control embryos at 7 dpf and 12 dpf, Figure S3 showing CYP1A immunofluorescence in OGE-exposed embryos, Figure S4 showing minimal CYP1A immunofluorescence in OGE-exposed herring embryos, Figure S5 showing the heart beat in an embryo exposed to OGE6.25, and Movie S3 showing the heart beat in an embryo exposed to OGE100. This material is available free of charge via the Internet at http://pubs.acs.org.

**Literature Cited**


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