Available online at www.sciencedirect.com
SCIENCE DIRECT®
Toxicology and Applied Pharmacology 196 (2004) 191–205
Toxicology and Applied Pharmacology
www.elsevier.com/locate/ytaap

Defects in cardiac function precede morphological abnormalities in fish embryos exposed to polycyclic aromatic hydrocarbons☆

John P. Incardona,* Tracy K. Collier, and Nathaniel L. Scholz

Ecotoxicology and Environmental Fish Health Program, Environmental Conservation Division, Northwest Fisheries Science Center, National Oceanic and Atmospheric Administration, Seattle, WA 98112, USA

Received 19 August 2003; accepted 6 November 2003

Abstract

Fish embryos exposed to complex mixtures of polycyclic aromatic hydrocarbons (PAHs) from petrogenic sources show a characteristic suite of abnormalities, including cardiac dysfunction, edema, spinal curvature, and reduction in the size of the jaw and other craniofacial structures. To elucidate the toxic mechanisms underlying these different defects, we exposed zebrafish (*Danio rerio*) embryos to seven non-alkylated PAHs, including five two- to four-ring compounds that are abundant in crude oil and two compounds less abundant in oil but informative for structure–activity relationships. We also analyzed two PAH mixtures that approximate the composition of crude oil at different stages of weathering. Exposure to the three-ring PAHs dibenzothiophene and phenanthrene alone was sufficient to induce the characteristic suite of defects, as was genetic ablation of cardiac function using a cardiac troponin T antisense morpholino oligonucleotide. The primary etiology of defects induced by dibenzothiophene or phenanthrene appears to be direct effects on cardiac conduction, which have secondary consequences for late stages of cardiac morphogenesis, kidney development, neural tube structure, and formation of the craniofacial skeleton. The relative toxicity of the different mixtures was directly proportional to the amount of phenanthrene, or the dibenzothiophene–phenanthrene total in the mixture. Pyrene, a four-ring PAH, induced a different syndrome of anemia, peripheral vascular defects, and neuronal cell death, similar to the effects previously described for potent aryl hydrocarbon receptor ligands. Therefore, different PAH compounds have distinct and specific effects on fish at early life history stages.
© 2004 Elsevier Inc. All rights reserved.

Keywords: Petroleum; Oil spill; Fish development; Heart morphogenesis; *silent heart* gene; CYP1A

Introduction

Polycyclic aromatic hydrocarbons (PAHs) are pervasive contaminants in rivers, lakes, and nearshore marine habitats. This is particularly true of urbanized areas, where anthropogenic inputs are derived predominantly from the consumption of petroleum. The largest fraction enters marine waters as land-based runoff or atmospheric deposition (National Research Council, 2003). While PAH levels in urban watersheds declined during the 1970s and 1980s due to reductions in coal burning and industrial emissions (Heit et al., 1988; Hites et al., 1980), the last decade has produced new increases in aquatic PAH accumulation due to automobile use associated with urban sprawl (Van Metre et al., 2000) or diesel combustion by heavy vehicles (Lima et al., 2002). Moreover, new evidence suggests that petroleum hydrocarbons from oil spills can persist in nearshore sediments for decades or longer (Reddy et al., 2002). Residential populations are increasing in coastal areas of the US (Bartlett et al., 2000) and, as a result, an increase in nearshore contamination by PAHs is expected. This issue is particularly important for regions such as the Pacific Northwest, where major recovery efforts are currently underway for several threatened and endangered fish species.

While there is an extensive literature describing the effects of PAHs on adult or juvenile animals, few studies have addressed the effects of PAHs on embryonic and early larval development in fish. These early life history stages may be particularly susceptible to PAH exposure,

☆ Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.taap.2003.11.026.

* Corresponding author. Ecotoxicology and Environmental Fish Health Program, Environmental Conservation Division, Northwest Fisheries Science Center, National Oceanic and Atmospheric Administration, 2725 Montlake Boulevard East, Seattle, WA 98112. Fax: +1-206-860-3335.
E-mail address: john.incardona@noaa.gov (J.P. Incardona).

0041-008X/$ - see front matter © 2004 Elsevier Inc. All rights reserved.
doi:10.1016/j.taap.2003.11.026

EX5048-000001-TRB

especially for species that spawn or rear near human settlements. The 1989 Exxon Valdez oil spill contaminated nearshore and intertidal spawning grounds for Pacific herring (*Clupea pallasi*) and pink salmon (*Oncorhynchus gorbuscha*) with Alaska North Slope crude oil, prompting a series of field and laboratory studies that examined the effects of PAHs on herring and salmon development (Brown et al., 1996; Carls et al., 1999; Heintz et al., 1999; Hose et al., 1996; Kocan et al., 1996; Marty et al., 1997; McGurk and Brown, 1996; Middaugh et al., 1998; Norcross et al., 1996; Spies et al., 1996). Together with recent studies on other species (Couillard, 2002; Middaugh et al., 1996, 2002; Pollino and Holdway, 2002) and an analysis of the effects of PAH-rich creosote on herring development (Vines et al., 2000), these collective studies documented what appears to be a common suite of developmental defects in teleost embryos exposed to petroleum-derived PAH mixtures. Gross malformations resulting from PAH exposure included pericardial and yolk sac edema, jaw reductions, and presumptive skeletal defects described as lordosis or scoliosis (dorsal curvature). Reductions in larval heart rate (bradycardia) and cardiac arrhythmia were also observed. Increased weathering of crude oil, which shifts the composition from predominantly two-ring (naphthalenes) to three-ring PAHs (e.g., phenanthrenes), resulted in a greater toxic potency and a higher frequency of malformations (Carls et al., 1999; Heintz et al., 1999). Significant sublethal effects were also observed in the absence of malformations. For example, pink salmon that were exposed to weathered crude oil as embryos and then released as smolts returned from the sea as adults in significantly fewer numbers (Heintz et al., 2000). Despite these careful analyses and the consistent findings across species, the precise mechanisms leading to PAH-associated malformations and sublethal effects are unknown. Moreover, all of the previous studies used complex mixtures of PAHs, and it is unclear from the results whether individual components act through distinct mechanisms or share a common mode of action.

To address these issues, we evaluated the effects of model PAH compounds on the embryonic and early larval development of the zebrafish *Danio rerio*. In zebrafish, hundreds of genes involved in the formation of virtually every organ system have been identified by large-scale mutagenesis screening (Haffter et al., 1996). Consequently, the phenotypes resulting from loss of gene function through mutation can be compared to malformations resulting from embryonic exposure to contaminants. This "chemical genetic" approach has been used recently to identify specific mechanisms of developmental toxicity (Peterson et al., 2000, 2001). Moreover, the bioaccumulation of PAHs by zebrafish embryos and larvae is similar to that for temperate marine species (Petersen and Kristensen, 1998). In the present study, we analyze the effects of non-alkylated PAHs containing 2–4 rings. Our primary objectives were to (1) determine if individual PAHs representing the most abundant homologous series in petroleum were capable of inducing developmental defects in laboratory zebrafish like those described for embryos of wild fish species exposed to petrogenic PAH mixtures, and (2) gain insight into the mechanism(s) underlying any PAH-induced developmental defects.

Methods

Chemicals. Naphthalene (purity >99%), fluorene (99%), dibenzothiophene (>99%), phenanthrene (>99.5%), anthracene (>99%), pyrene (>99%), and chrysene (98%) were obtained from Sigma-Aldrich, St. Louis, MO. Stock PAH solutions were made in dimethyl sulfoxide (tissue culture grade, Sigma) at 10 mg/ml, except anthracene (5 mg/ml) and chrysene (1 mg/ml).

Zebrafish exposures. A zebrafish breeding colony (wild-type AB strain) was maintained using routine procedures (Westerfield, 2000). Maintenance of adult fish, collection of fertilized eggs, and PAH exposures were all performed in water adjusted to a conductivity of approximately 1500 μS/cm, pH 7.5–8 with Instant Ocean salts ("system water"). Fertilized eggs were collected and washed with system water within 2 h of spawning and conventionally staged (Kimmel et al., 1995). In a series of experiments, exposure regimens were initiated at times ranging from 4 to 8 h post-fertilization (hpf). Exposures were carried out in 12-well plastic tissue culture plates with 5–10 embryos per well in 2.5 ml at 28.5 °C. Several experiments carried out in volumes up to 10 ml in 100 mm plastic dishes produced similar results (data not shown). Exposure water was exchanged at 18- to 24-h intervals. In general, the exposures utilized nominal concentrations of all PAHs at or above their solubility limits and actual aqueous concentrations were not measured. It was empirically determined (data not shown) that high nominal concentrations were required to maintain steady aqueous levels due to the small exposure volumes and high PAH-binding capacity of the tissue culture plastic.

Morpholino antisense "knockdown". The morpholino oligonucleotide (5′-CATGTTTGCTCTGATCTGACACGCA-3′; GeneTools, Philomath, OR) spans the cardiac troponin T translation start site and flanking 5′ sequence (Sehnert et al., 2002). Embryos were injected with approximately 4 ng oligonucleotide at the 1–4 cell stage using an MPPI-2 pressure microinjector (Applied Scientific Instrumentation, Eugene, OR), and incubated in system water for examination of phenotype over the following 6 days.

Imaging of live embryos or larvae. Embryos generally were examined with a Nikon SMZ800 stereomicroscope and by differential interference contrast (DIC) microscopy

using a Nikon E600 compound microscope. Before hatching stages embryos were analyzed without anesthesia and were manually dechorionated for DIC microscopy. For analysis of post-hatching stages, larvae were anesthetized with MS-222 (Sigma) and were mounted in 3% methyl cellulose (Sigma) in system water for DIC microscopy. Digital still images were acquired from both microscopes with a Spot RT camera and Spot 3.2.6 software (Diagnostic Instruments, Inc., Sterling Heights, MI), and video analysis was performed with a Fire i400 digital camera (Unibrain, San Ramon, CA) and BTV Pro Carbon 5.4 software. Heart rates were counted over 15- to 30-s intervals in unanesthetized animals viewed with the stereoscope after acclimation to room temperature for at least 10 min.

Immunofluorescence and Alcian blue staining. For analysis of Na^+/K^+ ATPase labeling in the pronephros with monoclonal antibody α6F hybridoma supernatant (Takeyasu et al., 1988) (Developmental Studies Hybridoma Bank, University of Iowa), 75–80 hpf embryos were fixed in methanol–DMSO and processed as described elsewhere (Dent et al., 1989; Drummond et al., 1998), except the fixed embryos were not treated with hydrogen peroxide. Cardiac morphogenesis was examined in embryos fixed in 4% phosphate-buffered paraformaldehyde with monoclonal antibodies MF20 against myosin heavy chain and atrium-specific S46 (Yelon et al., 1999). Embryos were permeabilized by washing in water followed by 1-h incubation in blocking solution (PBS, 0.2% Triton X-100, 1% DMSO, 5% normal goat serum). Embryos were incubated overnight at 4 °C with agitation in α6F or MF20 and S46 hybridoma supernatants diluted 1:10 in blocking solution. After three 1-h washes in PBS + 0.2% Triton X-100, embryos were incubated several hours at room temperature in secondary antibodies diluted 1:1000 in blocking solution. Secondary antibodies (Molecular Probes, Eugene, OR) were AlexaFluor488-conjugated goat anti-mouse IgG (α6F) or goat–anti-mouse IgG_1 (S46), and AlexaFluor568-conjugated goat anti-mouse IgG_{2b} (MF20). After three 1-h washes in PBS + 0.2% Triton X-100, embryos were mounted in 50% glycerol in PBS and imaged with a Nikon E600 compound microscope and Spot RT camera. Alcian blue staining of the cartilaginous skeleton was performed as described (Kimmel et al., 1998) on 5–6 dpf larvae. Head skeletons were dissected after treatment with trypsin and flat-mounted for DIC microscopy.

Results

Differential toxicity of PAH compounds during embryonic and early larval development

We tested the effects of seven non-alkylated PAH compounds containing 2–4 rings (Fig. 1 and Table 1) on zebrafish development. To obtain data potentially comparable to those from studies prompted by the Exxon Valdez spill, we focused primarily on PAHs representing the homologous series most abundant in weathered Alaska North Slope crude oil (naphthalene, fluorene, dibenzothiophene, phenanthrene, and chrysene). We also included anthracene and pyrene to determine if similar defects could also be induced in fish embryos by these PAHs that are far less abundant in weathered Alaska crude oil and have identical ring numbers but different structure as phenanthrene and chrysene, respectively. Using static renewal exposure conditions, zebrafish embryos were incubated in water containing PAH compounds or vehicle (≤0.1% DMSO) beginning at 4–8 h post-fertilization (hpf), with a final replacement of exposure water at 72–78 hpf. To allow for high-throughput screening using zebrafish embryos reared in very small volumes, we used nominal exposure concentrations that were higher than the levels reported in the natural environment and generally well above their solubility limits. Although not measured, the actual PAH aqueous concentrations were probably much lower, in part because of the high affinity of plastic for PAHs. Representative examples of hatching-stage larvae at 4 days post-fertilization (dpf) are shown in Fig. 2. Larvae treated throughout embryogenesis with naphthalene (Fig. 2B), anthracene (Fig. 2F), or chrysene (Fig. 2H) had grossly normal anatomic features. In contrast, larvae treated with fluorene (Fig. 2C), dibenzothiophene (Fig. 2D), or phenanthrene (Fig. 2E) displayed dorsal curvature of the trunk and tail and significant growth reduction, particularly of the head. In addition, dibenzothiophene- and phenanthrene-treated

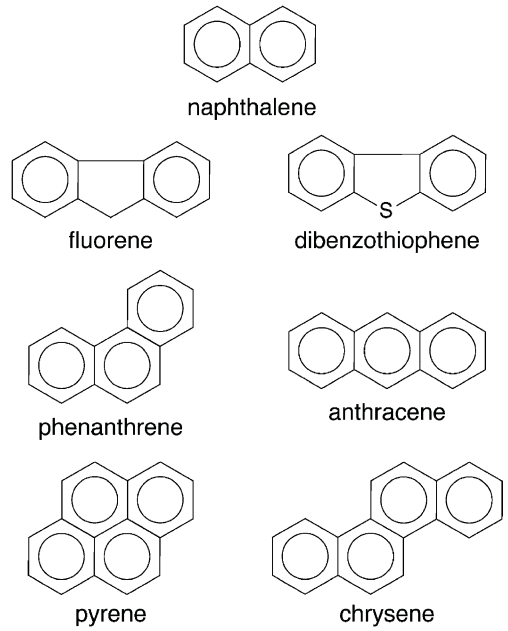

Fig. 1. Structures of the PAHs used in this study.

Table 1
PAHs used in the studies

PAH	MW	Water solubility (μM)	Mean log K_{ow}[a]
Naphthalene	128.2	247	3.34
Fluorene	166.2	12	4.22
Dibenzothiophene	184.3	7.9	4.44
Phenanthrene	178.2	7.2	4.53
Anthracene	178.2	0.4	4.53
Pyrene	202.2	0.67	5.07
Chrysene	228.3	0.009	5.77

[a] Meador et al. (1995).

embryos showed severe pericardial and yolk-sac edema, while fluorene treatment resulted in mild pericardial edema. Treatment with pyrene (Fig. 2G) resulted in mild pericardial edema and a less-pronounced dorsal curvature, and other distinct defects described in more detail below. Larvae treated with 0.2% DMSO (Fig. 2A), the highest level of solvent carrier achieved in the PAH exposures, were indistinguishable from larvae grown in system water only (data not shown). No lethality was observed with any PAH treatment by 96 hpf, despite the severe edema induced by some compounds. After placement in clean water and continued rearing to 6 or 7 dpf, 100% mortality was observed for fish treated with dibenzothiophene, phenanthrene, or pyrene, but not the other PAHs. Despite grossly normal development in the presence of naphthalene, anthracene, or chrysene, all PAH-exposed embryos showed delayed or failed inflation of the swim bladder.

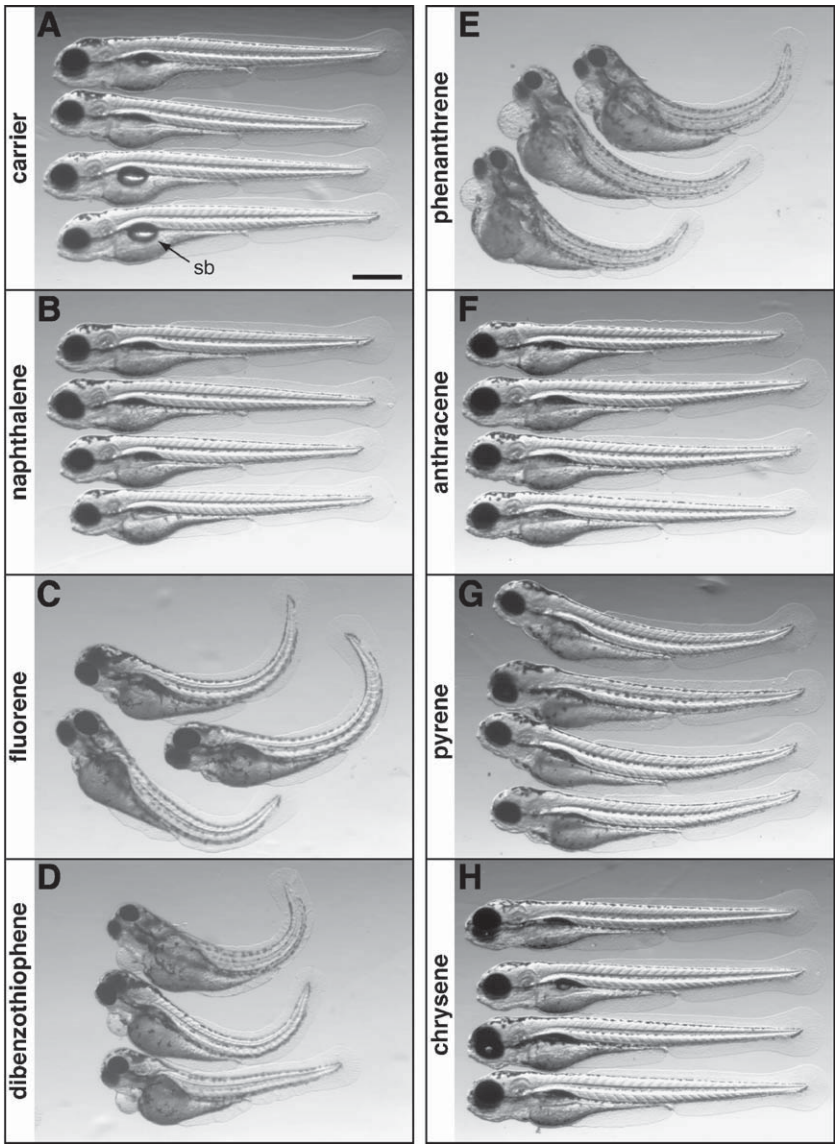

Fig. 2. Effects of individual non-alkylated PAHs on zebrafish development. Shown are 4 dpf larva after exposure to (A) 0.2% DMSO, (B) 78 μM naphthalene, (C) 60 μM fluorene, (D) 54 μM dibenzothiophene, (E) 56 μM phenanthrene, (F) 56 μM anthracene, (G) 5 μM pyrene, and (H) 8.8 μM chrysene. Swim bladder (*sb*) is indicated in A. Scale bar is 0.5 mm. Larvae shown are representative of at least three replicative experiments and approximately 25–100 treated embryos.

Three-ring PAH compounds selectively disrupt embryonic cardiac function

In embryos treated with the three-ring PAHs dibenzothiophene and phenanthrene, the earliest observed defect was loss or reduction in circulation at approximately 36 hpf (see below). We therefore compared PAH treatment to genetic disruption of cardiac function to determine whether cardiac dysfunction and loss of circulation were responsible for the suite of observed developmental abnormalities. The zebrafish mutant *silent heart* (*sih*) fails to develop a heart beat due to severely reduced expression of cardiac troponin T, an essential component of the sarcomere in cardiomyocytes (Sehnert et al., 2002). Targeted "knockdown" of cardiac troponin T expression by injection of *sih* antisense morpholino faithfully reproduces the *sih* phenotype (Sehnert et al., 2002) and resulted in a suite of defects nearly identical to those induced by three-ring PAHs (Fig. 3). At 48 hpf, embryos treated with 56 μM phenanthrene or injected with *sih* morpholino exhibited mild pericardial edema (not shown) and by 3 dpf began to show dorsal curvature of the body axis (Figs. 3A and B). In both cases, dorsal curvature was more severe by 4 dpf as edema accumulated (Figs. 3C and D). Notably, eye and jaw growths were similarly reduced by phenanthrene treatment or *sih* morpholino injection (Figs. 3E and F). This indicates that cardiac dysfunction alone is sufficient to cause the predominant developmental defects induced by three-ring PAHs.

A closer examination of the timing of phenanthrene- or dibenzothiophene-induced defects indicated that these compounds affect cardiac function and not primary cardiac morphogenesis. The onset of the heartbeat occurred normally in PAH-treated embryos at about 24 hpf (16–20 h of exposure; data not shown), but several hours thereafter embryos displayed bradycardia and arrhythmias characteristic of atrioventricular (AV) conduction block (see digital movies in Supplemental Material for the Web). Phenanthrene (Fig. 4B) or dibenzothiophene (data not shown) produced dose-dependent reductions in heart rate with AV conduction block occurring at the highest doses, which, in turn, was associated with gross morphological abnormalities at later developmental stages. At intermediate doses by 30–35 hpf (22–28 h of PAH exposure), both phenanthrene

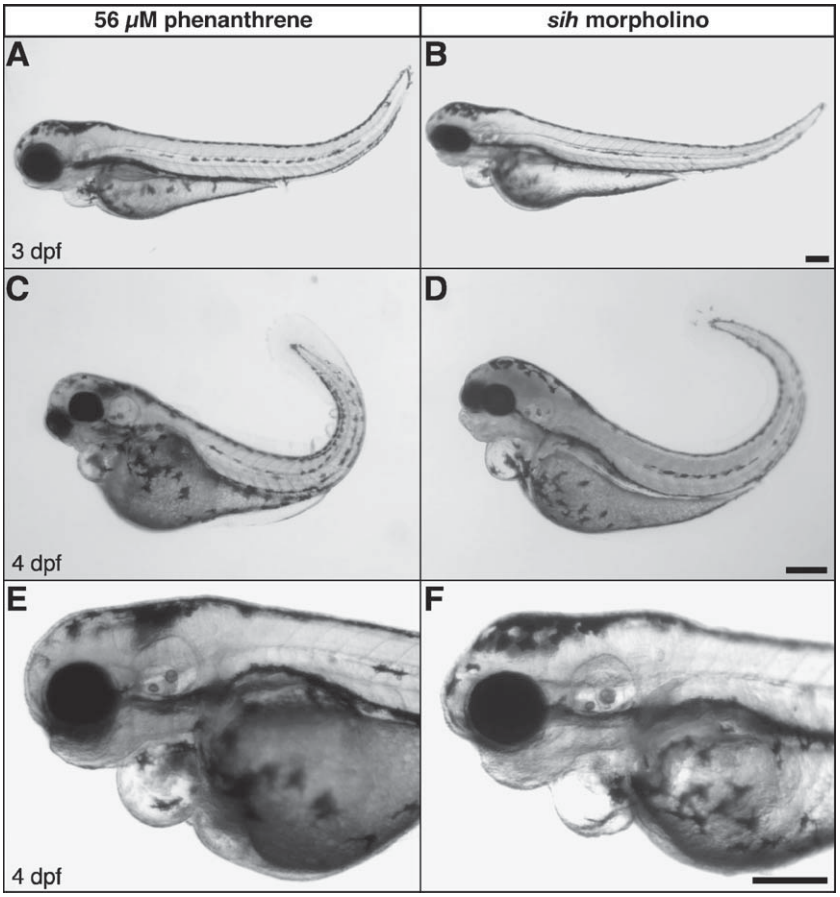

Fig. 3. Malformations induced by three-ring PAHs are indistinguishable from those associated with specific genetic disruption of heart development by *sih* antisense morpholino injection. Phenanthrene-treated (A, C, and E) and *sih* morpholino-injected (B, D, and F) are shown at the same stages of development, 3 dpf (A and B) and 4 dpf (C and D). (E and F) Higher magnifications of 4 dpf larvae. Scale bars are 0.2 mm.

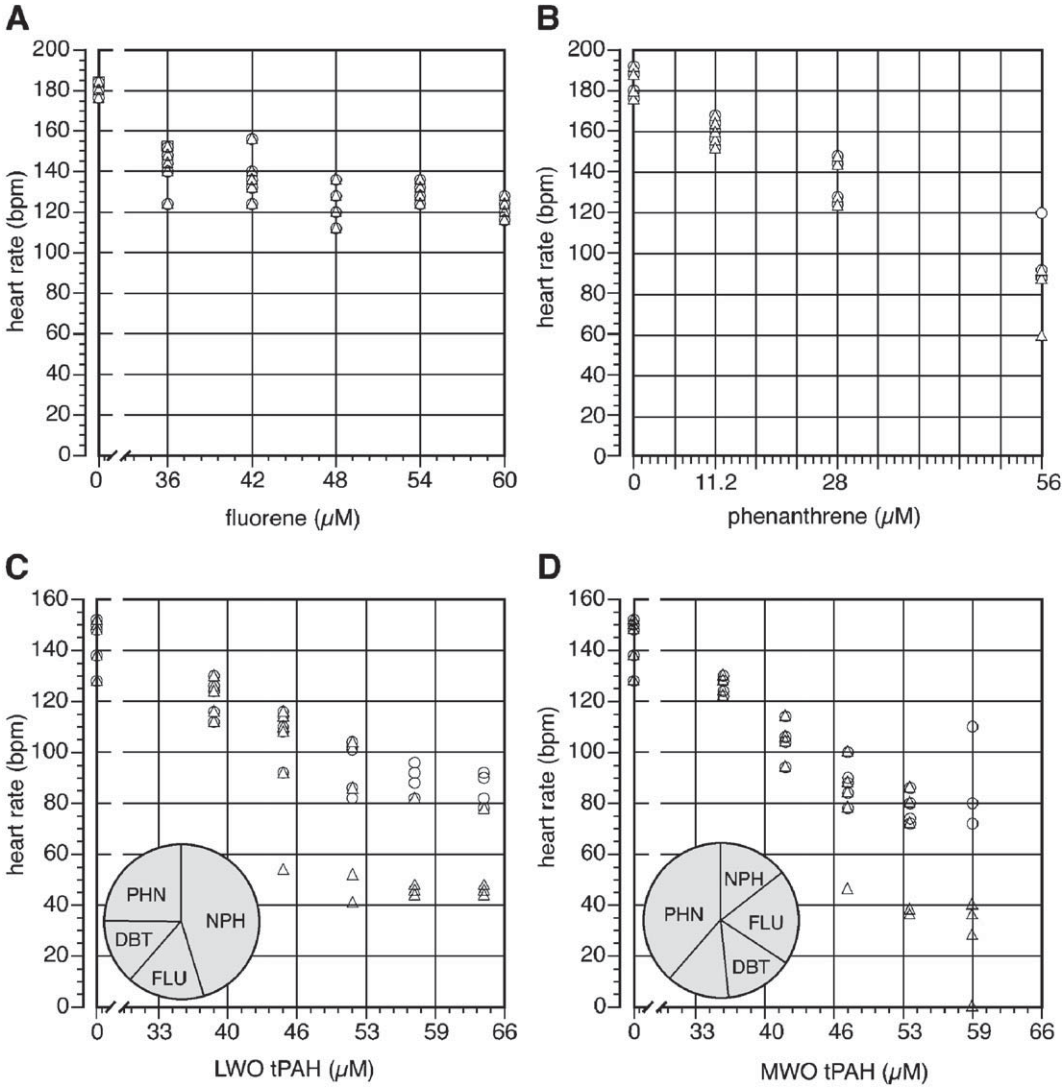

Fig. 4. Three-ring PAHs induce bradycardia and atrioventricular conduction block. Heart rates were counted without anesthesia in clutches of five larvae at 3 dpf after exposure to the indicated concentrations of PAHs. Atrial rates are indicated by circles and ventricular rates by triangles. Non-overlap of circles and triangles indicates an animal with 2:1 conduction block, or complete block (triangle with zero rate). Dose responses shown for (A) fluorene, (B) phenanthrene, (C) less-weathered oil mixture (LWO), and (D) more-weathered oil mixture (MWO). Pie chart insets indicate relative proportions of naphthalene (NPH), fluorene (FLU), dibenzothiophene (DBT), and phenanthrene (PHN) in the mixtures.

(28 μM) and dibenzothiophene (27 μM) induced a 2:1 AV conduction block, where the atrium beats twice for every ventricular contraction (Web Movies 1 and 2), although blood circulation was maintained. At higher doses, complete AV conduction block occurred, resulting in a more rapidly contracting atrium, a silent ventricle (Web Movie 3), and circulatory failure. These effects were reversible; heart rate began to increase within several hours of transfer to clean water. The AV conduction block also resolved in a few hours to overnight depending on the initial severity of the block (Web Movies 4–6). However, for embryos that were continuously exposed to doses ≥50 μM, cardiac activity ceased by 3 dpf. This resulted in the characteristic suite of morphologic abnormalities. In contrast, 60 μM fluorene did not induce complete AV block, although exposed embryos showed significant bradycardia (Fig. 4A), occasional 2:1 AV block, and a slowing of circulation (data not shown). Naphthalene induced a mild bradycardia (5–6% reduction) at 39 or 78 μM that was not dose-dependent, and anthracene and chrysene had no significant effect on heart rate (Table 2).

To determine whether PAH exposure disrupted the developmental patterning of the heart, we examined cardiac morphology with the chamber-specific antibodies MF20 and S46, which recognize myosin heavy chain in the ventricle and atrium, respectively (Yelon et al., 1999). The teleost heart begins as a linear tube that later loops at the atrioventricular boundary (between 24 and 48 hpf in zebrafish) to bring the ventricle to the right side of the atrium. As indicated by MF20/S46 immunofluorescence (Fig. 5) and

Table 2
Minimal or no effect of some PAHs on heart rate

Treatment	Heart rate (beats/min)
DMSO	183.2 ± 3
Naphthalene, 39 μM	173.6 ± 4.5 ($P = 0.056$)
Naphthalene, 78 μM	175.2 ± 2.7 ($P < 0.05$)
DMSO	165 ± 3
Chrysene, 22 μM	178 ± 7 ($P = 0.076$)
DMSO	201 ± 6
Anthracene, 56 μM	199 ± 4

direct light microscopic observations (not shown), formation of the cardiac chambers and initial looping of the heart occurred normally in phenanthrene- or dibenzothiophene-treated embryos. However, both cardiac chambers were dilated and had thinner walls in treated embryos (Fig. 5B). This was typically followed by a collapse and stretching of the chambers between their connections to either side of the distended pericardium (Fig. 5C). Collapse and generation of this string-like appearance of the heart is indicative of a failure to complete cardiac looping (Garrity et al., 2002). Severe AV conduction block induced by PAHs, even when reversed and circulation restored, had consequences for chamber size and later stages of cardiac looping (see Web Movie 6). These data indicate that three-ring PAHs, in particular dibenzothiophene and phenanthrene, can disrupt late stages of cardiac morphogenesis via persistent inhibition of cardiac conduction.

Narcosis is often considered a major toxic effect of PAH compounds, and narcotic compounds can affect cardiac function, although typically at much higher doses than those that produce anesthesia. To determine if the observed effects on cardiac function could be attributed to non-specific membrane effects on excitable cells, the basis for the nonpolar narcosis model (van Wezel and Opperhuizen, 1995), we assessed mechanosensory-evoked reflexes in PAH-treated embryos. One of the earliest behaviors that develop in zebrafish is the touch response, which is a precursor to the adult escape reflex. The touch response reflects the integration of mechanosensory input from Rohon–Beard neurons in the spinal cord and motor output via the hindbrain (Granato et al., 1996; Ribera and Nusslein-Volhard, 1998; Saint-Amant and Drapeau, 1998). In fish this response is abolished by general anesthetics such as MS-222. Qualitative analysis showed the touch responses of PAH-treated embryos were largely intact (Web Movies 7 and 8). Therefore, it does not appear that PAHs are generally narcotic in early zebrafish larvae, and there is no correlation between the effects of different PAHs on cardiac conduction and sensorimotor function.

The toxicity of PAH mixtures depends on the percentage of three-ring compounds, particularly phenanthrene

We tested the effects of two PAH mixtures prepared to mimic the composition of crude oil observed at two stages of weathering which represented conditions in Prince William Sound after the Exxon Valdez oil spill (Carls et al., 1999). Naphthalenes, fluorenes, dibenzothiophenes, phenanthrenes, and chrysenes made up 98.1% and 94.3% of less weathered oil (LWO) and more weathered oil (MWO),

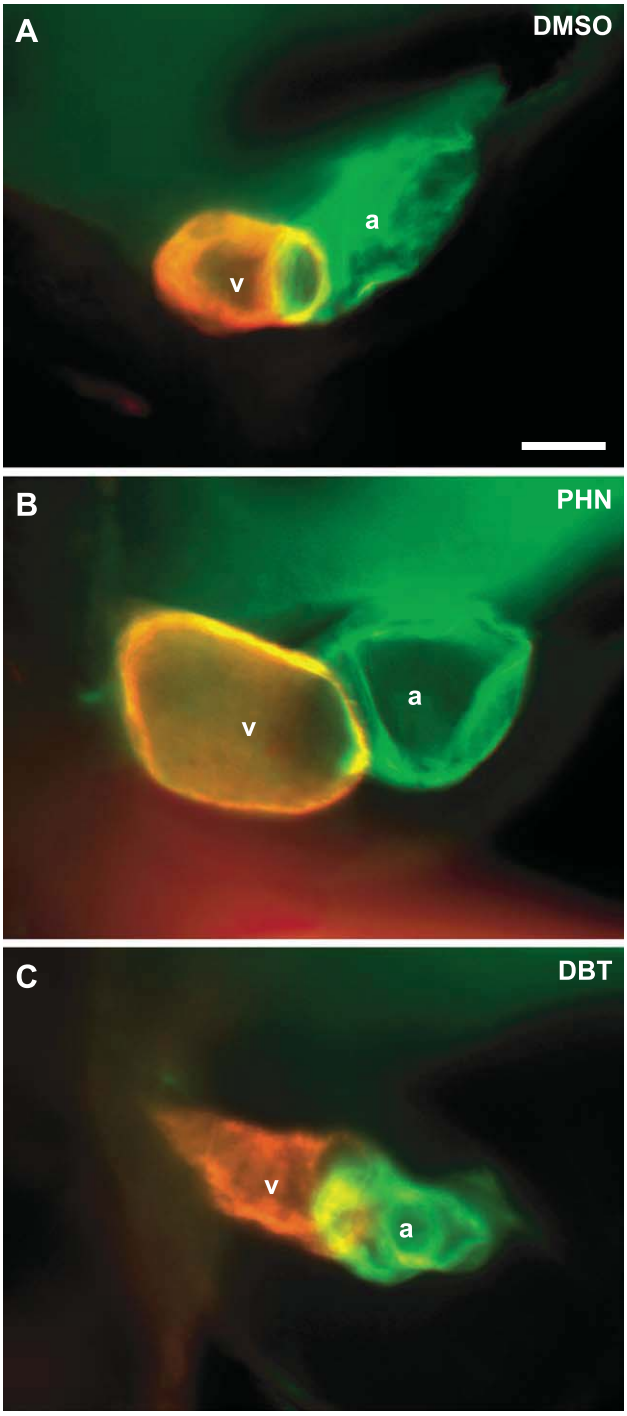

Fig. 5. Cardiac morphogenesis in PAH-treated embryos assessed with chamber-specific antibodies. Embryos treated with 0.2% DMSO (A), 56 μM phenanthrene (B), or 54 μM dibenzothiophene (C) were fixed at 52 hpf (44 h of exposure) and immunofluorescence performed with monoclonal antibodies S46 and MF20 to visualize the atrium (*a*, green) and ventricle (*v*, red), respectively. Lateral views are shown with anterior to the left. Scale bar is 50 μm.

respectively. Although alkylated homologues were more abundant than non-alkylated parent compounds, we tested a LWO-like mixture consisting of 44.1% naphthalene, 15.7% fluorene, 13.2% dibenzothiophene, 24.2% phenanthrene, and 0.9% chrysene; and a MWO-like mixture consisting of 13.0% naphthalene, 17.7% fluorene, 12.8% dibenzothiophene, 46.4% phenanthrene, and 4.4% chrysene. Note that the major difference between LWO and MWO is the relative proportion of naphthalene and phenanthrene. We also tested mixtures without chrysene to determine if the relatively small amount of this compound made a measurable contribution to the toxicity of the mixtures.

Using the same exposure protocol as above, we found that the LWO and MWO mixtures differed significantly in the ability to induce the malformation syndrome in a manner representative of the component three-ring PAHs. Both mixtures induced bradycardia and AV conduction blocks (Figs. 4C and D). However, at 64.5 μM, the LWO mixture induced only the partial AV block giving rise to the 2:1 rhythm and not circulatory failure (Fig. 4C), and was associated with only mild pericardial edema (data not shown) that readily resolved after transfer to clean water at 4 dpf (see Fig. 8A). In contrast, at 54 μM, the MWO mixture induced complete AV block (Fig. 4D) and the same cardiac degeneration and sequelae as observed with phenanthrene and dibenzothiophene alone (see Fig. 8B). Thus, the two PAH mixtures have markedly different effects on cardiac function at nearly the same total PAH concentration, consistent with our findings on individual PAHs and indicating that the relative amounts of specific PAHs are more important. Because the fluorene and dibenzothiophene concentrations are similar in both LWO and MWO mixtures, the toxicity of the MWO mixture can be attributed to the increase in phenanthrene (or the three-ring PAH total). Omission of chrysene in the mixtures had no apparent effect. Similar to what we observed for active three-ring compounds alone, larvae recovered cardiac function after transfer to clean water before obvious cardiac degeneration.

Three-ring PAH exposure has indirect effects on other tissues (kidney, neural tube, head skeleton) that are secondary to cardiovascular dysfunction

Because freshwater fish are hyperosmotic relative to their surroundings and must produce copious dilute urine, kidney dysfunction can lead to severe edema. The pronephric kidney of teleosts (Figs. 6A and B) consists of paired bilateral pronephric ducts joined to convoluted pronephric tubules that meet anteriorly at a midline glomerulus, which is derived by midline migration and fusion of bilateral pronephric primordia that coalesce around an ingrowing capillary. Completion of glomerular assembly in zebrafish occurs between 36 and 55 hpf and depends on hemodynamic forces provided by the early embryonic circulation (Drummond et al., 1998; Serluca et al., 2002). Assembly of the glomerulus fails in zebrafish mutants with severe cardiac dysfunction such as *sih* (Serluca et al., 2002). Therefore, for zebrafish embryos treated with concentrations of dibenzothiophene or phenanthrene sufficient to block cardiac func-

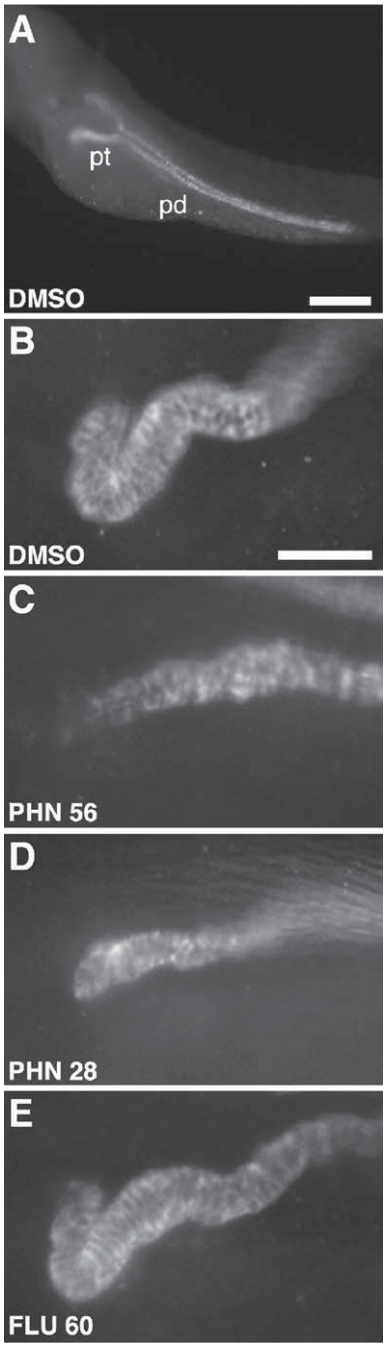

Fig. 6. Morphology of the pronephros is disrupted concomitant to circulatory defects induced by three-ring PAHs. Embryos treated with 0.2% DMSO (A and B), phenanthrene at 56 μM (C) or 28 μM (D), or 60 μM fluorene (E) were fixed at 80 hpf (72 h of exposure) and immunofluorescence carried out with α6F, which recognizes Na^+/K^+ ATPase in the pronephric tubule (*pt*) and pronephric duct (*pd*). Lower magnification dorsolateral view in A demonstrates the overall anatomy of the pronephros at 80 hpf, with anterior to the left. The glomerulus lies at the midline between the anterior portions of the paired pronephric tubules. (B–E) Higher magnifications of the pronephric tubule. Scale bars are 50 μm.

tion completely, the severe yolk sac edema observed by 4 dpf is likely to reflect failure of kidney morphogenesis. Embryos treated with levels of PAH sufficient to cause circulatory failure by 36 hpf showed a characteristic failure of glomerular assembly, as indicated by the appearance of bilateral pronephric cysts (data not shown) and straightening of the pronephric tubule with thinning of the epithelium (Fig. 6C). PAH-treated embryos with very weak circulation displayed marked but less severe defects in the pronephric tubular epithelium (Fig. 6D). Fluorene-treated embryos with bradycardia and mild pericardial edema did not show defects in the pronephric tubule (Fig. 6E).

Light microscopic examination of the trunk of PAH-treated (Fig. 7) and *sih* morpholino-injected embryos (data not shown) indicated that dorsal curvature of the body axis may be secondary to fluid imbalance in the neural tube. The lumen or central canal of the neural tube is normally only faintly discernable by DIC microscopy (arrowheads in Fig. 7A). In contrast, the central canal appeared more distinct and increasingly dilated in fish with more severe dorsal curvature (Figs. 7B and C). Dorsal curvature and distension of the central canal were observed in embryos that had a reduction of circulation that was sufficient to cause pericardial but not yolk sac edema (e.g., fluorene-treated; data not shown), suggesting that fluid balance in the neural tube is more sensitive to circulatory function than kidney morphogenesis. Examination of the skeleton in affected 6-day-old larvae by calcein staining confirmed that the dorsal curvature did not result from a primary defect in skeletogenesis (data not shown). If not too severe, the dorsal curvature was reversible with PAH depuration (see Figs. 8B and C), consistent with a functional rather than structural etiology.

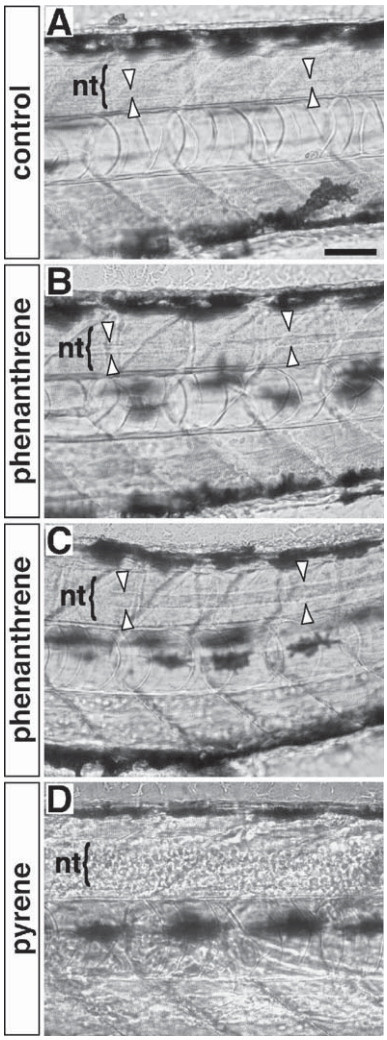

Fig. 7. Dorsal curvature is correlated with distention of the central canal of the neural tube in larvae treated with three-ring PAHs, but curvature in pyrene-treated larvae is associated with widespread cell death in the neural tube. Views of the trunk at the level of the 10th somite are shown at 84 hpf for a control larva (A) and larvae treated with 28 μM phenanthrene with moderate (B) and high (C) degrees of dorsal curvature. The same view is shown at 5 dpf for a larva treated with 5 μM pyrene (D). Brackets indicate the dorsoventral extent of the neural tube (*nt*), and arrowheads in A–C indicate the margins of the central canal. Scale bar is 50 μm.

Reductions in jaw size have been observed previously in larval fish exposed to PAHs. We analyzed head skeletons in larvae at 6 dpf after treatment up to 4 dpf with individual PAHs (data not shown) or the LWO–MWO mixtures (Fig. 8). Embryos treated with the MWO mixture to 4 dpf had moderate to severe pericardial edema (Fig. 8B), but recovered after transfer to clean water (Fig. 8C), with the exception of larvae that had irreversible cardiac degeneration (Fig. 8C, arrowhead). Although larvae that recovered appeared grossly normal, skeletal elements of the head were smaller (Figs. 8F and G). The most severe effects on the head skeleton were observed in larvae with no cardiac function and severe edema (Fig. 8H). Similar results were observed in *sih* morpholino-injected embryos reared to 5 dpf (data not shown), indicating that edema, absent circulation, or both affect the growth of skeletal precursors in the jaws. Upon higher magnification (data not shown), the size of individual chondrocytes appeared reduced rather than the total number of cells contributing to individual skeletal elements. Moreover, the overall size of the head skeleton was reduced disproportionately to body length (data not shown). Larvae exposed to LWO exhibited a moderate but transient bradycardia and mild pericardial edema, and had slightly smaller head skeletons (Fig. 8E). Overall, these data suggest that reduction of head skeletal elements is correlated with the degree of edema accumulation secondary to even transient cardiac dysfunction.

Pyrene does not affect cardiac conduction but induces anemia, peripheral vascular defects, and neuronal cell death

Embryos exposed to pyrene showed less severe pericardial edema and slight bradycardia by 80 hpf (data not shown). By 98 hpf slight dorsal curvature was observed (Fig. 2G). However, this appeared to reflect a different underlying pathophysiology. Despite a relatively unaffected heart rate and rhythm, pyrene-treated embryos at this stage

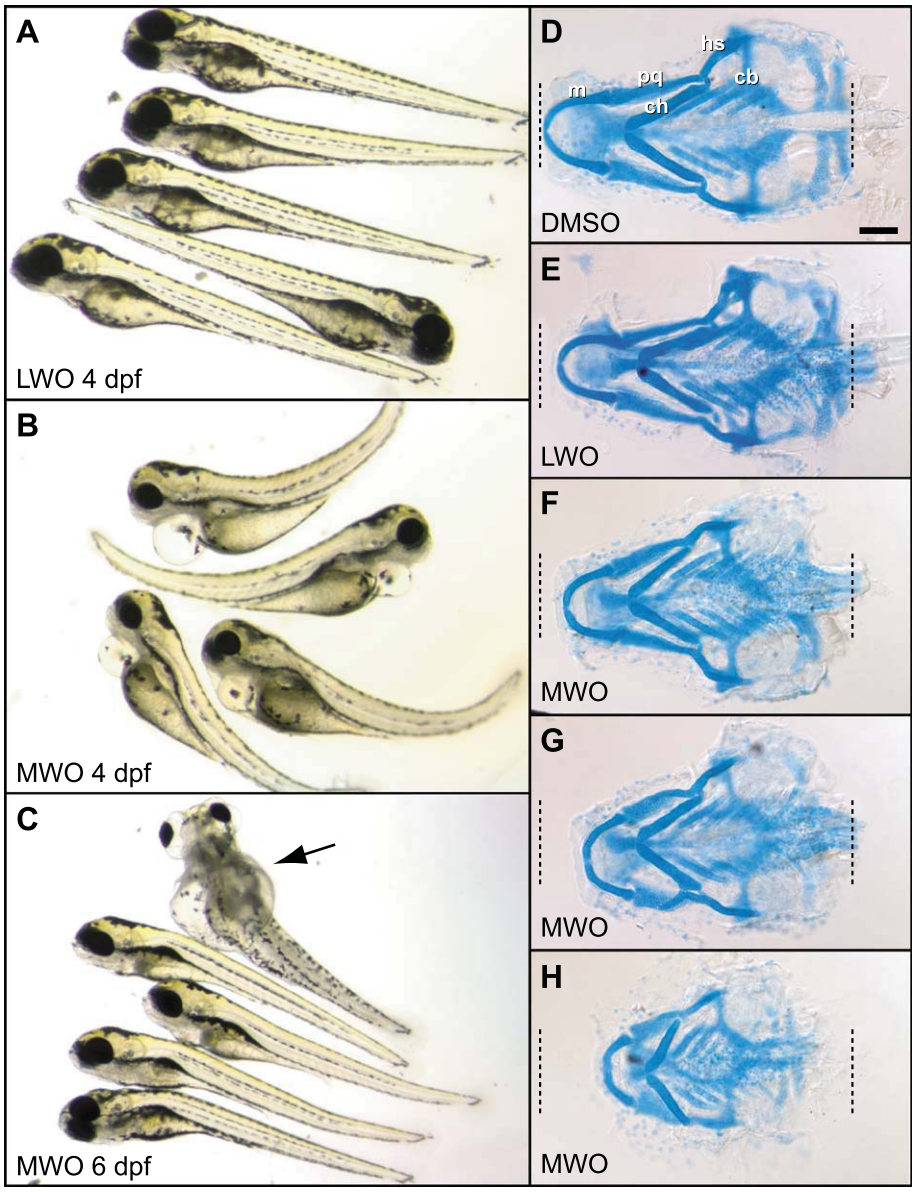

Fig. 8. Reduction of craniofacial skeletal elements correlates with the degree of pericardial edema. Larvae are shown at 4 dpf after treatment with 64 μM LWO mixture (A) and 43 μM MWO mixture (B), and at 6 dpf after 48 h of MWO mixture depuration (C). The four larvae in B are a subset of the five shown in C. At 6 dpf, cartilaginous skeletal precursors were stained with Alcian blue as described in Methods, and the head skeletons were dissected for flat mounting and microscopy. (D) Vehicle-treated control larva, (E) representative larva treated with LWO-mixture from (A), and (F–H) representative larvae treated with MWO-mixture from group in C. Arrow in C indicates larva corresponding to the head skeleton in H. Dashed lines in D–H indicate the anterior–posterior dimensions of the control skeleton.

showed severely reduced peripheral blood circulation in the head (Web Movies 9 and 10) and trunk (Web Movies 11 and 12). Moreover, a significant anemia was apparent (Web Movies 13 and 14), with markedly reduced numbers of circulating erythrocytes. Examination of affected embryos by DIC microscopy showed widespread cell death in the brain and trunk regions of the neural tube, indicated by the granular appearance of neural tissue (Fig. 7D and data not shown). We did not determine if cell death was necrotic or apoptotic, but hemorrhaging was not observed. It is unlikely that cell death in the neural tube is secondary to loss of peripheral circulation because this was not observed in embryos with complete circulatory failure at the same stage due to three-ring PAH treatment (Figs. 7B and C) or *sih* morpholino injection.

Discussion

In the context of aquatic toxicology, PAHs are generally thought to act as nonspecific or "baseline" toxicants through the nonpolar narcosis mode of action, or through activation of the aryl hydrocarbon receptor (AhR) pathway leading to cytochrome *P*450 1A (CYP1A) induction. In

addition, a wealth of studies have documented carcinogenic and immunotoxic effects of PAHs, though the former is usually associated with high molecular weight (≥5 rings) series (reviewed by Payne et al., 2003). In zebrafish embryos, we observed two distinct types of developmental toxicity for PAHs containing 2–4 rings: (1) cardiac dysfunction and its sequelae associated with some three-ring compounds, and (2) peripheral vascular defects, anemia, and neuronal cell death associated with pyrene, a four-ring compound. Edema and cardiovascular defects are common in fish embryos treated with either PAHs or AhR ligands such as dioxins, leading to a general view that these two classes of compounds act on fish early life history stages by a common pathway. Our findings and a series of studies describing the effects of dioxins on zebrafish embryos indicate that different PAH subclasses act through distinct toxic mechanisms, some novel, in embryonic and early larval stages of teleosts.

Remarkably, dibenzothiophene and phenanthrene are each individually capable of inducing a suite of defects that closely mirrors that observed in other teleost embryos exposed to complex PAH mixtures enriched in two- to four-ring compounds (e.g., Carls et al., 1999; Couillard, 2002; Heintz et al., 1999; Marty et al., 1997; Vines et al., 2000). However, it was previously unclear whether PAHs have direct and independent effects on different structures (e.g., heart, eye, jaw, body axis), or if the suite of PAH-induced developmental defects are functionally interrelated. Comparison to embryos with the *silent heart* phenotype indicates that most or all of the morphological defects induced by these three-ring PAHs are consequences of cardiac dysfunction. Because *sih*–cardiac troponin T is not expressed in tissues other than the heart, all aspects of the mutant phenotype are highly specific consequences of cardiac dysfunction.

Three-ring PAHs and cardiac function

Due to their lipophilicity and simple structure generally containing only carbon and hydrogen, low molecular weight PAHs (fewer than four rings) are widely considered to have low baseline toxicity attributed to nonpolar narcosis. The original principle underlying nonpolar narcosis (the functional equivalent of anesthesia) is nonspecific disruption of membrane integrity due to incorporation of hydrophobic organic compounds (for review, see Schultz, 1989; van Wezel and Opperhuizen, 1995). Nonpolar narcosis is thought to be achieved when membranes accumulate a certain mole fraction of contaminant, empirically determined in fish to reflect the total tissue levels or body burdens in the range of 2–8 mmol/kg wet weight (reviewed by Escher and Hermens, 2002; McCarty and Mackay, 1993). Studies in a variety of organisms have shown that PAH toxicity increases with increasing lipophilicity, as expressed by the log octanol–water partition coefficient (log K_{ow}), up to the point where toxicity declines in association with low water solubility and bioavailability (e.g., Sverdrup et al., 2002). This relationship appears to be true for compounds with log K_{ow}s of 2–5. Basic quantitative structure–activity relationships predict that many of the PAH solutions used in our studies could theoretically produce tissue PAH levels that would be lethal by nonpolar narcosis (Verhaar et al., 1992). Thus it may appear that some of our results are consistent with the nonpolar narcosis model, with toxicity increasing from naphthalene (K_{ow} 3.3) to phenanthrene (K_{ow} 4.5), but decreasing for chrysene (K_{ow} 5.8). However, several observations indicate that nonpolar narcosis is not the mode of toxic action at work for three-ring PAHs in fish embryos.

In principle, if narcotic or anesthetic compounds act by nonspecific disruption of membrane function, any excitable cell type should be affected. However, contemporary studies indicate that general anesthetics do not act nonspecifically on lipid bilayers, but selectively perturb the action of ligand-gated ion channels in the central nervous system, with minimal effects on cardiac function (reviewed by Franks and Lieb, 1994, 1998, 1999; Park, 2002; Patel, 2002). In contrast, our results indicate that three-ring PAHs are poor anesthetics that disrupt cardiac function selectively while having little effect on neuronal function. Doses of phenanthrene or dibenzothiophene that stop circulation do not immobilize or render embryos insensitive to mechanosensory stimulation, the hallmarks of narcosis. In this light it is important to recognize that the nonpolar narcosis syndrome in fish was actually defined using the general anesthetic MS-222, a neuronal sodium channel blocker (McFarland, 1959; McKim et al., 1987). Instead, the effects of the active three-ring PAHs on cardiac function are remarkably similar to compounds that cause bradycardia due to inhibition of repolarizing potassium currents in the heart (Milan et al., 2003), suggesting the cardiac-specific KCNH2 potassium channel subunit as a possible molecular target (Keating and Sanguinetti, 2001; Mitcheson et al., 2000). Another possible target is the L-type calcium channel α1C (C-LTCC) subunit, which is required for normal atrioventricular conduction in zebrafish (Rottbauer et al., 2001).

The specific and local effects of PAHs on AV conduction also argue against the nonpolar narcosis model. In a study of the bioaccumulation and depuration of radiolabeled PAHs by zebrafish embryos and larvae, naphthalene and phenanthrene accumulated to similar levels after 24–30 h of exposure (7–8 mmol/kg dry weight for embryos, 4–5 mmol/kg for larvae; Petersen and Kristensen, 1998). This is also the time at which we observed significant effects on cardiac conduction. These authors also noted "bilaterally bent chorda" in phenanthrene-treated animals while naphthalene-treated embryos were unaffected. This indicates that two-ring PAHs do not affect the same target in the cardiac conduction system as do three-ring PAHs, despite reaching essentially identical tissue levels. Using the published correlation between log K_{ow}s and bioconcentration factors determined for zebrafish (Petersen and Kristensen, 1998), we estimate that fluorene should accumulate to levels 96%

of that for dibenzothiophene, a difference unlikely to account for the inability of fluorene to readily induce the AV conduction block. Whether the absence of an effect for anthracene reflects structural specificity cannot be determined because the predicted bioconcentration factor indicates that anthracene tissue levels would be 10- to 20-fold lower than phenanthrene. In zebrafish bioaccumulation studies, radiolabeled four-ring pyrene reached levels of approximately 3 mmol/kg dry weight in zebrafish larvae, respectively, and also resulted in dorsal curvature (Petersen and Kristensen, 1998). However, our studies indicate that this is not due to effects on cardiac conduction. Overall, our data support a direct and specific effect of a subset of three-ring PAHs on cardiac conduction.

Pyrene toxicity and induction of the Ah receptor pathway

Pyrene induced a very different suite of defects at a different stage in development, including loss of peripheral circulation, anemia, delayed onset of pericardial edema, and cell death in the neural tube. These effects all manifested after 3 dpf. These distinct effects of pyrene are very similar to those described for 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), a potent ligand for the Ah receptor. A series of studies documenting the effects of TCDD in zebrafish described reductions in circulation in the head and trunk beginning around 3 dpf, with pericardial edema, failure of erythropoiesis, and apoptotic cell death in the neural tube (Belair et al., 2001; Dong et al., 2001, 2002; Henry et al., 1997; Teraoka et al., 2002). These effects were correlated with CYP1A induction as early as 36 hpf and were ameliorated to a similar degree by an Ah receptor antagonist or CYP1A inhibitor (Andreasen et al., 2002; Dong et al., 2001, 2002). Moreover, gene silencing with AhR or CYP1A antisense morpholinos demonstrated that TCDD-induced pericardial edema in zebrafish requires an active AhR pathway (Teraoka et al., 2003). Given the qualitative similarities between our results with pyrene and the recent work on TCDD, it is likely that pyrene acts via the AhR pathway. Irrespective of pyrene's mode of action, it appears that the three- and four-ring PAHs have fundamentally different effects on fish embryos.

Implications for the characterization of sublethal effects of PAH exposure in fish

Although the blockade of cardiac conduction by three-ring PAHs is reversible, these preliminary studies suggest that transient changes in conduction may have subtle and perhaps irreversible effects during later stages of heart development. Initial stages of cardiac looping were unaffected by PAHs, but significant remodeling occurs after looping, including development of the cardiac valves, formation of the trabeculae, and thickening of the ventricular myocardium (Hu et al., 2000). Some of these processes continue through several weeks of larval development in zebrafish (Hu et al., 2000), and genetic analysis indicates that many aspects of cardiac remodeling and maturation are dependent on function (reviewed by Glickman and Yelon, 2002). Although three-ring PAH-exposed fish had a normal cardiac rhythm at 48–72 hpf after depuration, the morphology of the heart was different. Because hemodynamic forces influence multiple aspects of myocardial formation (Hove et al., 2003; Sedmera et al., 1998, 1999; Taber et al., 1993), there are likely to be significant long-term effects of three-ring PAHs on this basis alone. Therefore, a more complete analysis of cardiac structure in PAH-treated fish, especially those reared for longer periods, is needed. In particular, an analysis of maturation of the trabeculae is important because these structures provide the main atrioventricular conduction pathway in the hearts of lower vertebrates (Sedmera et al., 2003). Also, defects in the cardiac trabeculae were observed in pink salmon larvae that had edema after exposure to weathered crude oil during embryogenesis (Marty et al., 1997). It is possible that PAH-exposed fish in the natural environment may experience sublethal reductions in cardiac function that translate, in turn, to impaired performance at later life history stages. Although affected fish might appear grossly normal, their physiology and behavioral performance could be impaired. This could explain, for example, the reduced rate of marine survival among pink salmon exposed to PAHs as embryos (Heintz et al., 2000).

Finally, our findings also raise the possibility that impacts of PAHs on kidney development could potentially contribute to sublethal effects that have not been anticipated previously. Although glomerular assembly fails in the absence of circulation, it is unknown whether there is a threshold hemodynamic force required to induce assembly, or if more moderate reductions in pressure would have consequences for kidney development that could affect individual survival. This is a particularly important question for freshwater and anadromous fishes. In addition, there are a host of other secondary effects that could conceivably contribute to reduced overall fitness. For example, reduced jaw structures could impact prey choices, growth, and overall size, factors which ultimately influence survival (Sogard, 1997). Similarly, it is unclear how transient changes in shape might affect subsequent development of the neural tube, where spatial relationships are important in the specification of spinal cord cell types (reviewed by Jessell, 2000). We anticipate that many of these questions can be answered efficiently using zebrafish as an experimental system for mechanistic studies of PAH toxicity in fish.

Note added in proof

The zebrafish *breakdance* mutant, which is characterized by 2:1 atrioventricular conduction block, was recently determined to disrupt the cardiac delayed rectifier K^+ channel (Langheinrich et al., 2003).

Acknowledgments

The authors would like to thank Carla Stehr for her work in establishing the zebrafish breeding colony at the NWFSC, Jana Labenia and Tiffany Kao for excellent technical assistance and zebrafish husbandry, David Baldwin for help with video microscopy acquisition and editing software, Dave Raible for the use of microinjection apparatus and discussion of data, Jim Lister for help with morpholino injections, Tor Linbo for advice on Alcian blue staining, Chuck Kimmel for advice on the head skeleton data, Frank Stockdale for generously providing the S46 antibody, and Didier Stainier, Brant Weinstein, and Calum McCrae for advice on analysis of zebrafish cardiovascular phenotypes. Monoclonal antibody MF20 was obtained from the Developmental Studies Hybridoma Bank developed under the auspices of the NICHD and maintained by the Department of Biological Sciences, University of Iowa, Iowa City, IA 52242. This work was supported by the National Research Council Research Associateships Program (J.P.I.) and a NWFSC Internal Grant (N.L.S.). Neither funding source was involved in the design or implementation of the described studies.

References

Andreasen, E.A., Spitsbergen, J.M., Tanguay, R.L., Stegeman, J.J., Heideman, W., Peterson, R.E., 2002. Tissue-specific expression of AHR2, ARNT2, and CYP1A in zebrafish embryos and larvae: effects of developmental stage and 2,3,7,8-tetrachlorodibenzo-*p*-dioxin exposure. Toxicol. Sci. 68, 403–419.

Bartlett, J.H.G., Mageean, D.M., O'Connor, R.J., 2000. Residential expansion as a continental threat to U.S. coastal ecosystems. Popul. Environ. 21, 429–468.

Belair, C.D., Peterson, R.E., Heideman, W., 2001. Disruption of erythropoiesis by dioxin in the zebrafish. Dev. Dyn. 222, 581–594.

Brown, E.D., Baker, T.T., Hose, J.E., Kocan, R.M., Marty, G.D., McGurk, M.D., Norcross, B.L., Short, J., 1996. Injury to the early life history stages of Pacific herring in Prince William Sound after the Exxon Valdez oil spill. In: Rice, S.D., Spies, R.B., Wolfe, D.A., Wright, B.A. (Eds.), Proceedings of the Exxon Valdez Oil Spill Symposium. American Fisheries Society, Anchorage, AK (USA), pp. 448–462 (25 Feb. 1993).

Carls, M.G., Rice, S.D., Hose, J.E., 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.

Couillard, C.M., 2002. A microscale test to measure petroleum oil toxicity to mummichog embryos. Environ. Toxicol. 17, 195–202.

Dent, J.A., Polson, A.G., Klymkowsky, M.W., 1989. A whole-mount immunocytochemical analysis of the expression of the intermediate filament protein vimentin in Xenopus. Development 105, 61–74.

Dong, W., Teraoka, H., Kondo, S., Hiraga, T., 2001. 2, 3, 7, 8-Tetrachlorodibenzo-*p*-dioxin induces apoptosis in the dorsal midbrain of zebrafish embryos by activation of arylhydrocarbon receptor. Neurosci. Lett. 303, 169–172.

Dong, W., Teraoka, H., Yamazaki, K., Tsukiyama, S., Imani, S., Imagawa, T., Stegeman, J.J., Peterson, R.E., Hiraga, T., 2002. 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin toxicity in the zebrafish embryo: local circulation failure in the dorsal midbrain is associated with increased apoptosis. Toxicol. Sci. 69, 191–201.

Drummond, I.A., Majumdar, A., Hentschel, H., Elger, M., Solnica-Krezel, L., Schier, A.F., Neuhauss, S.C., Stemple, D.L., Zwartkruis, F., Rangini, Z., Driever, W., Fishman, M.C., 1998. Early development of the zebrafish pronephros and analysis of mutations affecting pronephric function. Development 125, 4567–4655.

Escher, B.I., Hermens, J.L.M., 2002. Modes of action in ecotoxicology: their role in body burdens, species sensitivity, QSARs, and mixture effects. Environ. Sci. Technol. 36, 4201–4217.

Franks, N.P., Lieb, W.R., 1994. Molecular and cellular mechanisms of general anaesthesia. Nature 367, 607–614.

Franks, N.P., Lieb, W.R., 1998. Which molecular targets are most relevant to general anaesthesia? Toxicol. Lett. 100–101, 1–8.

Franks, N.P., Lieb, W.R., 1999. Background K+ channels: an important target for volatile anesthetics? Nat. Neurosci. 2, 395–396.

Garrity, D.M., Childs, S., Fishman, M.C., 2002. The heartstrings mutation in zebrafish causes heart/fin Tbx5 deficiency syndrome. Development 129, 4635–4645.

Glickman, N.S., Yelon, D., 2002. Cardiac development in zebrafish: coordination of form and function. Semin. Cell Dev. Biol. 13, 507–513.

Granato, M., van Eeden, F.J., Schach, U., Trowe, T., Brand, M., Furutani-Seiki, M., Haffter, P., Hammerschmidt, M., Heisenberg, C.P., Jiang, Y.J., Kane, D.A., Kelsh, R.N., Mullins, M.C., Odenthal, J., Nusslein-Volhard, C., 1996. Genes controlling and mediating locomotion behavior of the zebrafish embryo and larva. Development 123, 399–413.

Haffter, P., Granato, M., Brand, M., Mullins, M.C., Hammerschmidt, M., Kane, D.A., Odenthal, J., van Eeden, F.J., Jiang, Y.J., Heisenberg, C.P., Kelsh, R.N., Furutani-Seiki, M., Vogelsang, E., Beuchle, D., Schach, U., Fabian, C., Nusslein-Volhard, C., 1996. The identification of genes with unique and essential functions in the development of the zebrafish, *Danio rerio*. Development 123, 1–36.

Heintz, R.A., Short, J.W., Rice, S.D., 1999. Sensitivity of fish embryos to weathered crude oil: Part II. Increased mortality of pink salmon (*Oncoorhynchus gorbuscha*) embryos incubating downstream from weathered Exxon Valdez crude oil. Environ. Toxicol. Chem. 18, 494–503.

Heintz, R.A., Rice, S.D., Wertheimer, A.C., Bradshaw, R.F., Thrower, F.P., Joyce, J.E., Short, J.W., 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.

Heit, M., Tan, Y.L., Miller, K.M., 1988. The origin and deposition history of polycyclic aromatic hydrocarbons in the finger lakes region of New York USA. Water Air Soil Pollut. 37, 85–110.

Henry, T.R., Spitsbergen, J.M., Hornung, M.W., Abnet, C.C., Peterson, R.E., 1997. Early life stage toxicity of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin in zebrafish (*Danio rerio*). Toxicol. Appl. Pharmacol. 142, 56–68.

Hites, R.A., Laflamme, R.E., Windsor, J.G.J., Farrington, J.W., Deuser, W.G., 1980. Polycyclic aromatic hydrocarbons in an anoxic sediment core from the Pettaquamscutt River Rhode-Island USA. Geochim. Cosmochim. Acta 44, 873–878.

Hose, J.E., McGurk, M.D., Marty, G.D., Hinton, D.E., Brown, E.D., Baker, T.T., 1996. Sublethal effects of the Exxon Valdez oil spill on herring embryos and larvae: morphological, cytogenetic, and histopathological assessments, 1989–1991. Can. J. Fish. Aquat. Sci. 53, 2355–2365.

Hove, J.R., Koster, R.W., Forouhar, A.S., Acevedo-Bolton, G., Fraser, S.E., Gharib, M., 2003. Intracardiac fluid forces are an essential epigenetic factor for embryonic cardiogenesis. Nature 421, 172–177.

Hu, N., Sedmera, D., Yost, H.J., Clark, E.B., 2000. Structure and function of the developing zebrafish heart. Anat. Rec. 260, 148–157.

Jessell, T.M., 2000. Neuronal specification in the spinal cord: inductive signals and transcriptional codes. Nat. Rev., Genet. 1, 20–29.

Keating, M.T., Sanguinetti, M.C., 2001. Molecular and cellular mechanisms of cardiac arrhythmias. Cell 104, 569–580.

Kimmel, C.B., Ballard, W.W., Kimmel, S.R., Ullmann, B., Schilling, T.F., 1995. Stages of embryonic development of the zebrafish. Dev. Dyn. 203, 253–310.

Kimmel, C.B., Miller, C.T., Kruze, G., Ullmann, B., BreMiller, R.A., Larison, K.D., Snyder, H.C., 1998. The shaping of pharyngeal cartilages during early development of the zebrafish. Dev. Biol. 203, 245–263.

Kocan, R.M., Hose, J.E., Brown, E.D., Baker, T.T., 1996. Pacific herring (*Clupea pallasi*) embryo sensitivity to Prudhoe Bay petroleum hydrocarbons: laboratory evaluation and in situ exposure at oiled and unoiled sites in Prince William Sound. Can. J. Fish. Aquat. Sci. 53, 2366–2387.

Langheinrich, U., Vacun, G., Wagner, T., 2003. Zebrafish embryos express an orthologue of HERG and are sensitive toward a range of QT-prolonging drugs inducing severe arrhythmia. Toxicol. Appl. Pharmacol. 193, 370–382.

Lima, A.L.C., Eglinton, T.I., Reddy, C.M., 2002. High-resolution record of pyrogenic polycyclic aromatic hydrocarbon deposition during the 20th century. Environ. Sci. Technol. 37, 53–61.

Marty, G.D., Short, J.W., Dambach, D.M., Willits, N.H., Heintz, R.A., Rice, S.D., Stegeman, J.J., Hinton, D.E., 1997. Ascites, premature emergence, increased gonadal cell apoptosis, and cytochrome *P*4501A induction in pink salmon larvae continuously exposed to oil-contaminated gravel during development. Can. J. Zool. 75, 989–1007.

McCarty, L.S., Mackay, D., 1993. Enhancing ecotoxicological modeling and assessment. Body residues and modes of toxic action. Environ. Sci. Technol. 27, 1718–1728.

McFarland, W.N., 1959. A study of the effects of anaesthetics on the behaviour and physiology of fishes. Publ. Inst. Mar. Sci., Univ. Tex. 6, 23–55.

McGurk, M.D., Brown, E.D., 1996. Egg-larval mortality of Pacific herring in Prince William Sound, Alaska, after the Exxon Valdez oil spill. Can. J. Fish. Aquat. Sci. 53, 2343–2354.

McKim, J.M., Schmieder, P.K., Carlson, R.W., Hunt, E.P., Niemi, G.J., 1987. Use of respiratory–cardiovascular responses of rainbow trout (*Salmo gairdneri*) in identifying acute toxicity syndromes in fish: 1. Pentachlorophenol, 2,4-dinitrophenol, tricaine methanesulfonate and 1-octanol. Environ. Toxicol. Chem. 6, 295–312.

Meador, J.P., Stein, J.E., Reichert, W.L., Varanasi, U., 1995. Bioaccumulation of polycyclic aromatic hydrocarbons by marine organisms. Rev. Environ. Contam. Toxicol. 143, 79–165.

Middaugh, D.P., Chapman, P.J., Shelton, M.E., 1996. Responses of embryonic and larval inland silversides, *Menidia beryllina*, to a water-soluble fraction formed during biodegradation of artificially weathered Alaska North Slope crude oil. Arch. Environ. Contam. Toxicol. 31, 410–419.

Middaugh, D.P., Shelton, M.E., McKenney Jr., C.L., Cherr, G., Chapman, P.J., Courtney, L.A., 1998. Preliminary observations on responses of embryonic and larval Pacific herring, *Clupea pallasi*, to neutral fraction biodegradation products of weathered Alaska North Slope oil. Arch. Environ. Contam. Toxicol. 34, 188–196.

Middaugh, D.P., Chapman, P.J., Shelton, M.E., McKenney Jr., C.L. , Courtney, L.A., 2002. Effects of fractions from biodegraded Alaska North Slope crude oil on embryonic inland silversides, *Menidia beryllina*. Arch. Environ. Contam. Toxicol. 42, 236–243.

Milan, D.J., Peterson, T.A., Ruskin, J.N., Peterson, R.T., MacRae, C.A., 2003. Drugs that induce repolarization abnormalities cause bradycardia in zebrafish. Circulation 107, 1355–1358.

Mitcheson, J.S., Chen, J., Lin, M., Culberson, C., Sanguinetti, M.C., 2000. A structural basis for drug-induced long QT syndrome. Proc. Natl. Acad. Sci. U.S.A. 97, 12329–12333.

National Research Council, 2003. Oil in the Sea III: Inputs, Fates, and Effects. National Academies Press, Washington, DC, p. 446.

Norcross, B.L., Hose, J.E., Frandsen, M., Brown, E.D., 1996. Distribution, abundance, morphological condition, and cytogenetic abnormalities of larval herring in Prince William Sound, Alaska, following the Exxon Valdez oil spill. Can. J. Fish. Aquat. Sci. 53, 2376–2387.

Park, K.W., 2002. Cardiovascular effects of inhalational anesthetics. Int. Anesthesiol. Clin. 40, 1–14.

Patel, S., 2002. Cardiovascular effects of intravenous anesthetics. Int. Anesthesiol. Clin. 40, 15–33.

Payne, J.F., Mathieu, A., Collier, T.K., 2003. Ecotoxicological studies focusing on marine and freshwater fish. In: Dauben, P.E.T. (Ed.), PAHs—An Ecotoxicological Perspective. Wiley, West Sussex, UK, pp. 191–224.

Petersen, G.I., Kristensen, P., 1998. Bioaccumulation of lipophilic substances in fish early life stages. Environ. Toxicol. Chem. 17, 1385–1395.

Peterson, R.T., Link, B.A., Dowling, J.E., Schreiber, S.L., 2000. Small molecule developmental screens reveal the logic and timing of vertebrate development. Proc. Natl. Acad. Sci. U.S.A. 97, 12965–12969.

Peterson, R.T., Mably, J.D., Chen, J.N., Fishman, M.C., 2001. Convergence of distinct pathways to heart patterning revealed by the small molecule concentramide and the mutation heart-and-soul. Curr. Biol. 11, 1481–1491.

Pollino, C.A., Holdway, D.A., 2002. Toxicity testing of crude oil and related compounds using early life stages of the crimson-spotted rainbowfish (*Melanotaenia fluviatilis*). Ecotoxicol. Environ. Saf. 52, 180–189.

Reddy, C.M., Eglinton, T.I., Hounshell, A., White, H.K., Xu, L., Gaines, R.B., Frysinger, G.S., 2002. The West Falmouth oil spill after thirty years: the persistence of petroleum hydrocarbons in marsh sediments. Environ. Sci. Technol. 36, 4754–4760.

Ribera, A.B., Nusslein-Volhard, C., 1998. Zebrafish touch-insensitive mutants reveal an essential role for the developmental regulation of sodium current. J. Neurosci. 18, 9181–9191.

Rottbauer, W., Baker, K., Wo, Z.G., Mohideen, M.A., Cantiello, H.F., Fishman, M.C., 2001. Growth and function of the embryonic heart depend upon the cardiac-specific L-type calcium channel alpha1 subunit. Dev. Cell 1, 265–275.

Saint-Amant, L., Drapeau, P., 1998. Time course of the development of motor behaviors in the zebrafish embryo. J. Neurobiol. 37, 622–632.

Schultz, T.W., 1989. Nonpolar narcosis: a review of the mechanism of action for baseline aquatic toxicity. In: Cowgill, U.M., Williams, L.R. (Eds.), Aquatic Toxicology and Hazard Assessment: 12th Volume, ASTM STP 1027. American Society for Testing and Materials, Philadelphia, pp. 104–109.

Sedmera, D., Pexieder, T., Hu, N., Clark, E.B., 1998. A quantitative study of the ventricular myoarchitecture in the stage 21–29 chick embryo following decreased loading. Eur. J. Morphol. 36, 105–119.

Sedmera, D., Pexieder, T., Rychterova, V., Hu, N., Clark, E.B., 1999. Remodeling of chick embryonic ventricular myoarchitecture under experimentally changed loading conditions. Anat. Rec. 254, 238–252.

Sedmera, D., Reckova, M., deAlmeida, A., Sedmerova, M., Biermann, M., Volejnik, J., Sarre, A., Raddatz, E., McCarthy, R.A., Gourdie, R.G., Thompson, R.P., 2003. Functional and morphological evidence for a ventricular conduction system in zebrafish and *Xenopus* hearts. Am. J. Physiol.: Heart Circ. Physiol. 284, H1152–H1160.

Sehnert, A.J., Huq, A., Weinstein, B.M., Walker, C., Fishman, M., Stainier, D.Y., 2002. Cardiac troponin T is essential in sarcomere assembly and cardiac contractility. Nat. Genet. 31, 106–110.

Serluca, F.C., Drummond, I.A., Fishman, M.C., 2002. Endothelial signaling in kidney morphogenesis: a role for hemodynamic forces. Curr. Biol. 12, 492–497.

Sogard, S.M., 1997. Size-selective mortality in the juvenile stage of teleost fishes: a review. Bull. Mar. Sci. 60, 1129–1157.

Spies, R.B., Rice, S.D., Wolfe, D.A., Wright, B.A., 1996. The effects of the Exxon Valdez oil spill on the Alaskan coastal environment. In: Rice, S.D., Spies, R.B., Wolfe, D.A., Wright, B.A. (Eds.), Proceedings of the Exxon Valdez Oil Spill Symposium. American Fisheries Society, Anchorage, AK (USA), pp. 1–16 (25 Feb. 1993).

Sverdrup, L.E., Nielsen, T., Krogh, P.H., 2002. Soil ecotoxicity of polycyclic aromatic hydrocarbons in relation to soil sorption, lipophilicity, and water solubility. Environ. Sci. Technol. 36, 2429–2435.

Taber, L.A., Hu, N., Pexieder, T., Clark, E.B., Keller, B.B., 1993. Residual strain in the ventricle of the stage 16–24 chick embryo. Circ. Res. 72, 455–462.

Takeyasu, K., Tamkun, M.M., Renaud, K.J., Fambrough, D.M., 1988.

Ouabain-sensitive (Na+ + K+)-ATPase activity expressed in mouse L cells by transfection with DNA encoding the alpha-subunit of an avian sodium pump. J. Biol. Chem. 263, 4347–4354.

Teraoka, H., Dong, W., Ogawa, S., Tsukiyama, S., Okuhara, Y., Niiyama, M., Ueno, N., Peterson, R.E., Hiraga, T., 2002. 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin toxicity in the zebrafish embryo: altered regional blood flow and impaired lower jaw development. Toxicol. Sci. 65, 192–199.

Teraoka, H., Dong, W., Tsujimoto, Y., Iwasa, H., Endoh, D., Ueno, N., Stegeman, J.J., Peterson, R.E., Hiraga, T., 2003. Induction of cytochrome *P*450 1A is required for circulation failure and edema by 2,3,7,8-tetrachlorodibenzo-*p*-dioxin in zebrafish. Biochem. Biophys. Res. Commun. 304, 223–228.

Van Metre, P.C., Mahler, B.J., Furlong, E.T., 2000. Urban sprawl leaves its PAH signature. Environ. Sci. Technol. 34, 4064–4070.

van Wezel, A.P., Opperhuizen, A., 1995. Narcosis due to environmental pollutants in aquatic organisms: residue-based toxicity, mechanisms, and membrane burdens. Crit. Rev. Toxicol. 25, 255–279.

Verhaar, H.J.M., Vanleeuwen, C.J., Hermens, J.L.M., 1992. Classifying environmental-pollutants: 1. Structure–activity-relationships for prediction of aquatic toxicity. Chemosphere 25, 471–491.

Vines, C.A., Robbins, T., Griffin, F.J., Cherr, G.N., 2000. The effects of diffusible creosote-derived compounds on development in Pacific herring (*Clupea pallasi*). Aquat. Toxicol. 51, 225–239.

Westerfield, M., 2000. The Zebrafish Book. University of Oregon Press, Eugene, OR.

Yelon, D., Horne, S.A., Stainier, D.Y., 1999. Restricted expression of cardiac myosin genes reveals regulated aspects of heart tube assembly in zebrafish. Dev. Biol. 214, 23–37.