Published in final edited form as:

Aquat Toxicol. 2014 March ; 148: 16–26. doi:10.1016/j.aquatox.2013.12.028.

Multigenerational effects of benzo[a]pyrene exposure on survival and developmental deformities in zebrafish larvae

Jone Corrales, Cammi Thornton, Mallory White, and Kristine L. Willett*

Department of Pharmacology and Environmental Toxicology Research Program, School of Pharmacy, University of Mississippi, University, MS 38677, USA

Abstract

In the aquatic environment, adverse outcomes from dietary polycyclic aromatic hydrocarbon (PAH) exposure are poorly understood, and multigenerational developmental effects following exposure to PAHs are in need of exploration. Benzo[a]pyrene (BaP), a model PAH, is a recognized carcinogen and endocrine disruptor. Here adult zebrafish (F0) were fed 0, 10, 114, or 1012 µg BaP/g diet at a feed rate of 1% body weight twice/day for 21 days. Eggs were collected and embryos (F1) were raised to assess mortality and time to hatch at 24, 32, 48, 56, 72, 80, and 96 hours post fertilization (hpf) before scoring developmental deformities at 96 hpf. F1 generation fish were raised to produce the F2 generation followed by the F3 and F4 generations. Mortality significantly increased in the higher dose groups of BaP (2.3 and 20 µg BaP/g fish) in the F1 generation while there were no differences in the F2, F3, or F4 generations. In addition, premature hatching was observed among the surviving fish in the higher dose of the F1 generation, but no differences were found in the F2 and F3 generations. While only the adult F0 generation was BaP-treated, this exposure resulted in multigenerational phenotypic impacts on at least two generations (F1 and F2). Body morphology deformities (shape of body, tail, and pectoral fins) were the most severe abnormality observed, and these were most extreme in the F1 generation but still present in the F2 but not F3 generations. Craniofacial structures (length of brain regions, size of optic and otic vesicles, and jaw deformities), although not significantly affected in the F1 generation, emerged as significant deformities in the F2 generation. Future work will attempt to molecularly anchor the persistent multigenerational phenotypic deformities noted in this study caused by BaP exposure.

Keywords

multigeneration; survival; hatching; deformities; zebrafish; benzo[a]pyrene

1. Introduction

Polycyclic aromatic hydrocarbons (PAHs), such as benzo[a]pyrene (BaP), are ubiquitous environmental contaminants derived from the incomplete combustion of organic compounds. While forest fires and ocean floor seeps are natural sources of PAHs (Latimer and Zheng, 2003), the more numerous and damaging anthropogenic sources include diesel-

© 2014 Elsevier B.V. All rights reserved

*Corresponding author at: Box 1848, 305 Faser Hall, Department of Pharmacology, University of Mississippi, University, MS 38677, USA. Tel.: +1 662 915 6691; fax: +1 662 915 5148. kwillett@olemiss.edu (K.L. Willett)..

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

and gasoline-powered vehicles, coal-fired power plants, residential heating, cooking, and tobacco smoking. PAHs enter the aquatic environment via urban runoff and dry and wet depositions of atmospheric PAHs (Boström et al., 2002; Latimer and Zheng, 2003). Once in the aquatic environment PAHs are taken up by organisms from the water or through the diet (Hylland, 2006). In the 2011 CERCLA's Priority List of Hazardous Substances, BaP was ranked # 8 in front of PAHs as a mixture (#9) and benzo(b)fluoranthene (#10), an increase in priority since CERCLA's 2007 report. Moreover, in the 2012 IARC Monographs, BaP was classified as Group 1 (an animal and human carcinogen) (<http://monographs.iarc.fr/ENG/Classification/>).

Waterborne PAH exposure caused toxicity and altered the rate of development in fish early life-stages (Barron et al., 2004; Hawkins et al., 2002; Incardona et al., 2004; Colavecchia et al., 2004). *Fundulus heteroclitus*, a model fish species used in ecotoxicological studies, had elevated deformity indices (heart elongation, pericardial edema, tail shortening, and hemorrhaging) after exposure to binary mixtures of PAHs including BaP, β -naphthoflavone (BNF), α -naphthoflavone (ANF), fluoranthene (FL), piperonyl butoxide (PBO), and 2-aminoanthracene (AA) (Wassenberg and Di Giulio, 2004). Cardiac deformities were also apparent after BaP exposure in the absence of other PAHs (Wills et al., 2009). In pink salmon (*Oncorhynchus gorbuscha*) and rainbow trout (*O. mykiss*), dissolved PAHs from crude oil caused higher mortality, alterations in time to hatch, growth reduction, spinal deformities, jaw deformities, yolk sac edema, and impaired swimming (Carls and Thedinga, 2010; Barron et al., 2004; Hawkins et al., 2002). In *Sebastiscus marmoratus*, a teleost of the scorpion fish family, skeletal deformities including incidence of spinal curvature and craniofacial defects were caused by BaP as well as altered expression of genes involved in bone formation (He et al., 2011).

Recently zebrafish (*Danio rerio*) has become a preferred toxicity model due to its rapid life cycle, high fecundity, transparent development, and because the embryos are amenable to genetic manipulation using transgenic approaches and morpholino gene knockdowns (Sipes et al., 2011). Cardiac dysfunction resulting in pericardial edema and heart looping after exposure to PAHs has been extensively examined in zebrafish by Incardona et al. (2004, 2005, 2006, 2011). Zebrafish larvae treated with dibenzothiophene, phenanthrene, or fluorene not only displayed curvature of the trunk and tail and growth reduction, but also showed pericardial edema (Incardona et al., 2004). Moreover, atrioventricular conduction block was the primary effect of dibenzothiophene and phenanthrene with secondary defects on cardiac morphology, kidney development, neural tube structure, and craniofacial skeleton (Incardona et al., 2004). BaP exposure also caused defects in heart morphology and dysfunction (Incardona et al., 2011).

However, none of the studies described above examined developmental malformations across generations. In fish, multigenerational studies have primarily focused on reproductive repercussions associated with endocrine disruptors [e.g. bisphenol A (Sohoni et al., 2001; Staples et al., 2011), nonylphenol (Holdway et al., 2008), 2,3,7,8-tetrachlorodibenzo-p-dioxin (King-Heiden et al., 2005), 17 α -ethinylestradiol (Zha et al., 2008), and pulp and paper mill effluents (Parrott et al., 2010)] with little emphasis on multigenerational developmental deformities. Moreover, all the previously mentioned studies were waterborne exposures. Alternatively, dietary exposures provide an avenue to investigate this environmentally relevant exposure route (Peterson et al., 2003). Although some work on the reproductive impacts of dietary persistent organic pollutants and brominated flame retardants (Berg et al., 2011; Halden et al., 2011; Nourizadeh-Lillabadi et al., 2009; Chou et al., 2010) has been done, studies looking at dietary and multigenerational effects of PAH exposure on fish developmental deformities are lacking.

In the work presented here multigenerational developmental defects (F1, F2, F3) were measured subsequent to a parental dietary BaP exposure (F0). We found that (1) a parental dietary exposure to BaP had adverse effects on the immediate offspring and (2) some of these adverse effects persisted across generations.

2. Materials and methods

2.1. Zebrafish care

AB line wild-type zebrafish were purchased from Zebrafish International Resource Center (ZFIN, Eugene, OR) and raised under the approved IACUC protocol. Fish were kept in Aquatic Habitats ZF0601 Zebrafish Stand-Alone System (Aquatic Habitats, Apopka, FL) with zebrafish water (pH 7.0–7.5, 60 parts per million (ppm), Instant Ocean, Cincinnati, OH) at 25–28°C, 14:10 light-dark cycle. Fish were fed twice daily with TetraMin® Tropical Flakes and live brine shrimp. Sexually mature fish without any deformities or signs of disease were selected as breeders. Their eggs were collected and larvae were raised to 120 days post fertilization (dpf) to obtain the F0 generation for the dietary exposure described below.

2.2 Parental dietary exposure

Sexually mature (120 dpf) zebrafish were fed either acetone-treated (control) or BaP-treated (0.25, 2.5, or 25 µg/g fish nominally equivalent to 12.5, 125, or 1250 µg/g food, respectively) flake food; for actual BaP concentrations measured in the diet, see section 2.3. Comparable BaP concentrations have previously been used in fish including zebrafish, although we also extended the dose range to include a higher concentration (Alsop et al., 2007; Bailey et al., 2009). A preliminary exposure in our lab showed that feed rate and egg production did not vary between fish fed acetone-treated and non-treated food, therefore acetone was used as the BaP carrier solvent. Acetone was purchased from Fisher Scientific (Fair Lawn, NJ) and BaP from Supelco Analytical (Belfonte, PA). To prepare the treated flake food, 24 g of flake food were spiked with 18 ml of acetone containing BaP (0, 0.01667, 0.1667, or 1.667 µg/µL). The spiked flakes were immediately rotary evaporated to dryness and stored in amber vials at room temperature. Paired (2×2) zebrafish in ten replicate tanks per treatment group (N=10 replicate tanks for a total 40 fish/group) were allowed to acclimate for a week while maintained at 25.5–28°C and fed twice daily with TetraMin® Tropical Flakes and live brine shrimp. During the exposure, fish were fed 1% body weight twice daily of the corresponding dose of BaP-treated flake food and once daily live brine shrimp for 23 days. On days 20 and 21, eggs were collected to produce the F1 generation. On day 22, one fish per tank (five females and five males per group) were euthanized for histological examination. On day 23, the exposure was terminated and remaining fish were euthanized.

2.3. Extractions and chemical analysis

Treated flake food was extracted with methylene chloride to confirm the nominal concentrations of BaP. Approximately 10 mg of flakes were extracted right before day 0 and on days 7 and 14 with 2–3 mL of methylene chloride. A known concentration of a surrogate standard, benzo[a]pyrene-d12, was added to each sample to yield a final concentration of 0.2 µg/mL. Samples were vortexed for 30 sec and centrifuged for 7 min at 2000 rpm (668 × g). Samples were then blown to dryness with N₂ and brought back up with a known volume of hexane. A known concentration of internal standard, fluorene-d10, was added to each sample to determine extraction efficiency. Samples were run on the GC/MS under the selected ion mode to quantitate the concentration of BaP in each sample. BaP was not identified in the acetone-treated samples. Actual BaP concentrations of the treated flakes were: 10.44 ± 0.4, 113.6 ± 2.3, and 1012 ± 30.7 µg BaP/g flake equivalent to 0.209 ± 0.008,

2.27 ± 0.046 , and 20.25 ± 0.614 μg BaP/g fish, respectively. Percent recoveries ranged from 70–145%.

In addition to the BaP-treated flake food, chemical analysis was done in F1 fertilized eggs to assess potential BaP transfer into the embryos. Approximately 110 F1 fertilized eggs (8 hpf) collected on days 20 and 21 were stored at -80°C until chemical analysis. Eggs from the four treatment groups in triplicate were extracted 3 times with 3 ml of methylene chloride each time. A known concentration of the surrogate standard, benzo[a]pyrene-d12, was added to each sample to yield a final concentration of 0.2 $\mu\text{g}/\text{mL}$. Samples were vortexed for 15 sec and centrifuged for 7 min at 3000 rpm ($1509 \times g$) and passed over an alumina and sodium sulfate column to remove fat and water from the extract. Samples were then blown to dryness with N_2 and brought back up with a known volume of hexane. A known concentration of the internal standard, fluorene-d10, was added to each sample to yield a final concentration of 0.2 $\mu\text{g}/\text{mL}$ to determine extraction efficiency. Samples were run on the GC/MS under the selected ion mode to quantitate the concentration of BaP in each sample. BaP was not detected in the eggs despite good recovery concentrations (50–105%). This could be due to multiple reasons including but not limited to insufficient number of eggs, small amount of BaP deposition in the eggs, or BaP being rapidly metabolized by the mothers.

2.4. Production of F1, F2, F3 and F4 generations

After the parental exposure, F1 fish at 96 hpf were transferred to a recirculating system (Aquatic Habitats, Inc., Apopka, FL) and raised at a density of 20 ml/larva. From 0 hpf to 45 dpf, larvae were kept separately by parental tank. After 45 dpf, fish were mixed within treatments so as to minimize genetic bottlenecks within tanks. Larvae were fed twice daily Artemac Size 0 (powdered Artemia, brine shrimp) and starting on day 8 also live brine shrimp to increase survival. At approximately 20 dpf, fish were fed twice daily only live brine shrimp and after 30 dpf, they were fed twice daily TetraMin® Tropical Flakes and live brine shrimp. Fish were raised to approximately 120 dpf at which time 20 males and females were selected from each corresponding parental treatment group (0, 0.21, 2.3, or 20 μg BaP/g fish) to produce the F2 generation. The same protocol was followed to produce the F3 and F4 generations.

2.5. Mortality and time to hatch

At the time of egg collection, five fertilized eggs per tank were placed in a well of a 12-well plate containing 4 ml of 0.05% methylene blue. Each tank ($n = 10$) had nine replicate wells (45 embryos/larvae per tank) giving a total of 450 fertilized eggs per treatment group. To determine multigenerational effects of BaP on mortality and hatching in F1, F2, F3, and F4 generations, number of dead and hatched embryos and larvae were recorded at 24, 32, 48, 56, 72, 80, and 96 hpf.

2.6. Developmental deformities

At 96 hpf, photos were taken of a subset of 50 larvae per group to assess developmental deformities of F1, F2, and F3 generations. Five larvae per tank at a time were anesthetized in 300 mg/L tricaine methanesulfonate (MS-222) and 600 mg/L sodium bicarbonate. They were immediately placed on a microscope slide with a chamber containing 3% methyl cellulose and photos were captured with a MicroFire® camera (Optronics, Goleta, CA) attached to a Zeiss Stemi 2000-C Stereo Microscope (Jena, Germany) using Picture Frame™ Application 2.3 software (Optronics, Goleta, CA). Two photos were taken per fish: dorsal view and lateral view. Anatomical structures to determine morphological development were recorded as previously described by Brannen et al. (2010) and Panzica-Kelly et al. (2010) with modifications. Feature analysis included body length, body shape,

tail shape, pectoral fins, heart, swim bladder, abdomen, and craniofacial morphology (Supplementary Figure 1).

2.7. Image analysis

Blind to treatment measurements and scoring of the anatomical structures were recorded using ImageJ software (Schneider et al., 2012). First, the scale was set to the number of pixels per millimeter using a 1-mm micrometer. Then, the total body length along the spine and distance between the junctions separating the forebrain, midbrain and hindbrain were measured followed by the area (size) of the swim bladder, area of the pericardial and yolk sac edema when present, area and diameter of the optic vesicle (eye), and area of the otic vesicle. Scores were given to the structures following specific criteria (Supplemental Table 1).

2.8. Statistical analysis

Results were analyzed using GraphPad Prism 5.0 (La Jolla, CA) and presented as mean $\pm$ S.E. Data sets were first analyzed by the Kolmogorov-Smirnov test to determine if they were normally distributed. Mortality, hatching, and ordinal data of developmental deformities were analyzed using the 1-way ANOVA followed by Tukey's post hoc test or using Kruskal-Wallis followed by Dunn's post hoc test if the Kolmogorov-Smirnov test was not passed. Deformity incidence by treatment across score classifications was analyzed by 2-way ANOVA. Statistical significance was accepted at $p = 0.05$ for all tests.

3. Results

3.1. Overview of multigenerational BaP impacts

Mortality was significantly increased in the higher dose groups of BaP (2.3 and 20 μ g BaP/g fish) in the F1 generation (Fig. 1A) while there were no differences in the F2, F3, or F4 generations (Supplemental Fig. 2). Time to hatch in the higher doses significantly decreased in the F1 and F4 generations, but no differences were found in the F2 and F3 generations (Fig. 1B, Supplemental Fig. 2).

Multigenerational phenotypic impacts were caused in three generations (F1, F2, and F3) following a dietary challenge that corresponded to a parental, grandparental, and great-grandparental BaP exposure (Table 1). In summary, body morphology deformities were most extreme in the F1 generation although still present in the F2 generation and absent in the F3 generation. Craniofacial structures, although not significantly affected in the F1 generation, emerged as significant deformities in the F2 generation.

From this point on the parental 0.21, 2.3 and 20 μ g BaP/g fish treatment groups will be referred to as low dose, medium dose, and high dose groups, respectively.

3.2. Multigenerational BaP impacts on mortality

At 48 hpf, the F1 generation medium dose group showed a significant increase in percent mortality (55.2%) compared to control (27%) (Fig. 1A). Eight hours later (56 hpf), the medium and high dose groups had a significantly higher mortality incidence (57.7% and 54.2%, respectively) than control. There were no deaths in the control group after 48 hpf, while between 48 and 96 hpf the percent mortality increased only slightly in the BaP groups. The lowest BaP dose group was intermediate and not significantly different than control or higher BaP groups. In the F2, F3, and F4 generations there were no significant differences in percent mortality between the treatment groups (Supplemental Fig. 2 A, B, and C). In addition, the percent mortality for the control group at 48 hpf was consistently between 24 and 29% in the F1 – F4 generations. Percent mortality which was highest at 96 hpf in the

higher BaP groups decreased from 57.1% in F1 generation to ~24% in the F2 – F4 generations.

3.3. Multigenerational BaP impacts on time to hatch

In the F1 generation, the number of embryos that hatched at 48 and 56 hpf was significantly higher in the high dose group than in the F1 control and medium dose groups (Fig. 1B). That is, at 48 hpf, 25.2% more fish had hatched in the high dose group than control group, and 39.2% than in the medium dose group. This difference declined to ~17% at 56 hpf. The low BaP dose group was intermediate and not significantly different from either control or higher BaP dose groups. By 72 hpf, between 99 and 100% of the fish that were alive had hatched in all groups.

In the F2 and F3 generations, there were no differences in time to hatch (Supplemental Fig. 2 D and E). However, in the F4 generation, at 48 hpf there were significantly more fish that had hatched in the medium dose group than in the control and low BaP dose groups (Supplemental Fig. 2F).

3.4. Impact on larvae growth in the F1, F2, and F3 generations

Representative effects on body length of the parental BaP dietary exposure are provided in Fig. 2. In the F1 generation, body length significantly decreased from 3.8 ± 0.02 mm in the control group to 3.5 ± 0.1 mm in the medium and high dose groups (Fig. 3A). In the F2 generation, body length was not different between groups (Fig. 3A).

Moreover, the medium dose group which was significantly smaller in the F1 generation than control was significantly longer in the F2 generation (3.8 ± 0.04 mm) than the F1 medium dose group. Body length defects were restored in the F2 generation and remained restored in the F3 generation without significant differences between groups (Fig. 3A).

3.5. External morphological deformities

In the F1 generation, the same fish that were smaller also had a significantly less than normal body shape (Fig. 3B), tail shape (Fig. 3C), and pectoral fins (Fig. 3D) than F1 control except the high dose group that had a lower percent incidence of normal body shape than control (62% compared to 80%) but was not statistically different.

In the F2 generation, morphological effects such as body shape were still apparent in larvae whose grandparents were fed BaP (Fig. 2C and D), and in fact, dietary BaP exposure had an effect on body shape, tail shape and pectoral fins (Fig 3B, C, and D). Incidence of normal body shape was significantly lower in the F2 medium and higher BaP dose groups (82%) than in control (100%), and mild deformity incidence (score 3) was significantly higher (18%) in the F2 medium dose group than in control (Fig. 3B). The F2 high dose group was the only group with moderate (score 2) and severe (score 1) body shape deformities but their incidences were not statistically significant. The F2 high dose group also had a significantly lower incidence of normal tail shape and normal pectoral fins (Fig. 3C and D). Moreover, moderate and severe deformities were only observed in the medium and high dose groups.

In the F3 generation, body morphology of larvae whose great grandparents were exposed to BaP was not affected (Fig. 2E and F) based on no differences in body length (Fig. 3A) or incidence of body shape, tail shape, and pectoral fin deformities (Fig. 3B, C, and D).

3.6. Impacts on the heart, swim bladder and yolk sac

In the F1 generation, significantly fewer normal hearts (= absence of pericardial edema) were observed in the medium dose group than in the control and low dose groups (Fig. 4A).

This meant that 12% of the F1 larvae in the medium dose group had pericardial edema. The F1 medium dose larvae also had the largest size (Supplemental Fig. 3A) and highest incidence of severe edema (score = 1). Regarding swim bladder defects, the F1 larvae in the high dose group had significantly less normal swim bladders than the F1 larvae in control and low dose groups (Fig. 4B; Supplemental Fig. 3B). While the control and low dose F1 groups had zero incidence of yolk sac edema, the F1 larvae in the medium dose group had a significantly higher incidence (16%) than any group including the F1 high dose group that had a 4% incidence (Fig. 4C). The size of the yolk sac edema was not significantly different due to high variability (Supplemental Fig. 3C).

In the F2 generation, incidence of pericardial edema and yolk sac edema were not different between groups (Fig. 4A and C). Unexpectedly, the incidence of swim bladders not inflated was significantly higher in the F2 control (34%) than the F2 high dose group (6%) (Fig. 4B). It is worth noting that while the F2 high dose group had the lowest incidence of non-inflated (deflated) swim bladders, it had the highest incidence (not significant) of absent swim bladders.

Similar to the F2 generation, there were not significant differences in the incidence of pericardial edema or yolk sac edema in the F3 generation (Fig. 4A and C). Swim bladder deformities were not different between treatment groups, but remarkably, most of the larvae in all the groups had non-inflated swim bladders (Fig. 4B).

3.7. Craniofacial deformities

There were no significant differences in the scored incidence of craniofacial defects in the F1 generation (Fig. 5A, B, and C). The only statistically significant craniofacial deformity was a non-dose dependent decrease in optic vesicle size in the F1 medium dose group (Supplemental Fig. 4D).

In the F2 generation, normal brain incidence (score 4; Fig. 5A) was significantly lower in the high dose group (84.5%) than in control (100%). Normal incidence of upper face structures were also significantly lower (88%) in the F2 high dose group (Fig. 5B). Incidence of jaw deformities increased but was not significantly different between the F2 generation treatment groups (Score of 1; Fig. 5C).

In the F3 generation, incidence of brain deformities was not different between treatment groups (Fig. 5A), but when measuring the specific brain regions or structures some statistical differences were found but without clear dose-dependent relationships between sizes or lengths (Supplemental Fig. 4B, C and E). F3 upper face and lower face structures were mostly normal, and there were no significant differences in incidence (Fig. 5B and C). Therefore, at this time it is still preliminary to suggest that the subtle statistical changes measured in F3 brains are BaP-related.

4. Discussion

A parental (F0) BaP dietary exposure in zebrafish proved to be a successful model to evaluate subsequent multigenerational deformities in offspring (F1, F2, and F3 generations) that were never fed a BaP-treated diet (Table 1). Body morphology deformities (body or trunk, tail and pectoral fin shape) were the most severely impacted anatomical features in two generations (F1 and F2). In the F2 generation, craniofacial structures emerged as significant deformities. Developmental deformities in offspring never exposed to BaP indicate the potential for genetic and epigenetic mechanisms of toxicity. Now that multigenerational phenotypes are established, hypotheses can be made and tested as to the

molecular pathways that are altered by BaP multigenerational exposure. Some of the potential mechanisms involved in each type of developmental defect are highlighted below.

PAHs are taken up by aquatic organisms from the water or through the diet (Hylland, 2006). The environmental relevance of dietary exposure to PAHs is based on population level negative impacts that exposure to PAHs has via food webs (Peterson et al., 2003). Sediments represent a sink and source for total PAHs in the aquatic environment, and one review reported sediment concentrations ranging from 0.069–172 µg/g (Kimbrough and Dickhut, 2006).

Despite the environmentally relevant exposure route, laboratory dietary BaP exposure studies in fish are scarce. In one, zebrafish were fed ad libitum twice a day 0, 19, or 110 µg BaP/g diet for 260 days as part of a larger experiment to investigate effects on retinoids and reproduction (Alsop et al., 2007). BaP did not affect reproduction (number of eggs produced or fertilization success), and no deformities were observed in 36 hpf embryos. Inconsistencies in results between Alsop et al.'s experiment and the work in this study can be explained by fundamental differences in experimental design: for example, here fish were fed 10, 114, or 1012 µg BaP/g diet at a feed rate of 1% body weight twice/day for 21 days before collecting eggs. By contrast, they fed fish up to 110 µg BaP/g diet ad libitum for 245 days before collecting eggs. Moreover, they evaluated developmental deformities by “visual inspection” at 36 hpf at which time point development (organogenesis) is still occurring. Other effects of dietary BaP exposures to fish have been investigated (Couillard et al., 2009; Roesijadi et al., 2009; Yuen and Au, 2006), but none of these studies investigated offspring development or multigenerational effects.

With respect to developmental effects of PAHs, most previous studies in fish have utilized direct waterborne exposures of embryos to mixtures of PAHs (Garner and Di Giulio, 2012; Incardona et al., 2011; Carls and Meador, 2009; Barron et al., 2004; Colavecchia et al., 2004; Kocan et al., 1996). Here embryos were not directly exposed, and BaP was not detected in F1 embryos after parents were fed a BaP-treated diet for 21 days indicating that maternal deposition was potentially absent. Alternatively, if BaP was rapidly metabolized in the mothers, BaP metabolites could have been deposited in the embryos. In a *F. heteroclitus* study, ten embryos were pooled to determine BaP and BaP metabolite concentrations, and while BaP and BaP 9,10-dihydrodiol were recovered, all of the other metabolites (BaP-7,8,9,10-tetrahydrotetrol, BaP-7,8-dihydrodiol, BaP-1,6-dione, BaP-3,6-dione, BaP-6,12-dione, BaP-9OH and BaP-3-OH) remained below detection limits (Wills et al., 2009). Zebrafish embryos are smaller than *F. heteroclitus* embryos, and therefore, more than ten times as many zebrafish embryos were extracted, however, BaP was undetectable in our embryos. BaP metabolites were not analyzed here, and conclusions cannot be drawn about their presence or role in toxicity.

The negative impact on survival of fish embryos and larvae due to exposure to PAHs is well-documented in the literature. We found that mortality significantly increased in the F1 generation after parents (F0) had been fed medium and high doses of BaP. This result was consistent with previous findings that waterborne exposure to PAHs in embryos and larvae increased mortality (Carls and Thedinga, 2010; Barron et al., 2004; Hawkins et al., 2002; Carls et al., 1999). The same was true when zebrafish embryos were exposed to BaP alone (Fang et al., 2013; Bugiak et al., 2010). While we did not see a significant effect on mortality in the F2, F3, and F4 generations, 1 µg/L BaP negatively affected the survival in F2 fathead minnow embryos after a waterborne exposure (White et al., 1999).

Time to hatch after exposure to PAHs including BaP can either decrease (premature hatching) or increase (delayed hatching). It appears that the effect on time to hatch might be

PAH dose-dependent. In our study, we found that the number of embryos that hatched early in the F1 high dose group was significantly higher (premature hatching) than the control and medium dose groups. Because maternal BaP deposition from diet in F1 embryos was undetected, the embryo exposure may be most similar to lower PAH concentration exposures (waterborne or sediment) in embryos where time to hatch also decreased in Pacific herring, fathead minnow, and pink salmon embryos (Carls et al., 1999; Colavecchia et al., 2004; Carls and Thedinga, 2010). On the other hand, after direct exposure to higher PAH concentrations time to hatch increased (delayed hatching) (Colavecchia et al., 2004; Carls and Thedinga, 2010). Time to hatch also increased (delayed hatching) after a direct waterborne exposure to BaP alone (0.24 and 24 $\mu\text{g/L}$) (Fang et al., 2013). Mechanisms that control hatching include proteolytic activity of hatching enzymes, zinc metalloproteases, which are released from hatching gland cells (Inohoya et al., 1997). Zebrafish hatching depends on zebrafish hatching enzyme 1 (ZHE1) (Sano et al., 2008). The chorion consists of two primary glycoproteins, zona perlucida glycoproteins 2 and 3, and ZHE1 is responsible for cleaving them to soften the chorion which will then rupture by the embryo's contractile movements (Okada et al., 2010). Expression of ZHE1 was detected at 11.5 hpf and strongest at 24 hpf while undetectable after hatching (Sano et al., 2008). So, pollutants can potentially affect hatching by at least three mechanisms: downregulating ZHE1 expression, reducing ZHE1 activity, or disturbing the embryo's contractile movements. For example, metal oxide nanoparticles (CuO, ZnO, Cr₂O₃, and NiO) inhibited ZHE1 activity (Lin et al., 2013). In addition, trimethyltin chloride and perfluorooctanesulfonic acid stimulated the spontaneous tail movements at 20 and 25 hpf, respectively, however time to hatch was not determined (Chen et al., 2011; Huang et al., 2010a). Ultimately, changes in time to hatch are suggested to have long term effects on embryo fitness (Danzmann et al., 1989; Pakkasmaa and Jones, 2002). For example, embryos that hatch early could be developmentally behind and less fit for survival.

The developmental abnormalities presented here are consistent with those reported in PAH exposed fish. Developmental deformities such as growth reduction, spinal curvature (body and tail shape), pericardial edema, cardiac dysfunction, yolk sac edema, and craniofacial deformities significantly increased after embryonic exposure in Pacific herring, pink salmon, and zebrafish to mixtures of PAHs (Barron et al., 2004; Incardona et al., 2004). In this study, body and tail shape were the most severe deformities in F1 and F2 generations and may represent a relatively non-specific response to contaminants. For example, incidence of skeletal abnormalities such as spinal curvature is associated with high concentrations of heavy metals (Huang et al., 2010b), organophosphate pesticides (Jacobson et al., 2010), carbamate insecticides (DeMicco et al., 2010), pharmaceuticals (Parrot and Bennie, 2009), and PAHs (Danion et al., 2011). After exposure to triclosan, among the most frequently detected of pharmaceuticals and personal care products (PPCPs) in the aquatic environment worldwide, spinal curvature was reported in medaka, *Oryzias latipes* and zebrafish larvae (Nassef et al., 2010; Oliveira et al., 2009). Likewise, fathead minnow larvae showed spinal deformities after exposure to a mixture of PPCPs (100–300 ng/L) (Parrot and Bennie, 2009).

The molecular pathway(s) involved in the development of skeletal deformities due to environmental pollutants are only partly elucidated. The skeletal system is made up of both bone and cartilage tissues and confers multiple mechanical and metabolic functions, such as providing support site for muscle attachment, protecting vital organs (e.g., brain) or cells (e.g., bone marrow), and serving as a reserve of ions (Sims and Baron, 2000). The skeletal deformities reported in the zebrafish larvae in our manuscript were not differentiated between bone and cartilage tissue because determining changes in the rate of bone formation or ossification were not the goal of this study. In a previous study, calcified skeletal structures (bone tissue) were not detected by fluorescent signal in embryos until 120 hpf (Du et al., 2001). However, calcification of the cranium can occur in larvae 3 mm long and that

of the axial skeleton in larvae 3.8 mm long (Cubbage and Mabee 1996; Bird and Mabee 2003). In our study, the control larvae were on average 3.8 mm long in the F1 generation; the generation most impacted by the skeletal deformities. Therefore, it is possible that bone formation had started in the larvae in our study, and thus, skeletal deformities might have affected both bone and cartilage tissue.

Vertebrate bone can be cellular (osteocyte-containing bone) or acellular (osteocyte-deprived bone) (Witten et al., 2001). Osteocytes are present in bone of all tetrapod species from amphibians to reptiles, birds, and mammals. Fish, however, can have cellular or acellular bone and various stages between cartilage and bone and between bone and dentin depending on their phylogenetic lineage (Witten et al., 2001; Shahar and Dean, 2013). While zebrafish (lower Actinopterygii taxa) bone is primarily cellular, neoteleost (higher Actinopterygii taxa) bone is acellular (Kranenbarg et al., 2005). The molecular mechanisms involved in skeletal deformities due to pollutants involve an imbalance of mineral ions in both birds (Thompson et al., 2006) and fish (Danion et al., 2011). This indicates that mechanisms of action are conserved throughout vertebrates that have cellular or acellular bone, which might be indicative of a signaling deficit beyond osteoblasts. In mammalian embryos, TCDD delayed ossification (Sparschu et al., 1971, cited by Hart et al., 1991) and reduced calcium deposition in bone (Allen and Leamy, 2001). A similar mechanism of action is believed to be responsible for skeletal deformities in fish. Spinal curvature in European sea bass (*Dicentrarchus labrax*) was the result of vertebral bone demineralization during PAH exposure induced by disturbances of the phospho-calcic metabolism, which requires mobilization of mineral ions from the skeleton, and particularly the vertebrae (Danion et al., 2011). However, the genomic and epigenetic changes that would explain a spinal curvature across generations remain unknown.

In this study, incidence of normal hearts (no pericardial edema) was significantly lower in the F1 medium dose group. There are at least two potential reasons that the highest dose larvae did not have the highest incidence of edema. On one hand, the most impacted embryos at the highest dose may have already died by the 96 hpf assessment time point. Alternatively, the other skeletal defects in these larvae discussed previously could have masked the incidence or the ability to accurately assess pericardial edema. Over the long term, the middle BaP treatment dose group may be more representative of sub-lethal developmental abnormalities. Despite the higher effects in the middle dose group, cardiac deformities are prominent among PAH-induced defects (Garner and Di Giulio, 2012; Zhang et al., 2012; Bugiak and Weber, 2010; Billiard et al., 2006; Incardona et al., 2004). Additional model PAHs, such as β - and α -naphthoflavone, also caused an increase in the occurrence of pericardial edema in zebrafish (Billard et al., 2006), and cardiovascular defects were observed when co-exposure to BaP and α -naphthoflavone in zebrafish decreased ventricular length and chamber with increased ventricular wall thickness and increased blood vessel diameter (Bugiak and Weber, 2010). In an effort to understand the molecular mechanism involved in cardiotoxicity following BaP exposure, a morpholino knockdown of aryl hydrocarbon receptor 2 (*ahr2*) showed that an increase in pericardial edema size, bradycardia, and myocardia is *ahr2* dependent in zebrafish (Incardona et al., 2011). In contrast, benzo[k]fluoranthene caused severe pericardial edema and heart looping defects in an *ahr2*-independent manner (Incardona et al., 2011). Moreover, a morpholino knockdown of glutathione transferase pi class 2 (*GSTp2*) showed that *GSTp2* has a protective role in the occurrence of pericardial edema after co-exposures to BaP and fluoranthene and benzo[k]fluoranthene and fluoranthene (Garner and Di Giulio, 2012). In a microarray study, changes in cardiovascular gene expression triggered by the activation of the aryl hydrocarbon receptor preceded phenotypic cardiac deformities after exposure to a TCDD (Carney et al., 2006). All these studies focused on single-generation exposures,

therefore, the involvement of genomic changes in xenobiotic enzymes and the aryl hydrocarbon receptor in multigenerational studies need to be elucidated in future studies.

Swim bladder deformities (i.e., inflated, non-inflated, or absent) were significant in the F1 high dose group. Then, in the F3 generation most of the larvae in all treatment groups had non-inflated swim bladders, and this observation led us to conclude that phenotypic change in swim bladder might not serve well as an indicator of multigenerational contaminant exposure. In general, swim bladder deformities have not been characterized in exposures to PAHs in fish. However, non-inflated swim bladders were among the morphological defects observed in zebrafish after a dietary exposure to the endocrine disruptor TCDD (0.08–2.16 ng TCDD/fish/day) (King-Heiden et al., 2005). Swim-up behavior is critical for larvae to first inflate their swim bladder, decrease body density, and attain neutral buoyancy (Robertson et al., 2007). Follow-up studies are needed to anchor swim bladder phenotypic deformities to a molecular mechanism of action that might elucidate potential effects to the nervous, muscular, and/or vascular systems.

A possible explanation for the lack of significance in craniofacial abnormalities in the F1 generation, where mortality was highest and fish had the most severely deformed bodies, is that those embryos with brain deformities did not survive, as was suggested to explain the non-dose dependence of cardiac edema. A 7-day waterborne embryo exposure to BaP (0–1.4 µg/L) increased incidence of neurocranial and craniofacial skeletal defects in *S. marmoratus* (He et al., 2011). Craniofacial deformities also were documented in early life stages of rainbow trout directly exposed to 100 µg/L phenanthrene or 100 µg/L retene (Hawkins et al., 2002). Molecular results indicated down-regulation in expression of Sonic hedgehog, which plays an important role in the proliferation of chondrocytes, and thus bone formation (He et al., 2011). After a parental and continued embryonic waterborne BaP exposure, changes in the transcriptome of 96 hpf zebrafish were determined in a previous preliminary study by the authors (In preparation). Pathway analysis results of differentially expressed genes in the transcriptome of the exposed zebrafish identified the reelin signaling in neurons pathway to be impacted; however, brain deformities were not assessed in the embryos. Reelin is linked to neurological disorders in mammals such as Alzheimer's and schizophrenia (Raber et al., 2004; Guidotti et al., 2000), but we speculate that reelin also may be linked to disorders in the central nervous system in fish. Lead is detrimental to the developing nervous system, and thus, lead exposures are good models to study brain function. For example, in lead-exposed zebrafish reelin expression was altered, but brain morphology was unaltered (Peterson et al., 2013). In the dietary exposure to BaP presented here, brain deformities were not observed until the F2 generation; however, it is possible that genes important to the nervous system such as reelin were already altered in the F1 generation. It will also be interesting to investigate how genetic changes correlate with brain deformities.

The principal result of this experiment, multigenerational adverse effects on developmental phenotypes, invites reevaluating the priority of multigenerational studies which more closely reflect deleterious impacts at an ecosystem level. On the contrary, single generation studies harbor organismal level impacts alone. Here, we showed for the first time that a parental dietary exposure alone can cause development deformities in fish two generations removed from the exposure. In addition, the environmental relevance of these findings on populations is potentially compounded by the interactive effects that the many classes of pollutants can have on aquatic organisms. Future results from this dietary exposure are directed to anchoring phenotypic deformities to genotypic changes such as changes in the transcriptome and methylome, which will allow us to develop adverse outcome pathways from the molecular level in an individual to phenotypic effects across multiple generations.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We wish to thank graduate and undergraduate students Xiefan Fang, Frank Booc, Khalid Alharthy, Adam Hawkins, Kathryn Mislan, Mariane Landim Silva, and Jonathan Huwe for their critical role in assisting with fish husbandry duties during the exposure, experimental take-down, molecular laboratory work, and data collection. Research reported in this publication was supported by the National Institute of Environmental Health Sciences of the National Institutes of Health under award number: R21ES019940. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health.

References

- Allen DE, Leamy LJ. 2,3,7,8-Tetrachlorodibenzo-p-dioxin affects size and shape but not asymmetry of mandibles in mice. *Ecotoxicology*. 2001; 10:167–176. [PubMed: 11383474]
- Alsop D, Brown S, Van Der Kraak G. The effects of copper and benzo[a]pyrene on retinoids and reproduction in zebrafish. *Aquatic Toxicology*. 2007; 82:281–295. [PubMed: 17433458]
- Bailey GS, Reddy AP, Pereira CB, Harttig UI, Baird W, Spitsbergen JM, Hendricks JD, Orner GA, Williams DE, Swenberg JA. Nonlinear Cancer Response at Ultralow Dose: A 40800-Animal ED001 Tumor and Biomarker Study. *Chemical Research in Toxicology*. 2009; 22:1264–1276. [PubMed: 19449824]
- Barron MG, Carls MG, Heintz R, Rice SD. Evaluation of fish early life-stage toxicity models of chronic embryonic exposures to complex polycyclic aromatic hydrocarbon mixtures. *Toxicological Sciences*. 2004; 78:60–67. [PubMed: 14691206]
- Berg V, Lyche JL, Karlsson C, Stavik B, Nourizadeh-Lillabadi R, Hårdnes N, Skaare JU, Alestrøm P, Lie E, Ropstad E. Accumulation and effects of natural mixtures of persistent organic pollutants (POP) in zebrafish after two generations of exposure. *Journal of Toxicology and Environmental Health, Part A*. 2011; 74:407–423. [PubMed: 21391088]
- Bird NC, Mabee PM. Developmental morphology of the axial skeleton of the zebrafish, *Danio rerio* (Ostariophysi: Cyprinidae). *Dev. Dynam.* 2003; 228:337–357.
- Billiard SM, Timme-Laragy AR, Wassenberg DM, Cockman C, Di Giulio RT. The role of the aryl hydrocarbon receptor pathway in mediating synergistic developmental toxicity of polycyclic aromatic hydrocarbons to zebrafish. *Toxicological Sciences*. 2006; 92:526–536. [PubMed: 16687390]
- Boström CE, Gerde P, Hanberg A, Jernström B, Johansson C, Kyrklund T, Rannug A, Törnqvist M, Victorin K, Westerholm R. Cancer risk assessment, indicators, and guidelines for polycyclic aromatic hydrocarbons in the ambient air. *Environmental Health Perspectives*. 2002; 110:451–488. [PubMed: 12060843]
- Brannen KC, Panzica-Kelly JM, Danberry TL, Augustine-Rauch KA. Development of a zebrafish embryo teratogenicity assay and quantitative prediction model. *Birth Defects Res. B*. 2010; 89:66–77.
- Bugiak BJ, Weber LP. Phenotypic anchoring of gene expression after developmental exposure to aryl hydrocarbon receptor ligands in zebrafish. *Aquatic Toxicology*. 2010; 99:423–437. [PubMed: 20615557]
- Carls MG, Meador JP. A perspective on the toxicity of petrogenic PAHs to developing fish embryos related to environmental chemistry. *Human and Ecological Risk Assessment: An International Journal*. 2009; 15:1084–1098.
- Carls MG, Rice SD, Hose JE. 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 pallasii*). *Environmental Toxicology and Chemistry*. 1999; 18:481–493.
- Carls MG, Thedinga JF. Exposure of pink salmon embryos to dissolved polynuclear aromatic hydrocarbons delays development, prolonging vulnerability to mechanical damage. *Marine Environmental Research*. 2010; 69:318–325. [PubMed: 20089299]

- Carney SA, Chen J, Burns CG, Xiong KM, Peterson RE, Heideman W. Aryl hydrocarbon receptor activation produces heart-specific transcriptional and toxic responses in developing zebrafish. *Molecular Pharmacology*. 2006; 70:549–561. [PubMed: 16714409]
- Chen J, Huang C, Zheng L, Simonich M, Bai C, Tanguay R, Dong Q. Trimethyltin chloride (TMT) neurobehavioral toxicity in embryonic zebrafish. *Neurotoxicology and Teratology*. 2011; 33:721–726. [PubMed: 21964161]
- Chou CT, Hsiao YC, Ko FC, Cheng JO, Cheng YM, Chen TH. Chronic exposure of 2,2',4,4'-tetrabromodiphenyl ether (PBDE-47) alters locomotion behavior in juvenile zebrafish (*Danio rerio*). *Aquatic Toxicology*. 2010; 98:388–395.
- Colavecchia MV, Backus SM, Hodson PV, Parrott JL. Toxicity of oil sands to early life stages of fathead minnows (*Pimephales promelas*). *Environmental Toxicology and Chemistry*. 2004; 23:1709–1718. [PubMed: 15230323]
- Couillard CM, Laplante B, Pelletier E. A fish bioassay to evaluate the toxicity associated with the ingestion of benzo[a]pyrene-contaminated benthic prey. *Environmental Toxicology and Chemistry*. 2009; 28:772–782. [PubMed: 19391678]
- Cubbage CC, Mabee PM. Development of the cranium and paired fins in the zebrafish *Danio rerio* (Ostariophysi, Cyprinidae). *Journal of Morphology*. 1996; 229:121–160.
- Danion M, Deschamps MH, Thomas-Guyon H, Bado-Nilles A, Le Floch S, Quentel C, Sire JY. Effect of an experimental oil spill on vertebral bone tissue quality in European seabass (*Dicentrarchus labrax* L.). *Ecotoxicology and Environmental Safety*. 2011; 74:1888–1895. [PubMed: 21831432]
- Danzmann RG, Ferguson MM, Allendorf FW. Genetic variability and components of fitness in hatchery strains of rainbow trout. *Journal of Fish Biology*. 1989; 35(Supplement A):313–319.
- DeMicco A, Cooper KR, Richardson JR, White LA. Developmental neurotoxicity of pyrethroid insecticides in zebrafish embryos. *Toxicological Sciences*. 2010; 113:177–186. [PubMed: 19861644]
- Du SJ, Frenkel V, Kindschi G, Zohar Y. Visualizing normal and defective bone development in zebrafish embryos using the fluorescent chromophore calcein. *Developmental Biology*. 2001; 238:239–246. [PubMed: 11784007]
- Fang X, Thornton C, Scheffler BE, Willett KL. Benzo[a]pyrene decreases global and gene specific DNA methylation during zebrafish development. *Environmental Toxicology and Pharmacology*. 2013; 36:40–50. [PubMed: 23542452]
- Garner LVT, Di Giulio RT. Glutathione transferase pi class 2 (GSTp2) protects against the cardiac deformities caused by exposure to PAHs but not PCB-126 in zebrafish embryos. *Comparative Biochemistry and Physiology, Part C*. 2012; 155:573–579. [PubMed: 22269188]
- Guidotti A, Auta J, Davis JM, Di-Giorgi-Gerevini V, Dwivedi Y, Grayson DR. Decrease in reelin and glutamic acid decarboxylase67 (GAD67) expression in schizophrenia and bipolar disorder: a postmortem brain study. *Archives of General Psychiatry*. 2000; 57:1061–1069. [PubMed: 11074872]
- Halden AN, Arnoldsson K, Haglund P, Mattsson A, Ulleras E, Sturve J, Norrgren L. Retention and maternal transfer of brominated dioxins in zebrafish (*Danio rerio*) and effects on reproduction, aryl hydrocarbon receptor-regulated genes, and ethoxyresorufin-O-deethylase (EROD) activity. *Aquatic Toxicology*. 2011; 102:150–161. [PubMed: 21356177]
- Hart LE, Cheng KM, Whitehead PE, Shah RM, Lewis RJ, Ruschkowski SR, Blair RW, Bennett DC, Bandiera SM, Norstrom RJ, Bellward GD. Dioxin contamination and growth and development in great blue heron embryos. *Journal of Toxicology and Environmental Health*. 1991; 32:331–344. [PubMed: 2002515]
- Hawkins SA, Billiard SM, Tabash SP, Brown RS, Hodson PV. Altering cytochrome P4501A activity affects polycyclic aromatic hydrocarbon metabolism and toxicity in rainbow trout (*Oncorhynchus mykiss*). *Environmental Toxicology and Chemistry*. 2002; 21:1845–1853. [PubMed: 12206424]
- He C, Zuo Z, Shi X, Li R, Chen D, Huang X, Chen Y, Wang C. Effects of benzo(a)pyrene on the skeletal development of *Sebastiscus marmoratus* embryos and the molecular mechanism involved. *Aquatic Toxicology*. 2011; 101:335–341. [PubMed: 21216343]

- Holdway DA, Heffernan J, Smith A. Multigeneration assessment of nonylphenol and endosulfan using a model Australian freshwater fish, *Melanotaenia fluviatilis*. *Environmental Toxicology*. 2008; 23:253–262. [PubMed: 18214909]
- Huang H, Huang C, Wang L, Ye X, Bai C, Simonich MT, Tanguay RL, Dong Q. Toxicity, uptake kinetics and behavior assessment in zebrafish embryos following exposure to perfluorooctanesulphonic acid (PFOS). *Aquatic Toxicology*. 2010a; 98:139–147. [PubMed: 20171748]
- Huang W, Cao L, Shan X, Xiao Z, Wang Q, Dou S. Toxic effects of zinc on the development, growth, and survival of red sea bream *Pagrus major* embryos and larvae. *Archives of Environmental Contamination and Toxicology*. 2010b; 58:140–150. [PubMed: 19504147]
- Hylland K. Polycyclic aromatic hydrocarbon (PAH) ecotoxicology in marine ecosystems. *Journal of Toxicology and Environmental Health, Part A*. 2006; 69:109–123. [PubMed: 16291565]
- Incardona JP, Carls MG, Teraoka H, Sloan CA, Collier TK, Scholz NL. Aryl hydrocarbon receptor-independent toxicity of weathered crude oil during fish development. *Environmental Health Perspectives*. 2005; 113:1755–1762. [PubMed: 16330359]
- Incardona JP, Collier TK, Scholz NL. Defects in cardiac function precede morphological abnormalities in fish embryos exposed to polycyclic aromatic hydrocarbons. *Toxicology and Applied Pharmacology*. 2004; 196:191–205. [PubMed: 15081266]
- Incardona JP, Day HL, Collier TK, Scholz NL. Developmental toxicity of 4-ring polycyclic aromatic hydrocarbons in zebrafish is differentially dependent on AH receptor isoforms and hepatic cytochrome P4501A metabolism. *Toxicology and Applied Pharmacology*. 2006; 217:308–321. [PubMed: 17112560]
- Incardona JP, Lindo TL, Scholz NL. Cardiac toxicity of 5-ring polycyclic aromatic hydrocarbons is differentially dependent on the aryl hydrocarbon receptor 2 isoform during zebrafish development. *Toxicology and Applied Pharmacology*. 2011; 257:242–249. [PubMed: 21964300]
- Inohoya K, Yasumasu S, Araki K, Naruse K, Yamazaki K, Yasumasu I, Iuchi I, Yamagami K. Species-dependent migration of fish hatching gland cells that commonly express astacin-like proteases in common. *Development, Growth and Differentiation*. 1997; 39:191–197.
- Jacobson SM, Birkholz DA, McNamara ML, Bharate SB, George KM. Subacute developmental exposure of zebrafish to the organophosphate pesticide metabolite, chlorpyrifos-oxon, results in defects in Rohon-Beard sensory neuron development. *Aquatic Toxicology*. 2010; 100:101–111. [PubMed: 20701988]
- Kimbrough KL, Dickhut RM. Assessment of polycyclic aromatic hydrocarbon input to urban wetlands in relation to adjacent land use. *Marine Pollution Bulletin*. 2006; 52:1355–1363. [PubMed: 16797601]
- King-Heiden T, Hutz RJ, Carvan MJ III. Accumulation, tissue distribution, and maternal transfer of dietary 2,3,7,8-tetrachlorodibenzo-*p*-dioxin: Impacts on reproductive success of zebrafish. *Toxicological Sciences*. 2005; 87:497–507. [PubMed: 15901917]
- Kocan RM, Hose JE, Brown ED, Baker TT. Pacific herring (*Clupea pallasii*) embryo sensitivity to Prudhoe Bay petroleum hydrocarbons: laboratory evaluation and in situ exposure at oiled and unoiled sites in Prince William Sound. *Canadian Journal of Fisheries and Aquatic Sciences*. 1996; 53:2366–2375.
- Kranenbarg S, van Cleynenbreugel T, Schipper H, van Leeuwen J. Adaptive bone formation in acellular vertebrate of sea bass (*Dicentrarchus labrax* L.). *The Journal of Experimental Biology*. 2005; 208:3493–3502. [PubMed: 16155222]
- Latimer, JS.; Zheng, J. The sources, transport, and fate of PAHs in the marine environment. In: Douben, PET., editor. *PAHs: An ecotoxicological perspective*. John Wiley & Sons; West Sussex, UK: 2003. p. 9-33.
- Lin S, Zhao Y, Ji Z, Ear J, Chang CH, Zhang H, Low-Cam C, Yamada K, Meng H, Wang X, Liu R, Pokhrel S, Mädler L, Damoiseaux R, Xia T, Godwin HA, Lin S, Nel AE. Zebrafish high-throughput screening to study the impact of dissolvable metal oxide nanoparticles on the hatching enzyme, ZHE1. *Small*. 2013; 9:1776–1785. [PubMed: 23180726]
- Nassef M, Kim SG, Seki M, Kang IJ, Hano T, Shimasaki Y, Oshima Y. *Chemosphere*. 2010; 79:966–973. [PubMed: 20207391]

- Nourizadeh-Lillabadi R, Lyche JL, Almaas C, Stavik B, Moe SJ, Aleksandersen M, Berg V, Jakobsen KS, Stenseth NC, Skare JU, Alestrøm P, Ropstad E. Transcriptional regulation in liver and testis associated with developmental and reproductive effects in male zebrafish exposed to natural mixtures of persistent organic pollutants (POP). *Journal of Toxicology and Environmental Health, Part A*. 2009; 72:112–130. [PubMed: 19184727]
- Okada A, Sano K, Nagata K, Yasumasu S, Ohtsuka J, Yamamura A, Kubota K, Iuchi I, Tanokura M. Crystal structure of zebrafish hatching enzyme 1 from the zebrafish *Danio rerio*. *Journal of Molecular Biology*. 2010; 402:865–878. [PubMed: 20727360]
- Oliveira R, Domingues I, Grisolia CK, Soares AMVM. Effects of triclosan on zebrafish early-life stages and adults. *Environmental Science and Pollution Research*. 2009; 16:679–688. [PubMed: 19283420]
- Pakkasmaa S, Jones M. Individual-level analysis of early life history traits in hatchery-reared lake trout. *Journal of Fish Biology*. 2002; 60:218–225.
- Panzica-Kelly JM, Zhang CX, Danberry TL, Flood A, DeLan JW, Brannen KC, Augustine-Rauch KA. Morphological score assignment guidelines for the dechorionated zebrafish teratogenicity assay. *Birth Defects Res. B*. 2010; 89:382–395.
- Parrott JL, Bennie DT. Life-cycle exposure of fathead minnows to a mixture of six common pharmaceuticals and triclosan. *Journal of Toxicology and Environmental Health, Part A*. 2009; 72:633–641. [PubMed: 19308848]
- Parrott JL, Hewitt LM, Kovacs TG, MacLatchy DL, Martel PH, van den Heuvel MR, Van Der Kraak GJ, McMaster ME. Responses in a fathead minnow (*Pimephelas promelas*) lifecycle test and in wild white sucker (*Catostomus commersoni*) exposed to a Canadian bleached kraft mill effluent. *Water Quality Research Journal of Canada*. 2010; 45:187–200.
- Peterson CH, Rice SD, Short JW, Esler D, Bodkin JL, Ballachey BE, Irons DB. Long-term ecosystem response to the Exxon Valdez oil spill. *Science*. 2003; 302:2082–2086. [PubMed: 14684812]
- Peterson SM, Zhang J, Freeman JL. Developmental reelin expression and time point-specific alterations from lead exposure in zebrafish. *Neurotoxicology and Teratology*. 2013; 38:53–60. [PubMed: 23665418]
- Raber J, Huang Y, Ashford JW. ApoE genotype accounts for the vast majority of AD risk and AD pathology. *Neurobiology of Aging*. 2004; 25:641–50. [PubMed: 15172743]
- Robertson GN, McGee CAS, Dumbarton TC, Croll RP, Smith FM. Development of the swimbladder and its innervation in the zebrafish, *Danio rerio*. *Journal of Morphology*. 2007; 268:967–985. [PubMed: 17702001]
- Roesijadi G, Rezvankhah S, Perez-Matus A, Mitelberg A, Torruellas K, Van Veld PA. Dietary cadmium and benzo(a)pyrene increased intestinal metallothionein expression in the fish *Fundulus heteroclitus*. *Marine Environmental Research*. 2009; 67:25–30. [PubMed: 19046598]
- Sano K, Inohaya K, Kawaguchi M, Yoshizaki N, Iuchi I, Yasumasu S. Purification and characterization of zebrafish hatching enzyme; an evolutionary aspect of the mechanism of egg envelop digestion. *The FEBS Journal*. 2008; 275:5934–5946. [PubMed: 19021768]
- Schneider CA, Rasband WS, Eliceiri KW. NIH Image to ImageJ: 25 years of image analysis. *Nat. Methods*. 2012; 9:671–675. [PubMed: 22930834]
- Shahar R, Dean MN. The enigmas of the bone without osteocytes. *BoneKey Reports Article*. 2013; 343:1–9.
- Sims, N.; Baron, C. Bone cells and their function. In: Canalis, E., editor. *Skeletal Growth Factors*. Lippincott Williams and Wilkins; Philadelphia, PA: 2000. p. 1-16.
- Sipes NS, Padilla S, Knudsen TB. Zebrafish – as an integrative model for twenty-first century toxicity testing. *Birth Defects Research Part C*. 2011; 93:256–267.
- Sohoni P, Tyler CR, Hurd K, Caunter J, Hetheridge M, Williams T, Woods C, Evans M, Toy R, Gargas M, Sumpter JP. Reproductive effects of long-term exposure to bisphenol A in the fathead minnow (*Pimephales promelas*). *Environmental Science and Technology*. 2001; 35:2917–2925. [PubMed: 11478243]
- Staples CA, Hall AT, Friederich U, Caspers N, Klecka GM. Early life-stage and multigeneration toxicity study with bisphenol A and fathead minnows (*Pimephales promelas*). *Ecotoxicology and Environmental Safety*. 2011; 74:1548–1557. [PubMed: 21700340]

- Thompson HM, Fernandes A, Rose M, White S, Blackburn A. Possible chemical causes of skeletal deformities in grey heron nestlings (*Ardea cinerea*) in North Nottinghamshire, UK. *Chemosphere*. 2006; 65:400–409. [PubMed: 16545424]
- Wassenberg DM, Di Giulio RT. Synergistic embryotoxicity of polycyclic aromatic hydrocarbon aryl hydrocarbon receptor agonist with cytochrome P4501A inhibitors in *Fundulus heteroclitus*. *Environmental Health Perspectives*. 2004; 112:1658–1664. [PubMed: 15579409]
- White PA, Robitaille S, Rasmussen JB. Heritable reproductive effects of benzo[a]pyrene on the fathead minnow (*Pimephales promelas*). *Environmental Toxicology and Chemistry*. 1999; 18:1843–1847.
- Wills LP, Zhu S, Willett KL, Di Giulio RT. Effect of CYP1A inhibition on the biotransformation of benzo[a]pyrene in two populations of *Fundulus heteroclitus* with different exposure histories. *Aquatic Toxicology*. 2009; 92:195–201. [PubMed: 19269699]
- Witten PE, Hansen A, Hall BK. Features of mono- and multinucleated bone resorbing cells of the zebrafish *Danio rerio* and their contribution to skeletal development, remodeling, and growth. *Journal of Morphology*. 2001; 250:197–207. [PubMed: 11746460]
- Yuen BBH, AU DWT. Temporal changes of ethoxyresorufin-O-deethylase (EROD) activities and lysosome accumulation in intestine of fish on chronic exposure to dietary benzo[a]pyrene: Linking EROD induction to cytological effects. *Environmental Toxicology and Chemistry*. 2006; 25:2593–2600. [PubMed: 17022398]
- Zha J, Sun L, Zhou Y, Spear PA, Ma M, Wang Z. Assessment of 17 α -ethinylestradiol effects and underlying mechanisms in a continuous, multigeneration exposure of the Chinese rare minnow (*Gobiocypris rarus*). *Toxicology and Applied Pharmacology*. 2008; 226:298–308. [PubMed: 18054974]
- Zhang Y, Wang C, Huang L, Chen R, Chen Y, Zuo Z. Low-level pyrene exposure causes cardiac toxicity in zebrafish (*Danio rerio*) embryos. *Aquatic Toxicology*. 2012; 114–115:119–124.

Highlights

- Parental dietary benzo[a]pyrene exposure caused deformities in offspring.
- Non-exposed F1, F2, and F3 generations had developmental deficits.
- Body morphology (skeletal deformity) was the most severe in F1 and F2 generations.
- Craniofacial deformities emerged in the F2 generations.

Figure 1.

Mortality and percent hatching in F1 generation following a parental (F0) dietary exposure of BaP. At time 0, 45 fertilized eggs per tank (10 tanks/treatment group) were randomly selected. Number of dead embryos and larvae and number of hatched larvae were documented at 24, 32, 48, 56, 72, 80, and 96 hpf. Percent cumulative mortality (A) and percent cumulative hatching (B) were calculated. Different letters represent significant differences between treatment groups at each time point (ANOVA, $p < 0.05$).

Figure 2.

Representative photos of 96 hpf larvae documenting developmental deformities across F1, F2, and F3 generations after a parental (F0) dietary exposure of BaP (n=50 fish per treatment group). Dorsal view photos (A, C, and E) are useful for evaluating pectoral fin deformities (A, red arrow 0.21 BaP). Body length and body shape defects were apparent in F1 and F2 generations (A, B, C, and D). Optic vesicle (eye) size (A, red arrowhead 20 BaP), brain size (E, red arrowhead), pigmentation (E, red arrow), absent jaw (F, red arrowhead), and absent otolith (F, red arrow) are examples of the deformities observed.

Figure 3.

Body morphology deformities. Body length was measured and compared within and across generations (A). Percent incidence of deformities in body shape (B), tail shape (C), and pectoral fins (D) were compared within each generation. N = 10 tanks per treatment group; 5 larvae per tank. Bars represent the mean $\pm$ SE. Different letters indicate significant differences (two-way ANOVA, $p < 0.05$). For body length (A), the bars that have no letters indicate that they were not different from any treatment group or generation. For body shape, tail shape, and pectoral fins, score 4 = normal, 3 = mild deformity, 2 = moderate deformity, and 1 = severe deformity.

Figure 4.

Impacts on the heart, swim bladder, and yolk sac. Percent incidence of pericardial edema affecting the heart (A), swim bladder size or absence (B), and yolk sac edema (C) was determined for each treatment group in F1, F2, and F3 generations. For pericardial edema and swim bladder, score 4 = normal, 3 = mild deformity, 2 = moderate edema or swim bladder not inflated, and 1 = severe edema or swim bladder absent. N = 10 tanks per treatment group; 5 larvae per tank. Bars represent the mean $\pm$ SE. Different letters indicate significant differences between groups per score category (A and B, two-way ANOVA, $p < 0.05$) or significant differences between groups (C, Kruskal-Wallis, $p < 0.05$).

Figure 5.

Craniofacial structure deformities. Percent incidence of larvae having brain (A), upper face (B), and lower face (C) deformities was calculated. Brain deformities included enlargement or reduction of the brain regions or absence of brain junctions. Upper face included optic vesicle (eye) and otic vesicle deformities, and lower face deformities comprised of enlargement, reduction, irregular shape, or absence of the upper and lower jaws. Score 4 = normal, 3 = mild deformity, 2 = moderate deformity, and 1 = severe deformity. N = 10 tanks per treatment group; 5 larvae per tank. Bars represent the mean $\pm$ SE. Different letters indicate significant differences between groups per score category (two-way ANOVA, $p < 0.05$).

Table 1

Summary of developmental deformities observed at 96 hpf across F1, F2, and F3 generations. (+) significant change, (–) no significant change.

Anatomical feature	F1	F2	F3*
Body length	+	–	–
Body shape	+	+	–
Tail shape	+	+	–
Pectoral fins	+	+	–
Heart			
% incidence of edema	+	–	–
pericardial edema size	–	–	–
Swim bladder			
% deformity incidence	+	+	–
size	–	–	–
Yolk sac			
% incidence of edema	+	–	–
edema size	–	–	–
Craniofacial structures			
Brain			
% deformity incidence	–	+	–
forebrain length	–	–	+
midbrain length	–	+	+
hindbrain length	–	–	–
Upper face			
% deformity incidence	–	+	–
optic vesicle size	+	–	–
optic vesicle diameter	–	–	–
otic vesicle size	–	–	+
Jaw			
% deformity incidence	–	–	–

* F3 craniofacial deformities were not dose-dependent.