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Knockdown of AHR1A but not AHR1B exacerbates PAH and PCB-126 toxicity in zebrafish (*Danio rerio*) embryos

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Abstract

Various environmental contaminants are known agonists for the aryl hydrocarbon receptor (AHR), which is highly conserved across vertebrate species. Due to gene duplication events before and after the divergence of ray- and lobe-finned fishes, many teleosts have multiple AHR isoforms. The zebrafish (*Danio rerio*) has three identified AHRs: AHR1A and AHR1B, the roles of which are not yet well elucidated, and AHR2, which has been shown to mediate the toxicity of various anthropogenic compounds including dioxins, polychlorinated biphenyls (PCBs), and polycyclic aromatic hydrocarbons (PAHs). In this study, we sought to explore the role of the two AHR1 isoforms in PAH- and PCB-induced toxicity in zebrafish embryos utilizing morpholino gene knockdown of the AHR isoforms. Knockdown of AHR1B did not affect the toxicity of PAH mixtures or PCB-126, whereas knockdown of AHR1A exacerbated the cardiac toxicity caused by PAH mixtures and PCB-126. Knockdown of AHR1A did not impact the mRNA expression of CYP1A, CYP1B1, and CYP1C1 in exposed embryos, but it did result in increased CYP1 activity in exposed embryos. As has been shown previously, knockdown of AHR2 resulted in protection from PAH- and PCB-induced cardiac deformities and prevented CYP1 enzyme activity in exposed embryos. Co-knockdown of AHR1A and AHR2 resulted in an intermediate response compared to knockdown of AHR1A and AHR2 individually; co-knockdown did not exacerbate nor protect from PAH-induced deformities and embryos exhibited an intermediate CYP1 enzyme activity response. In contrast, co-knockdown of AHR1A and AHR2 did protect from PCB-126-induced deformities. These results suggest that AHR1A is not a nonfunctional receptor as previously thought and may play a role in the normal physiology of zebrafish during development and/or the toxicity of environmental contaminants in early life stages.

Keywords

Aryl hydrocarbon receptor; polycyclic aromatic hydrocarbon; PCB-126; zebrafish; cardiotoxicity; morpholino

1. Introduction

Environmental contaminants such as 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), coplanar non-ortho-substituted polychlorinated biphenyls (PCBs), and certain polycyclic aromatic

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hydrocarbons (PAHs) are known agonists for the aryl hydrocarbon receptor (AHR) (reviewed in Schmidt and Bradfield, 1996). Unlike TCDD and PCBs, the concentration of PAHs in aquatic environments is increasing in the environment due to increased urbanization and run-off (Van Metre and Mahler, 2005). Fish early life stages are particularly sensitive to TCDD, PCB, and PAH toxicity, and the developing heart is a target organ for the toxicity of all three classes of compounds. Early life exposure to these compounds has been shown to result in cardiac deformities including reduced cardiomyocyte number, impaired blood flow, pericardial effusion, and an elongated atrium that results in the “stringy heart phenotype” in various fish species (Carls et al., 2008; Peterson et al., 1993; Scott and Hodson, 2008; Wassenberg and Di Giulio, 2004).

The AHR is a member of the basic-helix-loop-helix per-ARNT-SIM (bHLH-PAS) family of transcription factors. It is constitutively present in the cytoplasm and is bound by various cofactors including HSP90 (heat shock protein 90) and XAP2/AIP (X-associated protein 2/ AHR-interacting protein) (Hahn, 2002). Upon ligand binding by an agonist, the AHR dissociates from its cofactors, translocates into the nucleus, and forms a heterodimer with the aryl hydrocarbon receptor nuclear translocator (ARNT), which is also a member of the bHLH-PAS family. The AHR-ARNT heterodimer then binds to xenobiotic response elements (XREs) and upregulates gene expression (Hankinson, 1995; Schmidt and Bradfield, 1996). Amongst the genes upregulated by the AHR, cytochrome P450 (CYP) 1A is well characterized, highly inducible, and often used as a marker of AHR agonism. The AHR also upregulates other phase I and II metabolic enzymes including various other CYPs, aldehyde dehydrogenase 3, NAD(P)H:quinone oxidoreductase (Nqo1), glutathione S-transferases (GSTs), and UDP-glucuronosyltransferases (UDPGTs) (Denison and Nagy, 2003; Nebert et al., 2000). Additionally, the AHR upregulates the aryl hydrocarbon receptor repressor (AHRR), which forms a negative feedback loop by binding to XREs, competing with the AHR, and downregulating the pathway (Karchner et al., 2002).

One AHR has been identified in mammals and has been shown to mediate many of the toxic effects of TCDD and PAHs; AHR deficient mice are protected from the developmental toxicity induced by TCDD and the carcinogenicity of benzo[a]pyrene (BaP) (Fernandez-Salguero et al., 1996; Shimizu et al., 2000). Three AHRs (AHR1A, AHR1B, and AHR2) have been identified in zebrafish (*Danio rerio*) (Andreasen et al., 2002; Karchner et al., 2005; Tanguay et al., 1999). AHR2 has been shown to mediate the toxicity of TCDD, PCB, and certain PAHs (Billiard et al., 2006; Incardona et al., 2006; Prasch et al., 2003; Van Tiem and Di Giulio, 2011), but the roles of the AHR1 isoforms are not well elucidated. AHR1B has moderate affinity for TCDD but does not appear to be inducible by TCDD, while AHR1A is not capable of binding TCDD or mediating TCDD-dependent gene expression and also appears to have a nonfunctional transactivation domain (Fraccalvieri et al., 2013; Karchner et al., 2005). Yet Incardona *et al.* (2006) demonstrated that knockdown of AHR1A was slightly protective against pyrene-induced developmental toxicity in developing zebrafish, delaying the onset of liver abnormalities and pericardial edema, suggesting that AHR1A is capable of interacting with certain PAHs.

The purpose of the current study was to examine the role of AHR1A and AHR1B in mediating PAH and PCB-126 (3,3',4,4',5-pentachlorobiphenyl) cardiac toxicity in developing zebrafish. Morpholinos were used to knockdown the various AHRs, and then cardiac deformities, CYP1 activity, and CYP1 gene expression were examined in response to PAHs and to PCB-126. Based on previous studies examining the AHR agonist affinity and binding capacity, activity, and induction of zebrafish AHR1 isoforms, we hypothesized the following: 1) AHR1B knockdown would provide a protective role against PAH- and PCB-126-induced toxicity and 2) AHR1A knockdown would have no impact on or provide a minimally protective role against AHR agonist toxicity. However, our results indicate that

AHR1B knockdown had no effect on PAH- and PCB-126 induced deformities. Surprisingly, AHR1A knockdown exacerbated PAH- and PCB-126-induced toxicity and increased CYP1 activity but did not affect CYP1 gene expression. These results suggest that AHR1A may interact with the classical AHR2 pathway in such a way that it serves a protective role when present (such as sequestering ligands away from AHR2 or acting as a repressor of the pathway) and/or may have a role in the normal development of zebrafish embryos.

2. Materials and Methods

2.1 Fish Husbandry

Adult EkkWill zebrafish (*D. rerio*; EkkWill Waterlife Resources, Ruskin, FL, USA) were maintained in a recirculating AHAB system (Aquatic Habitats, Apopka, FL, USA) in 60 mg/L salt water (Instant Ocean, Foster & Smith, Rhinelander, WI, USA). Fish were kept at 28°C on a 14 h:10 h light:dark cycle. Adults were fed brine shrimp in the morning and a mixture of Zeigler's Adult Zebrafish Complete Diet (Aquatic Habitats) and Cyclop-eeze (Argent Chemical Laboratories, Redmond, WA, USA) in the afternoon.

Embryos were collected after natural spawning of adult zebrafish and were maintained in 30% Danieau (Nasevicius and Ekker, 2000) in an incubator under the same temperature and photoperiod as adults. All adult care and breeding techniques were non-invasive and were approved by the Duke University Institutional Animal Care & Use Committee (A279-08-10).

2.2 Chemicals and Dosing

BaP, benzo[k]fluoranthene (BkF), and fluoranthene (FL) were purchased from Absolute Standards, Inc. (Hamden, CT, USA), and PCB-126 was purchased from AccuStandard (New Haven, CT, USA). Dimethyl sulfoxide (DMSO), 7-ethoxyresorufin (7-ER), and tricaine methanesulfonate (MS-222) were purchased from Sigma-Aldrich (St. Louis, MO, USA). BkF, BaP, FL, and PCB-126 stocks were dissolved in DMSO, protected from light, and kept at -20°C until use.

At 24 hours post fertilization (hpf), all embryos were examined microscopically for normal development. Uniform incorporation of the morpholino in the injected embryos was determined by observation of even fluorescence (from the fluorescein tag) throughout the embryo. Embryos exhibiting normal development, and uniform incorporation of the morpholinos for injected embryos, were dosed in 20-mL glass scintillation vials containing 7.5 mL 30% Danieau. Each vial contained five embryos, and there were three vials per treatment in each experiment. Each experiment was performed at least three times. After dosing, embryos were placed back into a 28°C incubator and kept on the same photoperiod as adults.

For initial deformity experiments utilizing AHR1A and AHR1B morpholinos, embryos were exposed to DMSO, 50 µg/L BkF, 150 µg/L FL, 50 µg/L BkF + 150 µg/L FL, and 1 µg/L PCB-126. For subsequent deformity experiments involving AHR1A morpholino injections, embryos were exposed to DMSO as a control and the nominal concentrations listed in Table 1. For experiments involving knockdown of AHR2 and co-knockdown of AHR1A and AHR2, the same doses were used with the addition of 2 µg/L PCB-126. Final DMSO concentrations were < 0.03% across all treatments for all experiments. For experiments examining CYP1 activity via the 7-ethoxyresorufin-*O*-deethylase (EROD) assay, embryos were exposed to DMSO, 100 µg/L BaP, 50 and 100 µg/L BkF, and 1 and 2 µg/L PCB-126. The substrate 7-ER was added at a final concentration of 21 µg/L at the time of PAH or PCB-126 dosing.

For RT-PCR and confirmation of morpholino efficacy, embryos were dosed with DMSO or 50 µg/L BkF. For QPCR and examination of CYP1 mRNA expression, embryos were dosed with DMSO as a control and the individual and mixture exposures presented in Table 1. Embryos were dechorionated at 48 hpf and fixed in RNA*later* (Applied Biosystems, Foster City, CA, USA). Samples were stored at -80°C until RNA extraction.

2.3 Morpholino Injection

Morpholino antisense oligonucleotides were designed and produced by Gene Tools, LLC (Philomath, OR, USA). AHR2 knockdown was achieved using a previously designed morpholino shown to block translation of AHR2 (AHR2-mo: 5'-TGTACCGATACCCGCCGACATGGTT-3') (Teraoka et al., 2003). Due to the lack of antibodies for the AHR1 isoforms, splice-junction morpholinos were used to target AHR1A and AHR1B. Splice-junction morpholinos cause aberrant splicing of pre-mRNA, most commonly via deletion of the targeted exon or insertion of the targeted intron, and knockdown via splice-junction morpholino can be quantified using PCR. A splice-junction morpholino targeting the exon 2-intron 2 boundary of AHR1A has been used previously and was a generous gift from Dr. John Incardona (AHR1A-mo: 5'-CTTTTGAAGTGACTTTTGGCCCGCA-3') (Incardona et al., 2006). A splice-junction morpholino shown to effectively target the exon 7-intron 7 boundary of AHR1B was kindly provided by Dr. Robert Tanguay (AHR1B-mo, 5'-ACACAGTCGTCCATGATTACTTTGC-3') (Goodale et al., 2012). Gene Tools' standard control morpholino (Co-mo: 5'-CCTCTTACCTCAGTTACAATTATA-3') was used as a morpholino injection control. Morpholinos were fluorescein-tagged at the 3' end to monitor injection success. Morpholinos were diluted to either 100 and 250 µM working stocks in 30% Danieau.

Morpholinos (approximately 3 nL injection volume) were injected by hand into the yolk of zebrafish embryos at the 1-2 cell stage using a microinjection system consisting of a Nikon SMZ-1500 zoom stereomicroscope (Nikon Instruments Inc., Lewisville, TX, USA) and an MDI PM 1000 Cell Microinjector (MicroData Instrument Inc., S. Plainfield, NJ, USA). For injections with individual morpholinos, 100 µM or 250 µM stocks were injected into the embryos. For co-injection of AHR1A and AHR2, a mixture of 100 µM of each morpholino was used.

2.4 Deformity Assessment

Pericardial effusion was quantified as a metric for cardiac toxicity (Billiard et al., 2006). At 96 hpf (72 h post dosing), embryos were removed from the dosing solution, rinsed with 30% Danieau, and anesthetized with MS-222. Fish were placed in the left lateral position on depression slides in 3% methylcellulose. Fish were imaged under 50× magnification (Zeiss Axioskop, Thornwood, NY, USA), and the two dimensional area of the pericardial sac was traced and measured with IPLab Software (Scanalytics Inc., Fairfax, VA, USA). Deformity values are expressed as a percentage of the 2D pericardial area of non-injected (NI) control embryos.

2.5 In vivo EROD Assay

In addition to deformity assessment, CYP1 enzyme activity was measured at 96 hpf via a modified *in vivo* EROD assay (Matson et al., 2008; Nacci et al., 1998). The 7-ER added at the time of dosing is a substrate for CYP1 metabolism; CYP1s cleave 7-ER into a fluorescent product, resorufin. Though present in various tissues, the fluorescence of resorufin is most evident in the gastrointestinal tract of developing zebrafish and was measured under fluorescent microscopy (Billiard et al., 2006; Otte et al., 2010). Fluorescence was measured under 50× magnification using a rhodamine red filter set

(510–560 nm excitation and 590 nm emission; Zeiss Axioskop) and quantified by IPLab software. EROD values are expressed as a percentage of the mean fluorescence in the gastrointestinal tract of NI control embryos.

2.6 Confirmation of AHR1A and AHR1B splice-mo efficacy by reverse transcription PCR analysis

Each sample consisting of five embryos was thawed on ice and then homogenized for 30 s with a sterile hand-held homogenizer. RNA extractions were performed according to the RNA-Bee protocol (Tel-Test Inc., Friendswood, TX, USA). RNA quality and quantity were analyzed spectrophotometrically with a NanoDrop ND-100 (NanoDrop Technologies, Wilmington, DE, USA). Quality was verified by an optical density (OD) absorption ratio 260 nm/280 nm > 1.6. cDNA was synthesized using Omniscript Reverse Transcriptase kits (Qiagen, Valencia, CA, USA) according to the manufacturer's instructions with 500 ng RNA, random hexamers, and RNaseOut (Invitrogen, Carlsbad, CA, USA). The reaction was carried out in a Biometra T1 thermocycler (Göttingen, Germany) for 1 h at 37°C.

RT-PCR was performed in a 25-μL reaction containing 62.5 ng template, 12.5 μL AmpliTaq Gold Master Mix (Applied Biosystems), 9 μL distilled H₂O, and 0.5 μL each 10 μM forward and reverse primer. The thermocycler conditions for the AHR1A morpholino were run according to Incardona *et al.* (2006) and were as follows: 94°C for 10 min followed by 35 cycles of 94°C for 15 s, 56.7°C for 30 s, 72°C for 45 s, and a final extension step at 72°C for 7 min. Primers spanning AHR1A exon 2 (F: 5'-CGCAAAGGAGGAAACCTGTC-3' and R: 5'-CCTGTAGCAAAAATTCCCCCT-3') were used in the PCR reaction. For confirmation of AHR1B morpholino efficacy, the thermocycler conditions were as follows: 95°C for 5 min; 35 cycles of 95°C for 15 s, 58°C for 15 s, and 72°C for 4 min; and finally, 72°C for 10 min. AHR1B forward primer was 5'-CTTTGTGTGTCGTTTCCGATGCC-3' and the reverse primer was 5'-GCACAGTAGAGCATATCAGCTGC-3' (Goodale *et al.*, 2012). Gel electrophoresis of 10 μL of PCR product was performed using agarose gels stained with SYBR Safe DNA gel stain (Invitrogen); 4% agarose gels were used for AHR1A final products, and 2% agarose gels were used for AHR1B final products. Gels were imaged with an AlphaImager HP (Cell Biosciences, Santa Clara, CA, USA).

2.7 QPCR

RNA extraction and cDNA synthesis were carried out as described above. cDNA was synthesized at a concentration of 25 ng/μL, which was subsequently diluted to 2 ng/μL. Each 25-μL QPCR reaction consisted of 12.5 μL SYBR Green PCR Master Mix (Applied Biosystems), 9.5 μL dH₂O, 200 nM each forward and reverse primer, and 4 ng cDNA template. The reactions were run on an Applied Biosystems 7300 Real-Time PCR System with cycling parameters of 10 min at 95°C and 40 replicates of 15 s at 95°C and 1 min at 60°C. Dissociation curves were calculated for each sample at the end of each profile to confirm formation of a single product during the reaction. All samples were run in duplicate, and technical replicates were averaged prior to normalization to β -actin 1, used as a housekeeping gene. The ABI PRISM 7300 Sequence Detection System Software, Version 1.1 (Applied Biosystems) was used to carry out data analysis.

The mRNA expression of CYP1A, CYP1B1, and CYP1C1 was examined in NI and AHR1A-mo-injected embryos after exposures to various PAHs and PCB-126. Primers for the three CYPs were published previously (Timme-Laragy *et al.*, 2007). β -actin 1 primers were also published previously (Van Tiem and Di Giulio, 2011). The average mRNA fold induction of each target gene was calculated by comparing the C_T (threshold cycle) of the target gene to that of β -actin (Livak and Schmittgen, 2001). β -actin 1 was determined to be a

valid housekeeping gene as its expression was not altered by morpholino injections or chemical treatments.

2.8 Statistical Analysis

The data were analyzed using JMP 8.1.1 (SAS Institute Inc., Cary, NC, USA). Deformity, EROD, and gene expression data were analyzed via two-way ANOVA to determine an overall effect of the various morpholino injections and chemical treatments followed by least square means (LSMeans) procedures. Tukey's post-hoc test was used to determine differences between groups. Deformity and EROD experiments were conducted three or four times with at least three replicates per treatment per experiment, and gene expression experiments were replicated twice with three samples per treatment per experiment. No differences between experimental replicates were observed for any test. Data are represented as mean $\pm$ standard error of the mean (SEM). Values were considered significantly different at $p < 0.05$.

3. Results

3.1 Preliminary experiments investigating the effect of AHR1A and AHR1B knockdown on PAH and PCB-126 toxicity

The efficacy of each morpholino was confirmed by RT-PCR (data not shown). As expected, exposure to either 50 $\mu\text{g/L}$ BkF or 150 $\mu\text{g/L}$ FL alone did not cause pericardial effusion in NI or any of the morpholino-injected embryos. Exposure to 50 $\mu\text{g/L}$ BkF + 150 $\mu\text{g/L}$ FL caused significant pericardial effusion of $149 \pm 5\%$, $151 \pm 7\%$, and $157 \pm 9\%$ of controls in NI, Co-mo, and AHR1B-mo embryos, respectively (Fig. 1A; all $p < 0.001$ vs. NI DMSO). AHR1B knockdown neither exacerbated nor ameliorated the cardiac toxicity caused by co-exposure to BkF + FL. Quite unexpectedly, AHR1A knockdown exacerbated PAH-induced pericardial effusion; AHR1A-mo embryos exposed to 50 $\mu\text{g/L}$ BkF + 150 $\mu\text{g/L}$ FL had a significantly greater pericardial area than NI controls and NI embryos of the same treatment ($210 \pm 14\%$, $p < 0.0001$ vs. NI controls and NI BkF + FL). Following 1 $\mu\text{g/L}$ PCB-126 exposure, NI embryos had an average pericardial area of $117 \pm 6\%$ of NI controls, Co-mo embryos had an area of $111 \pm 6\%$, and AHR1B-mo embryos had an area of $117 \pm 5\%$; none of which were significantly different than NI controls. However, AHR1A-mo embryos once again had a significantly increased pericardial area of $145 \pm 6\%$ (Fig. 1B; $p < 0.001$ vs. NI DMSO and NI 1 $\mu\text{g/L}$ PCB-126). There was no apparent difference between the effects of injection with 100 and 250 μM for each morpholino (data not shown) and thus, 100 μM AHR1A-mo was used for future experiments. Due to the apparent lack of effect of AHR1B knockdown and the unexpected effect of AHR1A knockdown, subsequent experiments focused on further investigation of the effect of AHR1A knockdown on PAH and PCB-126 toxicity.

3.2 Effect of AHR1A knockdown on PAH and PCB-126 toxicity

NI, Co-mo, and AHR1A-mo embryos were exposed to various individual PAHs, PAH mixtures, and PCB-126. The pericardial areas of AHR1A-mo embryos exposed to individual doses of AHR ligands (BkF and BaP) or a CYP1 inhibitor (FL) were not different than the areas of NI embryos exposed to the same treatments or to NI controls (Fig. 2).

AHR1A knockdown exacerbated the cardiac deformities caused by the three PAH mixtures examined. BaP + FL co-exposure resulted in an average pericardial area of $111 \pm 6\%$ in NI embryos, which was not significantly greater than NI controls (Fig. 2A). AHR1A knockdown resulted in embryos with moderate but significant pericardial effusion as compared to NI controls and NI BaP + FL-exposed embryos ($132 \pm 5\%$, $p < 0.001$ vs. NI DMSO and NI BaP + FL). NI embryos exposed to 100 $\mu\text{g/L}$ BkF + 100 $\mu\text{g/L}$ FL had an

average pericardial area of $122 \pm 6\%$ control levels, which was not significantly greater than controls, and AHR1A knockdown resulted in $177 \pm 10\%$ pericardial effusion, which was significantly greater than NI DMSO controls and NI embryos of the same treatment (Fig. 2B; $p < 0.0001$). AHR1A knockdown also exacerbated the deformities caused by co-exposure to a mixture of different concentrations of BkF + FL. Confirming the preliminary experiments, NI embryos exposed to $50 \mu\text{g/L}$ BkF + $150 \mu\text{g/L}$ FL had significant pericardial effusion compared to NI controls ($148 \pm 5\%$; $p < 0.0001$), while AHR1A knockdown resulted in even greater pericardial effusion (Fig. 2C; $214 \pm 11\%$; $p < 0.0001$ vs. NI DMSO and NI BkF + FL).

AHR1A knockdown also induced deformities in embryos exposed to $1 \mu\text{g/L}$ PCB-126 (Fig. 3). NI embryos exposed to PCB-126 had an average pericardial area of $110 \pm 4\%$, which was not significantly greater than the pericardial areas of control fish exposed to DMSO. AHR1A knockdown resulted in significant pericardial effusion in embryos exposed to $1 \mu\text{g/L}$ PCB-126 ($140 \pm 5\%$, $p < 0.001$ vs. NI DMSO and NI PCB-126).

3.3 Effect of AHR1A knockdown on PAH- and PCB-126-induced CYP1 gene expression

The effect of AHR1A knockdown on the expression of CYP1A, CYP1B1, and CYP1C1 in response to PAHs and PCB-126 was examined. Injection of the Co-mo or AHR1A-mo did not affect gene expression in DMSO-treated embryos. As determined by two-way ANOVA, there was an effect of treatment but not morpholino on expression of the three CYPs. FL alone did not upregulate any of the CYPs in NI, Co-mo, or AHR1A-mo embryos, while BkF and BaP alone significantly induced CYP expression in NI, Co-mo, and AHR1A-mo embryos to the same extent (data not shown for clarity of graphs). Exposure to $100 \mu\text{g/L}$ BaP + $500 \mu\text{g/L}$ FL significantly induced expression of all three CYPs in NI, Co-mo, and AHR1A-mo embryos (Fig. 4A, CYP1A - 33 ± 8 -, 34 ± 5 -, and 43 ± 6 -fold; Fig. 4B, CYP1B1 - 6 ± 1 -, 6 ± 1 -, and 8 ± 1 -fold; and Fig. 4C, CYP1C1 - 29 ± 4 -, 29 ± 4 -, and 25 ± 4 -fold, respectively; all $p < 0.001$ vs. NI DMSO). Exposure to $50 \mu\text{g/L}$ BkF + $150 \mu\text{g/L}$ FL also significantly upregulated expression of all three CYPs in NI, Co-mo, and AHR1A-mo embryos (Fig. 5A, CYP1A - 87 ± 6 -, 84 ± 10 -, and 80 ± 4 -fold; Fig. 5B, CYP1B1 - 19 ± 1 -, 19 ± 3 -, and 18 ± 1 -fold; and Fig. 5C, CYP1C1 - 75 ± 12 -, 74 ± 11 -, and 67 ± 8 -fold, respectively; all $p < 0.0005$ vs. NI DMSO). These increases in CYP expression in response to PAHs were not statistically different between NI, Co-mo, and AHR1-mo embryos.

CYP1A expression in NI, Co-mo, and AHR1A-mo embryos exposed to $1 \mu\text{g/L}$ PCB-126 was induced 60 ± 3 -, 55 ± 8 -, and 47 ± 3 -fold, respectively, above NI controls; CYP1B1 was upregulated 27 ± 5 -, 24 ± 4 -, and 18 ± 2 -fold, respectively; and CYP1C1 was induced 104 ± 22 -, 118 ± 17 -, and 111 ± 13 -fold, respectively (all $p < 0.002$ vs. NI DMSO) (Fig. 6). Although PCB-126-induced upregulation of CYP1A and CYP1B1 was lower in AHR1A-mo embryos compared to NI embryos, CYP induction was not significantly different between the different morpholino treatments.

3.4 Effect of AHR1A-mo, AHR2-mo, and AHR1A/2-mo on PAH and PCB-126 toxicity

After the unexpected finding that knockdown of AHR1A exacerbated PAH- and PCB-126-induced cardiac toxicity, we sought to examine the effect of co-knockdown of AHR1A and AHR2 (AHR1A/2-mo). Previous studies have shown that AHR2 knockdown prevents PAH-induced pericardial effusion in zebrafish embryos (Billiard et al., 2006; Scott et al., 2011; Van Tiem and Di Giulio, 2011). For these experiments, injection of the Co-mo once again did not result in any differences from NI embryos; these data are not shown for clearer graphical representation of the data.

As shown previously, exposure to each PAH individually did not result in pericardial effusion in any embryos. In NI embryos, 100 µg/L BkF + 100 µg/L FL did not cause significant pericardial effusion and thus, there was no gross toxicity from which AHR2-mo embryos could be protected; however, AHR2-mo embryos had a slightly, but not significantly, smaller average pericardial effusion (105±5%) than NI embryos of the same treatment (114±3%) (Fig. 7A). AHR1A knockdown caused significant pericardial effusion in embryos exposed to 100 µg/L BkF + 100 µg/L FL (167±10%, $p < 0.005$ vs. NI BkF + FL and NI DMSO). AHR1A/2 co-knockdown resulted in an average pericardial area of 124±2%, which was significantly less than the AHR1A-mo BkF + FL embryos ($p = 0.012$) but not different than NI DMSO or NI BkF + FL embryos.

Using a different concentration ratio of BkF and FL, NI embryos exposed to 50 µg/L BkF + 150 µg/L FL had significant pericardial effusion compared to NI DMSO embryos (146±6%, $p < 0.001$) (Fig. 7B). As expected based on previous studies, AHR2 knockdown prevented this toxicity; the average pericardial area of AHR2-mo embryos exposed to 50 µg/L BkF + 150 µg/L FL was 116±4%, which was not significantly different than NI controls. AHR1A knockdown caused more extreme pericardial effusion (182±8%, $p < 0.001$) than in NI embryos of the same treatment. AHR1A/2 co-knockdown did not protect nor exacerbate the pericardial effusion induced by BkF + FL (137±6%, $p < 0.001$ vs. NI DMSO, not different than NI BkF + FL).

To examine if the effect of AHR1A/2 co-knockdown was unique to the combination of the strong AHR agonist BkF in conjunction with FL, fish were also exposed to a combination of FL and another AHR agonist, BaP. Exposure to 100 µg/L BaP + 500 µg/L FL caused significant pericardial effusion in NI embryos compared to NI controls (124±5%, $p < 0.0001$) (Fig. 7C). AHR2 knockdown completely prevented this effusion; AHR2-mo embryos had an average pericardial area of 105±3% (not different from NI controls). AHR1A knockdown exacerbated BaP + FL-induced toxicity, resulting in embryos with an average pericardial area of 141±5% ($p < 0.0001$ vs. NI DMSO and NI BaP + FL). The same effect of AHR1A/2 co-knockdown that occurred in 50 µg/L BkF + 150 µg/L FL-exposed embryos was seen in BaP + FL-exposed embryos: AHR1A/2 co-knockdown neither prevented nor exacerbated the deformities compared to NI BaP + FL embryos (125±6%, $p < 0.0001$ vs. NI DMSO, not different than NI BaP + FL).

Embryos were also exposed to single doses of the strong AHR agonist PCB-126, which is capable of producing deformities without the addition of a CYP1 inhibitor. In these experiments, 1 µg/L PCB-126 did not appear to be as toxic as in previous experiments. However, NI embryos exposed to 2 µg/L PCB-126 had significant pericardial effusion, with an average pericardial area of 140±4% ($p < 0.0001$) (Fig. 8). AHR2 knockdown prevented the cardiac deformities caused by this dose of PCB-126; AHR2-mo embryos had an average pericardial of 110±2% (not different than NI DMSO). AHR1A knockdown resulted in embryos with significant pericardial effusion (164±12%, $p < 0.0001$ vs. NI DMSO and NI PCB-126). Unlike the effect seen with the PAH co-exposures, AHR1A/2 co-knockdown completely prevented the pericardial effusion caused by 2 µg/L PCB-126, just as AHR2 knockdown did. AHR1A/2 embryos had an average pericardial area of 111±3% ($p < 0.0001$ vs. NI 2 µg/L PCB-126, not different than NI DMSO).

3.5 Effect of AHR2-mo, AHR1A-mo, and AHR1A/2-mo on PAH- and PCB-126-induced CYP1 activity

Though knockdown of AHR1A did not impact CYPs at the mRNA level, we sought to evaluate the effect of AHR1A knockdown, AHR2 knockdown, and AHR1A/2 co-knockdown on CYP enzyme activity. NI and injected embryos were exposed to the AHR ligands (BaP, BkF, and PCB-126) individually without FL. CYP1 activity, as measured via

the EROD assay, varied depending on the agonist used, concentration of the agonist, and morpholino. CYP1 activity in NI embryos exposed to 100 µg/L BaP was significantly induced to 232±43% NI control levels ($p < 0.001$) (Fig. 9A). AHR2 knockdown prevented induction of CYP1 activity (112±20%). In contrast, AHR1A knockdown resulted in significantly increased CYP1 activity compared to activity in NI controls and NI BaP-exposed embryos (491±20%, $p < 0.001$ vs. NI DMSO and NI BaP). AHR1A/2 knockdown appeared to reduce BaP-induced CYP1 activity, but the induction of 149±14% was not statistically different from NI controls or NI BaP-exposed embryos.

NI and mo-injected embryos were also exposed to 50 and 100 µg/L BkF, which is a stronger AHR agonist than BaP. NI embryos exposed to 50 µg/L BkF exhibited CYP1 activity 732±49% of NI control levels ($p < 0.0001$) (Fig. 9B). AHR2 knockdown reduced induction of CYP1 activity compared to NI embryos of the same treatment (567±71%, $p < 0.001$), but the CYP1 activity was still significantly higher than NI controls ($p < 0.01$). AHR1A knockdown increased CYP1 activity to 989±130%, but this increase was not significantly different than NI 50 µg/L BkF-exposed embryos. AHR1A/2 knockdown resulted in 723±77% NI control CYP1 activity, which was not statistically different than NI 50 µg/L BkF levels but still higher than NI controls ($p < 0.01$). Exposure to 100 µg/L BkF significantly induced CYP1 activity in NI embryos (874±87%, $p < 0.001$), and AHR2 knockdown significantly reduced BkF-induced CYP1 activity compared to NI embryos of the same treatment (502±96%, $p < 0.001$ vs. NI DMSO and NI 100 µg/L BkF). AHR1A knockdown did not significantly increase or decrease CYP1 activity compared to NI 100 µg/L BkF-exposed embryos (831±67%) nor did AHR1A/2 co-knockdown (665±50%).

Exposure to 1 µg/L PCB-126 induced CYP1 activity to 314±14% control levels in NI embryos (Fig. 9C). AHR1A knockdown resulted in increased CYP1 activity (451±68%) compared to NI embryos of the same treatment ($p < 0.0001$ vs. NI DMSO and NI 1 µg/L PCB-126). AHR2 and AHR1A/2 knockdown both reduced CYP1 activity (205±20% and 255±18%, respectively) compared to NI treated embryos, but the activity was still higher than NI controls. NI embryos exposed to 2 µg/L PCB-126 exhibited significantly increased CYP1 activity above control levels (473±24%, $p < 0.0001$). AHR1A knockdown did not increase CYP activity compared to NI treated embryos (458±34%). AHR2 and AHR1A/2 knockdown both significantly reduced CYP1 activity (168±20% and 248±11%, respectively) compared to levels in 2 µg/L PCB-126-exposed NI embryos. In PCB-exposed embryos, AHR2 knockdown brought CYP1 activity back to NI control levels, whereas AHR1A/2 knockdown resulted in CYP1 activity that was still significantly higher than in NI control embryos.

4. Discussion

4.1 AHR1B knockdown does not impact PAH -or PCB-126-induced toxicity in developing zebrafish

Limited information about the AHR1 isoforms exists, but the findings that AHR1B knockdown had no impact on PAH- and PCB-126-induced cardiac toxicity while AHR1A knockdown exacerbated this toxicity was unexpected. The zebrafish AHR1s and AHR2 arose from a gene duplication event that occurred before the divergence of ray- and lobe-finned fishes and thus, before the teleost gene-duplication event that is responsible for the existence of many other paralogous genes in fish (Hahn, 2002). Karchner *et al.* (2005) determined that AHR1B is adjacent to AHR2 on chromosome 22 while AHR1A is located on chromosome 16. AHR1A is expressed in zebrafish embryos at 24 hpf but was not inducible by TCDD at 48 or 72 hpf (Karchner *et al.*, 2005). AHR1B mRNA expression was induced by 5 µg/L BaP in zebrafish following a 72-h developmental exposure (Huang *et al.*, 2012) but was not affected by 10 µg/L pyrene exposure (Zhang *et al.*, 2012). And using

velocity sedimentation analysis, it was shown to have high-affinity binding for [³H]TCDD and was shown to be transcriptionally active in response to TCDD in COS-7 cells with efficiency similar to AHR2 but sensitivity approximately eight-fold lower than AHR2 (Karchner et al., 2005). Furthermore, TCDD has been predicted to bind AHR1B based on molecular docking modeling (Goodale et al., 2012). Based on this information, we hypothesized that AHR1B knockdown would have similar effects to AHR2 knockdown, providing a protective role against PAH- and PCB-126-induced toxicity but perhaps to a lesser extent than AHR2 knockdown. However, AHR1B knockdown had no effect on the cardiac deformities caused by PAH mixtures and PCB-126 in our studies. This lack of apparent effect could be due to the fact that we only examined the gross morphological condition of embryos. AHR1B knockdown may result in a more subtle effect that could be elucidated via finer examination with histopathology, immunohistochemistry, or gene expression analysis. Alternatively, AHR1B could have some functional redundancy with AHR2. For example, Goodale *et al.* (2012) propose that the faint vascular CYP1A expression observed in TCDD-exposed AHR2 knockout zebrafish is due to AHR1B and that AHR1B is widely distributed like AHR2 but at lower levels. Due to the lack of effects of AHR1B knockdown in our study, co-knockdown with AHR2 was not performed but may provide further insight into the role of AHR1B in PAH toxicity. However, because AHR2 underlies much of the toxicities of certain PAHs and PCB-126 and appears to have a higher affinity for TCDD, it is apparent that AHR2 has a greater role in xenobiotic metabolism and toxicity than AHR1B in fish early life stages.

4.2 AHR1A knockdown exacerbates PAH- and PCB-126-induced toxicity in developing zebrafish

Andreasen *et al.* (2002) showed that AHR1A mRNA expression was detectable at 24 hpf in zebrafish and was slightly induced by TCDD. In adult tissues, AHR1A mRNA was most highly expressed in the liver and to a lesser extent in the heart and kidney but was not induced by TCDD exposure. In contrast, AHR2 was found to be expressed in all tissues and was significantly induced by TCDD in the heart. In the same study, AHR1A was shown to be able to dimerize with ARNT2b and recognize XREs *in vitro* but with DNA binding activity much lower than that of the AHR2-ARNT2b heterodimer. AHR1A was also not transcriptionally active in response to TCDD in COS-7 cells and did not exhibit specific binding with [³H]TCDD or [³H]BNF. These findings led to the hypothesis that AHR1A is a nonfunctional incipient pseudogene (Andreasen et al., 2002; Karchner et al., 2005). The lack of TCDD binding to AHR1A due to substitutions in the binding pocket of the protein was further confirmed by an *in silico*-based model (Goodale et al., 2012). In addition, using homology modeling of AHR ligand binding domains, Fraccalvieri *et al.* (2013) recently showed that the AHR1A binding cavity is smaller than that of AHR2 due to three side chain residues and that mutagenesis of two of the residues to those in AHR2 allowed AHR1A to bind TCDD. Based on these studies, we hypothesized that AHR1A knockdown would either have no effect on the toxicity induced by high molecular weight PAH mixtures and PCB-126 or would serve a minimally protective role against the toxicities induced by these compounds. Hence, the exacerbation of toxicity by AHR1A knockdown was unexpected.

Two studies have indicated that AHR1A is not a nonfunctional pseudogene as previously thought. Incardona *et al.* (2006) showed that pyrene-induced hepatic CYP1A expression was AHR1A-dependent and that AHR1A knockdown delayed the onset of liver abnormalities and pericardial effusion caused by high doses of pyrene by 18-24 h but did not impact pyrene-induced dorsal curvature. *In silico*-based modeling has shown that AHR1A was capable of binding leflunomide, a pyrimidine synthesis inhibitor and antirheumatic drug, in an orientation unique from the way in which the drug binds with AHR2 and AHR1B (Goodale et al., 2012). The ability of PAHs to bind to AHR1A has not been modeled, but

based on prior TCDD and BNF results it seems unlikely that parent PAH compounds are able to bind to AHR1A in the same manner in which they bind to AHR2. However, smaller PAH metabolites may be able to interact with the receptor. Furthermore, leflunomide-induced CYP1A expression in the liver was shown to be AHR1A-dependent, indicating that AHR1A is a functional receptor *in vivo* though its transactivation requirements are different than AHR2 (Goodale et al., 2012).

One possible explanation for the effect of AHR1A knockdown stems from the model that duplicate genes have acquired partitioning of function (“subfunctionalization”) of their orthologue through degenerate mutations (Lynch and Force, 2000). Various studies have indicated that in addition to mediating the toxic effects of dioxins, PCBs, and PAHs, the mammalian AHR plays a role in normal development. By six to thirteen months of age, AHR null (AHR^{-/-}) mice develop lesions in their hearts, uteri, skin, liver, and large intestines that are not seen in control mice (AHR^{+/-}) (Fernandez-Salguero et al., 1997). Furthermore, these AHR null mice have been shown to exhibit cardiomegaly, and some have fibrotic ventricular or atrial myocardium and enlarged coronary arteries due to smooth muscle hyperplasia. AHR null mice also have a reduced liver size that is due to failure of ductus venosus (DV) closure, which normally occurs in the first few days after birth (Lahvis et al., 2000). In untreated AHR null mice, the DV remains open, resulting in aberrant hepatic blood flow. Interestingly, treatment of AHR hypomorphs with TCDD during gestational development at a level that did not cause maternal toxicity rescued this deformity, leading to DV closure (Walisser et al., 2004). Though no gross deformities were seen in control AHR1A-mo embryos in this experiment, it is possible that AHR1A may serve a physiological role in the normal development of zebrafish, while AHR2 plays a larger role in xenobiotic metabolism and toxicity. Just as effects of AHR1B knockdown were not apparent in this study, subcellular effects of AHR1A knockdown could also have been missed and could be elucidated by histopathological examination. In a related scenario, the loss of AHR1A and its developmental function could be compensated for by AHR1B or AHR2 under untreated, unstressed conditions, while the costressor of PAH treatment could impair this compensatory mechanism, leading to the deformities seen in AHR1A-mo embryos exposed to PAH mixtures and PCB-126. This seems unlikely due to the fact that deformities were not further exacerbated by AHR1A/2 co-knockdown. However, AHR1A knockdown and PAH or PCB-126 exposure could function as co-stressors by some mechanism that is not compensated for by AHR2.

AHR1A and AHR2 seem to have opposing effects on CYP activity; AHR2 induces CYP activity while AHR1A seems to prevent or inhibit it in some way. Increased CYP enzyme activity arising from AHR1A knockdown could result in greater formation of toxic PAH metabolites that could in turn lead to increased cardiac toxicity. Moreover, even though AHR1A was shown to dimerize with ARNT2b and recognize XREs *in vitro* (Andreasen et al., 2002), it has since been discovered that ARNT1 also forms a heterodimer with AHR2 and is necessary for TCDD-induced cardiac toxicity in zebrafish embryos (Prasch et al., 2006). Therefore, the lack of AHR1A transactivation in response to TCDD *in vitro* could be a result of the absence of ARNT1 as a dimerization partner.

Various lines of evidence suggest that AHR1A may be acting in a manner similar to AHR2. An example from the mammalian literature shows that AHR2 deficient mice lack any overt signs of developmental deformities and exhibit increased CYP1A1 mRNA induction in the skin, stomach and spleen but not in the liver, lung, or heart (Hosoya et al., 2008). While we did not observe increased CYP1 mRNA expression in AHR1A-mo embryos exposed to DMSO or PAHs, we did observe greater CYP1 enzyme activity in AHR1A-mo embryos exposed to PAHs and PCB-126. This difference could be explained by the fact that the EROD assay measures the cumulative activity of the CYP1s until the time of the assay (96

hpf, 72 h post dosing) while QPCR was used to measure each of three CYPs (1A, 1B1, and 1C1 in this study) individually at one time point (48 hpf, 24 h post dosing). The lack of increased CYP1 mRNA expression could be due to species differences or the early developmental stage at which expression was examined. Also, due to the early time point, we were not able to examine tissue-specific differences in gene expression.

AHRR lacks a ligand-binding region but is capable of dimerizing with ARNT (Mimura et al., 1999) and downregulates the classic AHR2-mediated pathway by competing with the AHR2/ARNT complex for binding to XREs and also by XRE binding-independent mechanisms (Evans et al., 2008). Two AHRRs, initially termed AHRR1 and 2, now identified as AHRRa and AHRRb, have been identified in zebrafish (Evans et al., 2005; Jenny et al., 2009). Jenny *et al.* (2009) knocked down both AHRRa and AHRRb in zebrafish and found that CYP1 expression in response to TCDD exposure was not affected at 48 hpf but was increased at 72 hpf and that this enhancement of induction was due to AHRRb knockdown. In the current study, CYP1 expression was also unaffected by AHR1A knockdown. We did not examine gene expression at later time points; this would be informative for future experiments. Furthermore, AHRRa knockdown in untreated embryos resulted in a TCDD-like phenotype with embryos displaying pericardial effusion, cardiac deformities, and craniofacial malformations while AHRRb morphants were normal; however, knockdown of either repressor individually resulted in enhanced toxicity in TCDD-exposed embryos (Jenny et al., 2009). A similar enhancement of PAH- and PCB-126-induced deformities was evident in AHR1A-mo embryos in our study. If AHR1A acts as a repressor of AHR2 in some manner, then AHR1A knockdown would result in increased and inappropriate signaling from AHR2, resulting in increased toxicity in response to AHR ligand exposure.

4.3 Conclusions

In summary, the results presented herein indicate that AHR1A and AHR1B have distinct, though still not fully elucidated, roles in zebrafish development as well as response to xenobiotics. AHR1B knockdown via morpholino injection did not affect PAH- or PCB-126-induced cardiac toxicity. On the other hand, AHR1A knockdown unexpectedly exacerbated PAH- and PCB-126-induced toxicity in developing zebrafish embryos. AHR1A knockdown did not affect CYP1 mRNA expression but did enhance CYP1 activity. Concurrent knockdown of AHR1A and AHR2 did not exacerbate or prevent PAH-induced deformities but was protective with respect to PCB-126-induced deformities. Further experiments examining the interaction of the AHR1 isoforms with other members of the classical AHR2 pathway as well as examination of gene expression at different time points and further characterization of the effects of the AHR1 isoforms on cardiac structure and function will help to elucidate the developmental and/or toxicological role of AHR1 isoforms in embryonic zebrafish.

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- AHR1B knockdown did not affect the toxicity of PAH mixtures or PCB-126
- AHR1A knockdown exacerbated the cardiac toxicity of PAH mixtures and PCB-126
- AHR1A knockdown did not impact CYP1 mRNA expression in exposed embryos
- AHR1A knockdown resulted in increased CYP1 activity in exposed embryos

Fig. 1. Effect of PAHs (A) and PCB-126 (B) on deformities in non-injected (NI; white bars), control morpholino (Co-mo; grey bars), AHR1A-mo (black bars), and AHR1B-mo (patterned bars) embryos. Embryos were dosed at 24 hpf, and pericardial effusion was measured at 96 hpf. Values are expressed as percent NI DMSO pericardial area $\pm$ SEM ($n = 9$ per treatment; each n represents the average of five embryos). Groups not sharing a common letter are significantly different ($p < 0.05$; ANOVA, Tukey adjusted LSMeans).

Fig. 2. Effect of PAHs in non-injected (NI; white bars), control morpholino (Co-mo; grey bars), and AHR1A-mo (black bars) embryos. (A) 100 μg/L BaP and 500 μg/L FL; (B) 100 μg/L BkF and 100 μg/L FL; (C) 50 μg/L BkF and 150 μg/L FL. Embryos were dosed at 24 hpf, and pericardial effusion was measured at 96 hpf. Values are expressed as percent NI DMSO pericardial area ± SEM (n = 12 per treatment; each n represents the average of five embryos). Groups not sharing a common letter are significantly different ($p < 0.05$; ANOVA, Tukey adjusted LSMeans).

Fig. 3. Effect of PCB-126 in non-injected (NI; white bars), control morpholino (Co-mo; grey bars), and AHR1A-mo (black bars) embryos. Embryos were dosed at 24 hpf, and pericardial effusion was measured at 96 hpf. Values are expressed as percent NI DMSO pericardial area $\pm$ SEM ($n = 12$ per treatment; each n represents the average of five embryos). Asterisk (*) denotes treatment that is significantly different from NI controls ($p < 0.05$; ANOVA, Tukey adjusted LSMeans).

Fig. 4.

Effect of 100 µg/L BaP + 500 µg/L FL on CYP1 mRNA expression in non-injected (NI; white bars), control morpholino (Co-mo; grey bars), and AHR1A-mo (black bars) embryos. (A) CYP1A; (B) CYP1B1; (C) CYP1C1. Embryos were dosed at 24 hpf, and expression was measured at 48 hpf. Expression is shown as fold induction ± SEM compared to NI controls ($n = 6$ per treatment; each n represents the average of five embryos). An asterisk (*) represents a significant difference from NI control ($p < 0.05$; ANOVA, Tukey adjusted LSMeans).

Fig. 5. Effect of 50 µg/L BkF + 150 µg/L FL on CYP1 mRNA expression in non-injected (NI; white bars), control morpholino (Co-mo; grey bars), and AHR1A-mo (black bars) embryos. (A) CYP1A; (B) CYP1B1; (C) CYP1C1. Embryos were dosed at 24 hpf, and expression was measured at 48 hpf. Expression is shown as fold induction ± SEM compared to NI controls ($n = 6$ per treatment; each n represents the average of five embryos). An asterisk (*) represents a significant difference from NI control ($p < 0.05$; ANOVA, Tukey adjusted LSMeans).

Fig. 6. Effect of 1 µg/L PCB-126 on CYP1 mRNA expression in non-injected (NI; white bars), control morpholino (Co-mo; grey bars), and AHR1A-mo (black bars) embryos. (A) CYP1A; (B) CYP1B1; (C) CYP1C1. Embryos were dosed at 24 hpf, and expression was measured at 48 hpf. Expression is shown as fold induction ± SEM compared to NI controls (n = 6 per treatment; each n represents the average of five embryos). An asterisk (*) represents a significant difference from NI control ($p < 0.05$; ANOVA, Tukey adjusted LSM means).

Fig. 7. Effect of PAHs in non-injected (NI; white bars), AHR2-mo (diagonal striped bars), AHR1A-mo (black bars), and AHR1A/2-mo (horizontal striped bars) embryos. (A) 100 μg/L BkF and 100 μg/L FL; (B) 50 μg/L BkF and 150 μg/L FL; and (C) 100 μg/L BaP and 500 μg/L FL. Embryos were dosed at 24 hpf, and pericardial effusion was measured at 96 hpf. Values are expressed as percent NI DMSO pericardial area ± SEM (n = 9 per treatment; each n represents the average of five embryos). Groups not sharing a common letter are significantly different ($p < 0.05$; ANOVA, Tukey adjusted LSMeans).

Fig. 8.

Effect of 1 µg/L and 2 µg/L PCB-126 in non-injected (NI; white bars), AHR2-mo (diagonal striped bars), AHR1A-mo (black bars), and AHR1A/2-mo (horizontal striped bars) embryos. Embryos were dosed at 24 hpf, and pericardial effusion was measured at 96 hpf. Values are expressed as percent NI DMSO pericardial area $\pm$ SEM ($n = 9$ per treatment; each n represents the average of five embryos). Groups not sharing a common letter are significantly different ($p < 0.05$; ANOVA, Tukey adjusted LSMeans).

Fig. 9.

Effect of PAHs and PCB-126 on EROD induction in non-injected (NI; black line), AHR2-mo (grey line), AHR1A-mo (dotted line), and AHR1A/2-mo (hashed line) embryos. (A) 100 µg/L BaP; (B) 50 and 100 µg/L BkF; (C) 1 and 2 µg/L PCB-126. Embryos were dosed at 24 hpf, and EROD activity was measured at 96 hpf. Values are expressed as percent NI control EROD $\pm$ SEM ($n = 9$ per treatment; each n represents average of five embryos). Groups not sharing a common letter are significantly different ($p < 0.05$; ANOVA, Tukey adjusted LSMeans).

Table 1

Experimental regimes and corresponding results presentation

Experimental Endpoint	Morpholino	Individual PAH exposure (µg/L)	PAH co-exposure (µg/L)	PCB-126 Concentration (µg/L)	Presentation of Results
Preliminary deformity assessment	AHR1A AHR1B Co NI	50 BkF; 150 FL	50 BkF + 150 FL	1	Figure 1
Deformity assessment	AHR1A Co NI	50 BkF; 150 FL 100 BkF; 100 FL 100 BaP; 500 FL	50 BkF + 150 FL 100 BkF + 100 FL 100 BaP + 500 FL	1	Figure 2 (PAHs); Figure 3 (PCB)
CYP1 mRNA expression	AHR1A Co NI	50 BkF; 150 FL 100 BaP; 500 FL	50 BkF + 150 FL 100 BaP + 500 FL	1	Figures 4 and 5 (PAHs); Figure 6 (PCB)
Co-knockdown deformity assessment	AHR1A AHR2 Co NI	50 BkF; 150 FL 100 BkF; 100 FL 100 BaP; 500 FL	50 BkF + 150 FL 100 BkF + 100 FL 100 BaP + 500 FL	1 2	Figure 7 (PAHs); Figure 8 (PCBs)
EROD activity	AHR1A AHR2 Co NI	50 BkF 100 BkF 100 BaP	None	1 2	Figure 9

Co – Control morpholino; NI – Non-injected; BkF – Benzo[k]fluoranthene; FL – Fluoranthene; BaP - Benzo[a]pyrene