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Characterization of the recalcitrant CYP1 phenotype found in Atlantic killifish (*Fundulus heteroclitus*) inhabiting a Superfund site on the Elizabeth River, VA

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Abstract

Fundulus heteroclitus (Atlantic killifish) found at the Atlantic Wood Industries Superfund site on the Elizabeth River (ER) in Portsmouth, VA (USA), have been shown to be resistant to the teratogenic effects of creosote-contaminated sediments found at this highly contaminated site. Many of the polycyclic aromatic hydrocarbons (PAHs) found at the ER are known to activate the aryl hydrocarbon receptor (AHR), and are thought to mediate their toxic effects through this pathway. Activation of the AHR results in the induction of several Phase I and II metabolic enzymes. It has been previously shown that the AHR of killifish from the ER are refractory to induction by AHR agonists. To more fully characterize this altered AHR response, we exposed embryos from the ER and from a reference site on King's Creek, VA (KC) to two PAHs, benzo[*a*]pyrene (BaP) and benzo[*k*]fluoranthene (BkF), and to the dioxin-like compound (DLC), 3,3',4,4',5-pentachlorobiphenyl (PCB126). We compared their developmental and molecular responses by screening the embryos for CYP1A enzyme activity, cardiac deformities, and mRNA expression of *CYP1A*, *CYP1B1*, *CYP1C1*, and *AHR2*. Basal gene expression of both *CYP1A* and *CYP1B1* was 40% higher in the KC control embryos compared to those from the ER, while *AHR2* and *CYP1C1* were not significantly different between the populations. Exposure of KC embryos to BaP, BkF, and PCB126 induced CYP1A activity and cardiac deformities. In contrast, CYP1A activity was induced in ER embryos only in response to BkF exposure, although this induction in ER embryos was significantly lower than that observed in KC fish at comparable concentrations. ER embryos did not develop cardiac deformities in response to any of the chemicals tested. *CYP1A*, *CYP1B1* and *CYP1C1* mRNA were all significantly induced in the KC embryos after exposure to BaP, BkF and PCB126. Exposure to BaP and BkF in ER embryos resulted in a significant induction of *CYP1A* mRNA, albeit significantly lower than observed in KC fish. Interestingly, BaP exposure resulted in induction of *CYP1B1* at comparable levels in embryos from both populations. *CYP1s* were not induced in ER embryos in response to PCB126, nor was *CYP1C1* for any treatment examined. Additionally, *AHR2* was not significantly induced for any of

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the treatment groups. This study further characterizes the AHR response in killifish, and provides greater insight into the adapted ER phenotype. The ER adaptation involves the suppression of normal AHR-inducible gene expression for all three CYP1 genes, and therefore is likely an alteration in AHR signaling or control.

Keywords

Fundulus heteroclitus; Elizabeth River; cytochrome P450-1; polycyclic aromatic hydrocarbon; Benzo[a]pyrene; benzo[k]fluoranthene; 3,3',4,4',5-pentachlorobiphenyl

1. Introduction

Atlantic killifish (*Fundulus heteroclitus*) are teleosts that have been used for over a century in ecological, biochemical, and toxicological research (Atz, 1986; Burnett et al., 2007). These fish have limited migration patterns, allowing for the evaluation of multiple populations with different exposure histories within a small area (Nacci et al., 1999; Mulvey et al., 2002; Roark et al., 2005). Many killifish populations live in polluted estuaries along the Atlantic coast of North America, and have adapted to withstand the toxicity of multiple environmental contaminants, including metals, dioxin-like compounds (DLCs), and polycyclic aromatic hydrocarbons (PAHs) (Weis et al., 1987; Bello et al., 2001; Arzuaga and Elskus, 2002; Nacci et al., 2010).

Historically these resistant killifish populations have demonstrated reduced sensitivity to multiple toxic insults. Nacci et al. (2010) showed that a population of killifish on the Elizabeth River (ER) near Norfolk, VA had absolute tolerance to PCB126 (2- 200,000 ng/L) in both the F1 and F2 generations as measured by both lethal and sublethal effects. The ER killifish population inhabits an estuary contaminated with the wood preservative creosote, which primarily consists of PAHs. Unlike killifish from a reference site, embryos born to ER parents are resistant to the teratogenic effects of these PAHs (Ownby et al., 2002; Wassenberg and Di Giulio, 2004b). Killifish embryos and adults from the ER have developed alterations in their biochemistry that may contribute to their resistance, including increased expression of P-glycoproteins, manganese superoxide dismutase (MnSOD) and glutathione concentrations (Armknacht et al., 1998; Cooper et al., 1999; Meyer et al., 2003a). One of the features common to most of the adapted killifish populations is a recalcitrant CYP1A phenotype after exposure to AHR agonists (Van Veld and Westbrook, 1995; Elskus et al., 1999; Bello et al., 2001; Meyer et al., 2002; Wirgin and Waldman, 2004).

Although there are natural ligands for the AHR such as indoles and heme metabolites, one of the most potent known ligand for the receptor is 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD, dioxin) (Denison and Nagy, 2003; Mandal, 2005; Nguyen and Bradfield, 2008). Other xenobiotics that bind to the AHR include co-planar polychlorinated biphenyls such as PCB126, and certain PAHs (Billiard et al., 2004; Nguyen and Bradfield, 2008). Like many other fish species, killifish have two AHR paralogs that have differential tissue expression and functionality, AHR1 and AHR2 (Karchner et al., 1999; Powell et al., 2000; Hahn, 2002). AHR2 has recently been shown to mediate the toxicity of DLCs and PAHs in killifish (Clark et al., 2010), which is also the case for zebrafish (Andreasen et al., 2002; Prasch et al., 2003; Billiard et al., 2006).

When DLCs and PAHs bind to the AHR it migrates to the nucleus, binds with aryl hydrocarbon nuclear translocator (ARNT), and binds to the xenobiotic response elements (XREs) on a variety of genes including the cytochrome P450-1 (CYP1s) family of metabolic enzymes (Schmidt and Bradfield, 1996; Song and Pollenz, 2002; Pollenz et al., 2006; Dougherty and

Pollenz, 2008). Recent studies have shown that multiple fish species, including killifish, express five genes in four CYP1 subfamilies (CYP1A, CYP1B1, CYP1C1, CYP1C2, and CYP1D1) (Godard et al., 2000; Godard et al., 2005; Wang et al., 2006; Goldstone and Stegeman, 2008; Zanette et al., 2009). TCDD and the DLCs are potent inducers of CYP1A; however, these compounds are poor agonists for CYP1A and are not easily biotransformed or excreted (Billiard et al., 2002; Barron et al., 2004; Mandal, 2005). The teratogenic and carcinogenic effects of DLCs are mediated by the AHR in both mice and in aquatic organisms (Fernandez-Salguero et al., 1996; Mimura et al., 1997; Prasch et al., 2003). However chemical or molecular inhibition of CYP1A or CYP1B1 does not have an effect on the toxic response, suggesting that the mechanism of TCDD-induced toxicity is independent of CYP1 activity (Carney et al., 2004; Yin et al., 2008). In contrast to DLCs, PAHs are less potent AHR agonists; however, they are good CYP1A substrates and are easily metabolized. The biotransformation of PAHs by CYP1 enzymes aids in their detoxification and elimination; however, this process can also result in bioactivation and in the formation of reactive intermediate metabolites (Schlenk et al., 2008). The reactive metabolites of PAHs have been associated with mediating the carcinogenic effects of the compounds, and data suggests that bioactivation may also be necessary for PAH-induced teratogenesis (Levin et al., 1978; Buters et al., 1999; Burdick et al., 2003; Hodson et al., 2007). Similar to the DLCs, PAH-induced embryotoxicity can also be mediated by the AHR (Billiard et al., 2006). However, unlike TCDD, the CYP1A mediated biotransformation and excretion of PAHs seems to play a protective role concerning these compounds (Hawkins et al., 2002; Uno et al., 2004; Wassenberg and Di Giulio, 2004a; Billiard et al., 2006; Matson et al., 2008a). Like CYP1A, CYP1B1, CYP1C1 and CYP1C2 are also inducible by multiple AHR agonists and may also play a role in PAH metabolism and teratogenicity (Wang et al., 2006; Timme-Laragy et al., 2007; Zanette et al., 2009).

Studies with CYP1B1 knockout mice, show that like CYP1A, CYP1B1 is able to biotransform some PAHs to reactive and mutagenic metabolites including benzo[a]pyrene (BaP), 7,12-dimethylbenz[a]anthracene, and dibenzo[a,l]pyrene (Buters et al., 1999; Harrigan et al., 2004). Within fish, CYP1B1 is inducible at both embryonic and adult stages in response to AHR agonists such as TCDD, PCB126, 3-methylcholanthrene, and BaP (Willett et al., 2006; Jonsson et al., 2007b; Timme-Laragy et al., 2008; Yin et al., 2008; Zanette et al., 2009). Although there is no current information on the role of CYP1B1 mediating metabolism in fish, it is possible that it does play an important role in biotransformation. CYP1C1 and CYP1C2 were first identified in scup, and there has not been a comparable CYP1C isoform detected in mammals (Godard et al., 2005). CYP1C1 is induced strongly in killifish after exposure to PCB126; however, CYP1C2 is much less responsive (Zanette et al., 2009). Although CYP1Cs are inducible by AHR agonists, their role in biotransformation has yet to be identified (Wang et al., 2006; Jonsson et al., 2007b; Timme-Laragy et al., 2007). Current data indicate that CYP1D1 is not inducible by PCB126 or TCDD and a potential role in PAH metabolism has not yet been established (Goldstone et al., 2009; Zanette et al., 2009).

Previous research on the ER population has shown that, in addition to their resistance to the lethal and teratogenic effects of DLCs and PAHs, they are also refractory to the induction of CYP1A catalytic activity in adults, larvae and embryos (Meyer et al., 2002; Wassenberg and Di Giulio, 2004b; Vogelbein and Unger, 2006). Molecular analysis of the AHR pathway, revealed that adult ER killifish dosed with AHR agonists lacked mRNA inducibility of AHR2, CYP1A and the aryl hydrocarbon receptor repressor (AHRR) (Meyer et al., 2003b). It is not fully understood whether this adaptation represents primarily a suppression of normal AHR responsiveness, or a dose shift in the initiation of the response. Additionally, the inducibility (mRNA and protein) of the more recently identified CYP1B1 and CYP1C1 have not yet been characterized in this population.

In this study, we investigated the differential response of killifish from the ER and from a reference site (KC) to both DLC and PAH AHR agonists. We characterized the mRNA induction of *AHR2*, *CYP1A*, *CYP1B1*, and *CYP1C1* in response to the AHR agonists, PCB126, BkF and BaP. Additionally, we tested whether the observed recalcitrant CYP1A phenotype that has been described in the ER killifish, represents a suppression of CYP1A induction, a shift in the initiation of the CYP1A response, or a combination of the two. Through this work we have extended our knowledge of the refractory CYP1A phenotype observed in the ER population to other members of the CYP1 family of enzymes, CYP1B1 and CYP1C1.

2. Materials and Methods

2.1 Fish Care

Adult killifish were collected from both a reference site on King's Creek (KC), off of the Severn River in Gloucester County, Virginia, (37°17'52.4"N, 76°25'31.4"W) and from a contaminated site on the Elizabeth River in Portsmouth, VA (36°48'27.48"N, 76°17'35.77"W) between March and September of 2006 and 2007. Fish were housed in 25 ppt artificial seawater (ASW; Instant Ocean, Mentor, OH) in a recirculating system with 150-L tanks. Fish were maintained at a temperature of 23-25°C on a photoperiod of 14:10 L:D. Fish were fed TetraMin® Tropical Fish Food (Tetra Systems, Blacksburg VA), and newly hatched brine shrimp (*Artemia*, Brine Shrimp Direct, Ogden, UT). Killifish embryos were obtained via *in vitro* fertilization of pooled oocytes with milt from several males. At 2 hours post fertilization (hpf), eggs were treated with 0.3% hydrogen peroxide (H₂O₂) to prevent infection and rinsed three times with clean ASW. Eggs were examined at 24 hpf for normal development and placed individually into 20-mL glass scintillation vials with 10 mL of treatment solution.

2.2 Chemicals and Exposure

Dimethyl sulfoxide (DMSO) and ethoxyresorufin were purchased from Sigma-Aldrich (St. Louis, MO). BaP, BkF, and PCB126 standards were acquired from Absolute Standards (Hamden, CT). Embryos from each population were exposed individually from 24 to 144 hpf to either the DMSO vehicle control, BaP (10, 100, 200 and 400 µg/L), BkF (0.1, 1, 100 and 300 µg/L) or PCB126 (0.01, 0.1, and 1 µg/L). In all of the treatment groups DMSO concentration was maintained at less than 0.03%. *In ovo* EROD (7-ethoxyresorufin-*O*-deethylase) activity was measured 96 hpf and cardiac deformities were assessed treatment-blind by light microscopy at 144 hpf. Embryos used for RNA analysis were placed into RNAlater (Applied Biosystems, Foster City, CA) at 144 hpf, flash frozen in liquid nitrogen, and stored at -80°C until time of extraction.

2.3 In Ovo EROD Assay

An *in ovo* EROD assay was used to measure CYP1A activity in the developing embryo by the method outlined in Nacci et al. (1998) and modified by Wassenberg and Di Giulio (2004a). Although the contributions of CYP1B1 and CYP1C1 on EROD activity has not been fully elucidated, zebrafish embryos dosed with β-naphthoflavone (BNF) and injected with a morpholino against CYP1B1 did not show any decreased EROD activity compared to non-injected controls (Timme-Laragy et al., 2008). This suggests that the majority of EROD activity is a product of CYP1A. Embryos were dosed individually from 24 to 96 hpf with the treatment solution containing 21 µg/L ethoxyresorufin. At 96 hpf, resorufin, the fluorescent product of CYP1A metabolism of ethoxyresorufin, was visualized within the bi-lobed urinary bladder of developing embryos via fluorescent microscopy (Zeiss Axioskop, 50x magnification using rhodamine red filter set). EROD activity was measured as intensity of bladder fluorescence and quantified digitally using IPLab software (BD Biosciences, Rockville, MD). *In ovo* EROD values are expressed as a percentage of the mean fluorescence of DMSO exposed reference site embryos. Individuals with severely deformed bladders or with fluorescence in areas other

than the bladder (such as the pericardial sac in some embryos with severe pericardial effusion) were excluded from *in ovo* EROD measurement.

2.4 Deformity Assessment

Embryos were examined blindly by light microscopy for heart elongation (tube heart) and pericardial effusion 144 hpf. The severity of heart elongation was ranked as a 0, 1, or 2 representing normal, mild, and severe deformities, respectively, as outlined in Matson et al. (2008a). Previous experiments with killifish confirm a strong inverse correlation between deformity score and hatch success (Matson et al., 2008a).

2.5 Quantitative real-time PCR

Samples were homogenized for 30 s with a sterile hand-held homogenizer. The RNA extractions were carried out according to the RNA-Bee protocol (Tel-Test Inc., Friendswood, TX). RNA quantity was analyzed using a NanoDrop ND-100 (NanoDrop Technologies, Wilmington, DE). CDNA was synthesized using the Omniscript RT kits (Qiagen, Valencia, CA) according to the manufacturer's instructions with 500 ng of RNA, random hexamers, and RNase inhibitor. The reaction was performed in a thermocycler for 1 hour at 37°C, and the resulting cDNA was diluted to a concentration of 2 ng/μL.

β-actin, AHR2, CYP1A, and CYP1B1 primers were designed using PrimerQuest software (Integrated DNA Technologies, Inc, www.idtdna.com). CYP1C1 primers were published previously by Wang et al. (2006). Primer efficiencies were tested to confirm that the target genes amplified at the same rate as the housekeeping gene, with maximal efficiency. Primer sequences are provided in Table 1.

QPCR was performed with a 25 μl reaction containing 200 nM of each primer, 12.5 μl 2x SYBR Green PCR Master Mix (Applied Biosystems), 9.5 μl dH₂O, and 4 ng cDNA template. The reaction was carried out on an Applied Biosystems 7300 Real-Time PCR System with the following thermal profile: 10 minutes at 95°C, 40 replicates of 15 seconds at 95°C, 1 minute at 60°C. A dissociation curve was calculated for each sample at the end of the run to confirm that a single product was formed during the reaction. Each of the samples was run in duplicate. Data analysis was carried out using ABI PRISM 7300 Sequence Detection System Software (Applied Biosystems). Gene expression was calculated using relative quantification by the $2^{-\Delta\Delta C_T}$ method of Livak and Schmittgen (2001). Target gene expression, following normalization to β-actin, was compared to its appropriate reference to estimate average fold induction for each experimental group, and each target gene. The fold change calculated for each of the biological replicate pools of two larvae was averaged across treatments. Six replicates were used to determine the final fold change averages and standard error per treatment per time point. The primary purpose of this study was to examine the induction of each gene and not to compare basal levels of gene expression. Therefore, each gene was analyzed individually and induction was determined based on the control expression of that gene. Likewise, our goal was to determine the gene induction of the KC and ER killifish in comparison to their own basal levels; therefore gene induction was determined based on the population specific control expression of that gene.

2.6 Statistical Analyses

All of the data collected were analyzed using SPSS ver. 15 (Chicago, IL, USA). The EROD and deformity data were both determined not to be normally distributed according to the Kolmogorov-Smirnov test. For analysis, these data were rank transformed and examined using a non-parametric analysis of variance (ANOVA) to test for significant differences among treatments. The mRNA fold changes were analyzed by an ANOVA, and Dunnett's *post hoc*

analysis was used to compare the treatment groups to the controls. Statistical significance was accepted at $p \leq 0.05$ for all tests.

3. Results

3.1 Dose dependent EROD activity and cardiac toxicity

EROD activity was significantly induced in KC embryos for all of the concentrations of BaP (10-400 $\mu\text{g/L}$) that were examined ($p < 0.001$, Fig. 1). This response was maximized at 10 $\mu\text{g/L}$ and began to decrease at the 200 $\mu\text{g/L}$ dose. The ER fish did not induce EROD activity at any of the concentrations of BaP that we examined, indicating that CYP1 induction is suppressed in the ER fish in response to BaP. KC embryos exposed to 400 $\mu\text{g/L}$ suffered from cardiac deformities ($p < 0.001$), but ER fish did not manifest any teratogenic effects after exposure.

BkF significantly induced EROD activity in the KC embryos for all of the concentrations that were tested in this study (0.1-300 $\mu\text{g/L}$, $p < 0.001$, Fig. 1). CYP1 activity in these fish was maximized at 1 $\mu\text{g/L}$ and began to decrease at the 300 $\mu\text{g/L}$ dose. EROD activity was also induced in ER embryos, with the initiation of the response occurring at 1 $\mu\text{g/L}$ ($p < 0.001$). This activity was maximized at the 300 $\mu\text{g/L}$ dose, but was significantly lower than the response observed in the KC embryos ($p < 0.001$). This suggests that the CYP1 response is both shifted and suppressed in the ER embryos in response to BkF. KC embryos exposed to 100 and 300 $\mu\text{g/L}$ developed cardiac deformities ($p < 0.001$), while no deformities were observed in the ER embryos treated with BkF.

PCB126 induced EROD activity in KC embryos at all of the concentrations that were tested (0.01-1 $\mu\text{g/L}$, $p < 0.001$, Fig. 1). This induction was maximized at 0.1 $\mu\text{g/L}$ and began to decrease at the 1 $\mu\text{g/L}$ dose. The ER embryos did not significantly induce EROD activity in any of the PCB126 exposure groups. PCB126 was teratogenic at the 1 $\mu\text{g/L}$ dose in KC embryos ($p < 0.001$); however, no teratogenic effects were seen in the ER embryos at any concentration.

3.2 Dose dependent mRNA induction

In the KC and ER DMSO control embryos, the ΔC_T s (C_T (target gene) - C_T (β -actin)) for *CYP1A* and *CYP1B1* were significantly different ($p < 0.05$) (data not shown). This indicates a difference in basal mRNA expression levels between these two populations. The fold induction of both *CYP1A* and *CYP1B1* were 40% higher in the KC DMSO control embryos compared to those from the ER (data not shown). *AHR2* and *CYP1C1* were not significantly different between the populations. No significant induction of *AHR2* was observed in either population in response to any of the compounds that we examined (Fig. 2).

ER embryos had significantly lower BaP-induced mRNA induction of *CYP1A* and *CYP1C1* compared to KC embryos ($p < 0.001$, Fig. 3). In KC embryos, *CYP1A* and *CYP1C1* were induced in response to exposure to 10, 100, 200 and 400 $\mu\text{g/L}$ BaP ($p < 0.01$). While ER embryos showed significant induction of *CYP1A* at the 400 $\mu\text{g/L}$ BaP dose ($p < 0.01$), *CYP1C1* was not induced in ER embryos at any of the BaP doses examined. *CYP1B1* was induced in both populations to levels above control in response to BaP exposure ($p < 0.05$).

ER embryos exposed to BkF did not significantly increase gene expression of *CYP1B1* or *CYP1C1*, and had reduced *CYP1A* induction compared to the KC population ($p < 0.001$, Fig. 4). There was significant induction of *CYP1A* in KC embryos after exposure to 1, 100, and 300 $\mu\text{g/L}$ BkF ($p < 0.001$), and *CYP1B1* after exposure to 100 and 300 $\mu\text{g/L}$ ($p < 0.001$). Although *CYP1A* was induced in ER embryos, it was only significant at the 100 and 300 $\mu\text{g/L}$ doses ($p < 0.001$).

Exposure to PCB126 resulted in a significant induction of *CYP1A*, *CYP1B1*, and *CYP1C1* in KC embryos to levels above control at the 0.1 and 1 µg/L doses ($p < 0.001$) (Fig. 5). There was no induction of any of the metabolic enzymes examined in ER embryos in response to PCB126 exposure.

4. Discussion

In this study, we helped to further characterize the resistance and the altered CYP1 phenotype observed in a population of killifish that inhabits a PAH-contaminated Superfund site on the Elizabeth River in VA. These data provide new information about the inducibility of *CYP1B1* and *CYP1C1* in killifish embryos born to parents captured from both a reference site and the PAH-contaminated Elizabeth River. Evaluation of *CYP1A*, *CYP1B1* and *CYP1C1* mRNA showed that all of the enzymes are inducible in reference site killifish exposed to BaP, BkF, and PCB126; however, the magnitude of induction did vary between the compounds. *AHR2* was not significantly inducible in either population for any of the treatment groups that we examined. *CYP1A* mRNA was induced in ER embryos in response to BkF and BaP; however, their level of induction was still 40 times lower than what was observed in the KC embryos. *CYP1B1* was only induced in ER embryos exposed to BaP, and interestingly the level of induction was not significantly different between the two populations. *CYP1C1* was not induced in ER fish in response to any of the treatments examined. Additionally, exposure of ER embryos to PCB126 did not elicit significant mRNA alterations in any of the AHR regulated genes that we examined.

In addition to showing decreased *CYP1* mRNA induction when exposed to AHR agonists, the ER killifish had dramatically reduced induction of CYP1 enzymatic activity compared to fish from the KC reference site. However, the observed response was not identical between the three chemicals. ER fish exposed to PCB126 and BaP showed no significant increase in CYP1 activity above controls, but exposure to the highest concentrations of BkF did result in a 7-fold increase in enzyme activity above control as measured by the *in ovo* EROD assay. Although significant, this induction was still much lower than the 34-fold maximum induction observed in KC embryos. Embryos born to parents of the ER population were also completely resistant to the teratogenic effects of both DLCs and PAHs, specifically PCB126, BkF, and BaP, at all exposure concentrations used in this study.

The initial work in the ER killifish population hypothesized that the resistance of these fish was associated with increased expression of P-glycoproteins, elevated glutathione concentrations, and the recalcitrant CYP1A phenotype (Van Veld et al., 1991; Van Veld et al., 1992; Cooper et al., 1999). Interestingly, later research with reference fish showed that CYP1A played a protective role and that the inhibition of the enzyme exacerbated the toxic effects of PAHs. Chemical inhibitors of CYP1A, including fluoranthene, carbazole, dibenzothiophene, and α -naphthoflavone, cause a synergistic increase in cardiac deformities observed in zebrafish, trout (*Oncorhynchus mykiss*), and killifish embryos when co-exposed with PAHs that act as inducers of the enzyme (Hawkins et al., 2002; Wassenberg and Di Giulio, 2004a; Wassenberg et al., 2005; Billiard et al., 2006; Billiard et al., 2008; Matson et al., 2008b). Additionally, knocking down the mRNA translation of CYP1A using morpholino antisense oligo technology resulted in an increase in PAH-induced teratogenesis when compared with non-injected and control morpholino injected zebrafish (Billiard et al., 2006) and killifish embryos (Matson et al., 2008a). These data suggest that the CYP1A mediated biotransformation and/or elimination of PAHs is protective from teratogenesis. However, morpholino knock-down of the AHR2 resulted in protection from PAH induced toxicity (Billiard et al., 2006; Clark et al., 2010). Jonsson et al. (2007a), found that knocking down AHR2 significantly reduced *CYP1* gene induction and embryotoxicity in zebrafish embryos dosed with PCB126 and TCDD. This leads to the conclusion that blocking AHR2 expression

protects embryos from teratogenicity by an unknown mechanism that may be independent of the effects on the downstream CYPs.

The data presented in this study indicate that the ER killifish are protected from the teratogenic effects of BaP, BkF and PCB126. The ER embryos also show substantially reduced levels of CYP1 protein activity and mRNA induction of *CYP1A*, *CYP1B1*, and *CYP1C1*, relative to embryos from the KC population in response to two PAHs and PCB126. These data provide support for the hypothesis that the resistance of the ER fish may be explained by altered regulation of the AHR signaling pathway. The induction of the CYP family of enzymes can be regulated by multiple transcription factors including the AHR, the constitutive androstane receptor (CAR), the pregnane X receptor (PXR), the retinoic acid receptor (RXR), and the peroxisome proliferator-activated receptor (PPAR) (Xu et al., 2005; Monostory and Pascucci, 2008). Stimulation of the β -adrenergic receptor can also modulate CYP1 activity *in vitro* by cyclic-AMP mediated pathway (Abdulla and Renton, 2005). Solhaug et al. (2005) showed that inhibitors of p53, extracellular signal-regulated kinase (ERK) and p38 mitogen activated protein kinases (MAPKs) altered the expression of *CYP1A* and the metabolism of BaP in Hepa1c1c7 cells. Alterations in the responsiveness or function of any of these transcription factors or cell signaling pathways may be responsible for the refractive CYP1 phenotype observed in the ER embryos.

Within the KC embryos, the magnitude of CYP1 enzymatic activity began to decrease at the chemical doses for which we observed the onset of cardiac deformities. These data suggest that reduced enzyme function is correlated with the teratogenic effects of DLCs and PAHs, as suggested by Wassenberg et al. (2004a). The induction of mRNA did not show a similar pattern. The levels of mRNA either remained stable or continued to increase even at doses where embryos were suffering from severe cardiac malfunction. The differences observed between the induction of mRNA and the translation to functional enzymatic activity may be due to the fact that increasing amounts of the dosing compound interferes with enzymatic activity. Additionally, the induction of *CYP1A*, *CYP1B1* and *CYP1C1* were variable between the three different compounds that we examined. This variability may be explained by differences in the binding affinities of the chemicals for the AHR (Billiard et al., 2002; Nguyen and Bradfield, 2008). PCB126, which out of the three chemicals tested here has the highest affinity for the AHR, had the maximum induction for all of the CYP1 genes. BkF and BaP demonstrated similar mRNA induction levels; however, BkF induced higher enzymatic activity. This may demonstrate a level of disconnect between mRNA levels and protein activity.

Another explanation for the differences between these AHR regulated genes, and for the fact that we did not observe any significant induction of *AHR2*, may be the time point that we chose to perform our mRNA extractions. *AHR2* expression has been shown in zebrafish embryos to have transient and moderate (2-4 fold) induction in response to both TCDD and PAHs (Tanguay et al., 1999; Timme-Laragy et al., 2007). These differences in PAH-induced mRNA expression over time may be explained by the biotransformation and/or excretion of the parent compound, and thus the removal of the AHR ligand. Research in zebrafish also shows that the *CYP1s* play a physiological role and their expression levels vary throughout embryonic development (Jonsson et al., 2007a). It is possible that in killifish, 144 hpf is too late to observe any expression differences for *AHR2*.

This study also provides new information about the mRNA induction of *CYP1A*, *CYP1B1* and *CYP1C1* in the ER adapted killifish population. *CYP1A* was induced in ER embryos exposed to the PAHs, BkF and BaP; however, the level of induction in the ER fish in response to both compounds was significantly lower than the levels observed in the KC population. These data indicated that the ER adaptation can be characterized by a suppression of normal induction of AHR regulated genes. The only enzyme for which we did not observe this, was for the induction

of *CYP1B1* in response to BaP exposure. *CYP1B1* has been identified in multiple fish species and may be involved in normal mammalian and teleost embryonic development through its role in retinoic acid synthesis (Choudhary et al., 2005; Yin et al., 2008). In mammals it is also involved in PAH metabolism, and *CYP1B1* (–/–) mice are protected against the carcinogenic effects of the PAHs, dimethylbenz[a]anthracene and dibenzo[a,l]pyrene (Buters et al., 1999; Shimada and Fujii-Kuriyama, 2004). However, while inhibiting the activity of *CYP1B1* seems to be protective from the carcinogenic effects of PAHs, it does not appear to play a role in DLC or PAH-induced teratogenesis (Timme-Laragy et al., 2008; Yin et al., 2008). Therefore, it is unlikely that the ability of ER embryos to induce high levels of *CYP1B1* in response to BaP affects their resistance to PAH-induced embryotoxicity. Although the fold inductions were not different between the two populations, KC DMSO control embryos displayed a 40% higher level of *CYP1B1* expression compared to ER embryos, indicating in spite of equal fold inductions ER embryos have lower total amount of the enzyme.

CYP1C1 has been identified in killifish and has constitutive expression that is higher than *CYP1A* in the brain, spleen, eye and gonad (Wang et al., 2006). *CYP1C2* and *CYP1D1* have also recently been identified in this species; however they both show limited inducibility by AHR agonists (Goldstone et al., 2009; Zanette et al., 2009). Although the functionality of *CYP1C1* is not yet understood, researchers have induced its mRNA expression in zebrafish with PCB126, BNF and ANF (Jonsson et al., 2007b; Timme-Laragy et al., 2007). *CYP1C1* was also inducible in killifish adults and embryos after exposure to BaP (Wang et al., 2006). In this study, we found that while *CYP1C1* was inducible in KC embryos exposed to both PAHs and DLCs, it was the only enzyme that did not induce in the ER population in response to any of the doses that were examined. As the functionality of *CYP1C1* in fish becomes better understood, it may provide greater insight into the mechanism of resistance in ER embryos.

5. Conclusions

This study advances our understanding of the mechanisms of DLC and PAH resistance observed in ER killifish. We examined the mRNA induction of the newly characterized metabolic enzymes *CYP1B1* and *CYP1C1*, and showed that they are inducible in reference site killifish embryos after exposure to PAHs (BaP and BkF) as well as to the DLC, PCB126. We also examined the inducibility of these enzymes in embryos born to parents of the adapted ER population. In response to exposure to PCB-126, ER embryos did not significantly induce mRNA of any of the CYP1 enzymes. Furthermore, in response to BaP and BkF, the induction of *CYP1A* was significantly reduced in ER embryos compared to those from the KC reference site. *CYP1B1* was induced to comparable levels after BaP exposure in the KC and ER embryos; however, the basal level of expression is lower in the ER population. We observed no significant mRNA induction of *CYP1C1* in ER embryos exposed to DLCs or PAHs. We have confirmed that ER embryos are resistant to the teratogenic effects of DLCs and PAHs. Compared to reference site embryos, ER killifish have significantly reduced induction of CYP1 enzymatic activity in response to AHR agonists at equal levels of exposure. It is possible that full induction of the CYP1 enzymes in the ER fish may be achievable if higher concentrations of the inducer were used. However, solubility constraints for these PAHs precluded our using significantly higher concentrations. These data suggest that the ER adapted phenotype can be described as a suppression of AHR inducibility, and future studies in this population should focus on signaling events upstream of the CYP1 enzymes.

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Fig. 1.

Dose response curve of CYP1 enzymatic activity as measured by the *in ovo* EROD assay (96 hpf) and cardiac deformities (144 hpf) for BaP, BkF, and PCB126. Main effects and the interaction of population and treatment (BaP, BkF, and PCB126 independently) on EROD induction were significant ($p < 0.001$). There was a significant increase in EROD activity compared to controls in KC embryos exposed to each concentration of BaP, BkF and PCB126 ($p < 0.001$). There was no significant increase in EROD activity in BaP- or PCB126- dosed ER embryos; however, there was a significant increase in ER embryos exposed to 1, 100, and 300 μg/L BkF ($p < 0.001$). Main effects and interaction of population and treatment (BaP, BkF, and PCB126 independently) on cardiac deformities were significant ($p < 0.001$). There was a significant increase in cardiac deformities in KC embryos exposed to 400 μg/L BaP, 100 and 300 μg/L BkF, and 1 μg/L PCB126, relative to controls ($p < 0.001$). There were no significant increases in cardiac deformities in ER embryos dosed with BaP, BkF, or PCB126. EROD data is represented as average percent induction of control $\pm$ SEM; $n \geq 10$. Cardiac deformities represented as average deformity score $\pm$ SEM; $n \geq 10$. “*” indicates a significant difference from control among EROD data. “†” indicates a significant difference from control among deformity data. “#” indicates a significant difference between populations.

Fig. 2.

AHR2 mRNA induction in KC and ER embryos exposed to BaP, BkF and PCB126. Statistical analysis was performed using an ANOVA and Dunnett's *post hoc* test to determine treatments that differed from controls. The main effects and interaction of population and treatment were not significant for BaP, BkF or PCB126. Data represented as average fold induction $\pm$ SEM; $n \geq 6$ pools of 2 embryos.

Fig. 3. mRNA induction of the metabolic enzymes CYP1A, CYP1B1, and CYP1C1 in KC and ER embryos exposed to BaP. Statistical analysis was performed using an ANOVA and Dunnett's *post hoc* test to determine treatments that differed from controls. For *CYP1A* and *CYP1C1* the main effects and interaction of population and BaP treatment were significant ($p < 0.001$). The main effect of treatment was significant for *CYP1B1*. *CYP1A* and *CYP1C1* were induced at levels above control in KC embryos dosed with 10, 100, 200, and 400 µg/L BaP ($p \leq 0.01$). *CYP1B1* was induced at levels above control in KC and ER embryos dosed with 10, 100, 200, and 400 µg/L BaP ($p < 0.05$). *CYP1A* was induced in ER embryos exposed to 400 µg/L BaP ($p < 0.01$). *CYP1C1* was not significantly induced in ER embryos for any of the BaP treatments

examined. Data represented as average fold induction $\pm$ SEM; $n \geq 6$ pools of 2 embryos. “*” indicates a significant difference from control among mRNA data. “#” indicates a significant difference between populations.

Fig. 4. mRNA induction of the metabolic enzymes CYP1A, CYP1B1, and CYP1C1 in KC and ER embryos exposed to BkF. Statistical analysis was performed using an ANOVA and Dunnett's *post hoc* test to determine treatments that differed from controls. For *CYP1A*, *CYP1B1* and *CYP1C1* the main effects and interaction of population and BkF treatment were significant ($p < 0.001$). *CYP1A* was induced at levels above control in KC embryos dosed with 0.1, 1, 100, and 300 µg/L BkF ($p < 0.001$) and in ER embryos dosed with 100 and 300 µg/L BkF ($p < 0.001$). *CYP1B1* and *CYP1C1* were induced at levels above control in KC embryos dosed with 100 and 300 µg/L BkF ($p < 0.001$). *CYP1B1* and *CYP1C1* were not significantly induced in ER embryos for any of the BkF treatments examined. Data represented as average fold

induction $\pm$ SEM; $n \geq 6$ pools of 2 embryos. “*” indicates a significant difference from control among mRNA data. “#” indicates a significant difference between populations.

Fig. 5. mRNA induction of the metabolic enzymes CYP1A, CYP1B1, and CYP1C1 in KC and ER embryos exposed to PCB126. Statistical analysis was performed using an ANOVA and Dunnett's *post hoc* test to determine treatments that differed from controls. For *CYP1A*, *CYP1B1* and *CYP1C1* the main effects and interaction of population and PCB126 treatment were significant ($p < 0.001$). *CYP1A*, *CYP1B1* and *CYP1C1* were induced at levels above control in KC embryos dosed with 0.1 and 1 µg/L PCB126 ($p < 0.001$). *CYP1A*, *CYP1B1* and *CYP1C1* were not significantly induced in ER embryos for any of the PCB126 treatments examined. Data represented as average fold induction $\pm$ SEM; $n \geq 6$ pools of 2 embryos. “*” indicates a significant difference from control among mRNA data. “#” indicates a significant difference between populations.

Table 1

cDNA target genes, GenBank identification, and primers used for QPCR

Gene	GenBank ID	Forward Primer (5'-3') Reverse Primer (5'-3')
<i>B-ACTIN</i>	AY735154	ACCACACATTTCTCATACACTCGGG CGCCTCCTTCATCGTTCCAGTTT
<i>CYP1A</i>	AF026800	AAGAATGGAGGACACTGGATGACC AGATTACAGGACAACACGACAGCG
<i>CYP1B1</i>	AF235140	CCAAAGAATACACAGAGGCAACGG ATGAAGGCATCCAGGTAAGGCAT
<i>CYP1C1</i>	DQ133571	TCTGGACGCCTTCATCTACGA GTGACGTCCGATGTGGTTGA
<i>AHR2</i>	U29679	ACCCAAGAGTTCCCATAGTTCAGTCC GCTCCATCTGCTTCTGTCTGCT