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Effect of CYP1A inhibition on the biotransformation of benzo[a]pyrene in two populations of *Fundulus heteroclitus* with different exposure histories

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Abstract

Sediment contaminated with polycyclic aromatic hydrocarbons (PAHs) from a Superfund site on the Elizabeth River (ER), in Portsmouth, VA is teratogenic to embryonic killifish (*Fundulus heteroclitus*) from reference sites. However, embryos born to a population of ER killifish are resistant to PAH-induced teratogenicity. Mechanisms underlying the resistance are unclear; however, ER killifish are refractory to induction of metabolic enzyme cytochrome P4501A (CYP1A), at the level of mRNA, protein and activity. The contaminated ER sediment is comprised of a complex mixture of PAHs with different mechanisms of toxicity. While many are inducers of metabolic enzymes involved in both phase I and phase II of biotransformation, some PAHs can also inhibit phase I enzymatic activity. Previous research has shown that co-exposure to PAHs with different modes of action can result in synergistic embryotoxicity (Billiard et al. 2008). Two of the abundant PAHs at the ER are fluoranthene (FL), a CYP1A inhibitor, and benzo[a]pyrene (BaP), an agonist for the aryl hydrocarbon receptor (AHR). Based on the ER resistant phenotype and the PAH mixture in the ER sediment, we hypothesized that the inhibition of CYP1A activity affects the teratogenicity of PAHs through a biotransformation-mediated mechanism. To examine this hypothesis, we compared the responses of killifish embryos born to parents from the ER and from a reference site (King's Creek (KC), VA) after a water-borne exposure to BaP (0-400 µg/L) in the presence or absence of FL (0 - 500 µg/L). Embryos were dosed from 24 to 120 hours post fertilization (hpf) and were analyzed for induction of CYP1 enzymatic activity as measured by the ethoxyresorufin-O-deethylase (EROD) assay, cardiac deformities, and BaP metabolic profile. KC embryos showed significant induction of CYP1 protein activity at all BaP concentrations examined. Co-exposure to 500 µg/L of FL significantly decreased CYP1 activity and increased cardiac deformities. ER embryos showed no change in CYP1 activity or cardiac deformities for any treatment. Significantly greater concentrations of BaP and BaP 9,10 -dihydrodiol were recovered from ER embryos compared to those from KC. Co-exposure with FL did not significantly alter the amount of BaP or the metabolites recovered in either population. These findings suggest that the teratogenicity observed by co-exposure to BaP and FL cannot fully be explained by alteration in BaP metabolism. This study also indicates that the metabolic adaptation observed in the ER killifish cannot be explained simply by the refractory CYP1 phenotype.

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Keywords

Fundulus heteroclitus; PAH; Benzo[a]pyrene; Fluoranthene; Elizabeth River

1. Introduction

Polycyclic aromatic hydrocarbons (PAHs) are prevalent environmental contaminants produced as a byproduct of organic combustion (Douben 2003). Natural sources of PAHs include oil seeps, volcanoes and forest fires. However the sources of PAHs that are of the greatest concern are anthropogenic, including vehicle exhaust, power generation, and oil contamination (Latimer and Zheng 2003). Unlike the majority of priority pollutants, in the last decade the concentration of PAHs in aquatic environments has increased primarily due to an increased amount of these chemicals in stormwater runoff and atmospheric deposition (Van Metre et al. 2000, Walker et al. 2004). This increase in PAH contamination is predicted to continue with the expansion of coastal development (Billiard et al. 1999, Van Metre and Mahler 2005, Chalmers et al. 2007). Therefore, more research is needed to determine the impact of these compounds in environmentally relevant exposures of aquatic organisms.

Due to the nature of their production, polycyclic aromatic hydrocarbons (PAHs) occur in the environment as complex mixtures. Although the current risk assessment model assumes additive toxicity, research has shown that mixtures can result in synergistic embryotoxicity, characterized specifically by cardiac malformations and pericardial effusion (Wassenberg and Di Giulio 2004a, Wassenberg et al. 2005, Billiard et al. 2006). This synergy is most evident when embryos are co-exposed to mixtures containing a combination of PAHs that are agonists for the aryl hydrocarbon receptor (AHR) and inhibitors of the cytochrome P450-1 (CYP1) family of enzymes.

Some PAHs have been characterized as AHR agonists based upon their ability to bind the receptor and induce many downstream genes including the CYP1 family of enzymes (Billiard et al. 2002, Billiard et al. 2004, Wang et al. 2006, Willett et al. 2006). CYPs are the primary phase I metabolizing enzymes responsible for the first monooxygenation of PAHs, which can aid in formation of water soluble metabolites that are easily excreted from the organism (Yang 1988, Parkinson 1996). Although metabolism is important for detoxification, the process results in a variety of reactive metabolic intermediates that can alter cellular function. In the case of benzo[a]pyrene (BaP), primary metabolites include epoxides, phenols, dihydrodiols, and quinones (Miller and Ramos 2001). The most highly studied metabolic pathway of BaP is the oxidation between C-7 and C-8 to form BaP-7,8-epoxide (7,8-ox), which can be hydrolyzed by microsomal epoxide hydrolase (mEH) into BaP-7,8-dihydrodiol. This compound can then be further oxidized by CYP1A to form BaP-7,8-dihydrodiol-9,10-epoxide (BPDE), which can form a highly mutagenic DNA adduct making it the most carcinogenic form of BaP (Varanasi et al. 1986, Dunn et al. 1987, Maccubbin et al. 1987, Ericson and Balk 2000). However, many of the other metabolites of BaP are also reactive and could be playing a role in both the carcinogenic and teratogenic effects of the compound. The intermediate epoxides can bind directly to nucleic acids and proteins, while quinones can undergo electron redox cycling with their semiquinone radicals leading to the formation of the superoxide anion ($O_2 \cdot^-$), hydrogen peroxide (H_2O_2), and hydroxyl radical ($\cdot OH$) (Miller and Ramos 2001, Burdick et al. 2003).

The Elizabeth River (ER) is a sub-estuary of the James River, which is the southernmost tributary of the Chesapeake Bay. The ER was home to the Atlantic Wood Industries wood treatment facility from 1926-1992 (Huggett et al. 1992). The primary chemicals utilized by the company were the fungicide pentachlorophenol and creosote, a wood preservative made up primarily of PAHs. Concentrations of PAHs at the site are some of the highest observed

throughout the United States and have been measured in mean concentrations of 410 µg/g dry weight in the sediment (Bieri et al. 1986, Vogelbein et al. 2008). Two of the most abundant PAHs at the ER are BaP (an AHR agonist and CYP1 inducer) and fluoranthene (FL; a weak AHR agonist and non-competitive inhibitor of CYP1). Although the sediment is both acutely lethal and teratogenic to a variety of aquatic organisms, there is a population of *Fundulus heteroclitus* (killifish) that is thriving at the site. This population of killifish provides researchers with a unique opportunity to examine the mechanisms of PAH toxicity and elucidate the methods of adaptation to protect organisms from these effects.

Killifish are small teleost fish that inhabit marshes along the Atlantic coast. These fish have limited migration making them an excellent species for examining the effects of natural and anthropogenic environmental changes on biological responses (Bigelow and Schroeder 1953, Duvernell et al. 2008). Populations of killifish have been found thriving in a number of polluted estuaries including the ER, revealing their ability to develop resistance to a variety of toxicants (Nacci et al. 1999, Mulvey et al. 2002, Nacci et al. 2002a, Meyer and Di Giulio 2003, Wirgin and Waldman 2004, Burnett et al. 2007). Like many of the other adapted populations, the ER killifish are refractory to cytochrome P450-1(CYP1) induction when exposed to agonists for the aryl hydrocarbon receptor (AHR), such as PAHs.

Historically PAHs have been studied for their role in carcinogenesis; however, multiple studies indicate that PAHs can also be embryotoxic in a variety of fish species (Incardona et al. 2004, Wassenberg and Di Giulio 2004a, Incardona et al. 2006, Billiard et al. 2008). Similar to the carcinogenic properties of these compounds, it is possible that the teratogenic effects are the result of their biotransformation and the production of reactive metabolites. Research in medaka (*Oryzias latipes*) showed that some of the earliest embryonic tissues formed are capable of metabolizing and redistributing BaP throughout the yolk, biliary system and gastrointestinal tract (Hornung et al. 2007). Therefore, it is likely that factors influencing biotransformation will have an effect on teratogenicity.

We used both the refractive CYP1 phenotype of the ER and the chemical CYP1A inhibitor FL to examine the effect of CYP1 inhibition on the biotransformation and teratogenicity of BaP. Willett et al. (2001) found that killifish adults co-exposed to BaP and FL had 55% lower CYP1 activity than those exposed to BaP alone. Zebrafish embryos co-exposed to BaP and FL had decreased induction of CYP1 activity and a synergistic increase in pericardial effusion (Matson et al. 2008b). The first goal was to examine the synergistic teratogenicity observed in reference embryos exposed to PAH mixtures. In an attempt to explain this synergism, two alternate working hypotheses for this study are that CYP1 inhibition results in synergistic acute toxicity by shifting the metabolism of BaP or by preventing metabolism of the parent BaP, resulting in persistent activation of the AHR, similar to dioxin (Hahn 1998, Mandal 2005).

The second goal of this work was to examine the ability of the ER embryos to biotransform PAHs in order to further examine the role of the refractory phenotype and to examine how this adaptive inhibition of CYP1 activity differs from chemical inhibition. Our hypotheses were that this lack of CYP1 activity would either extend the half-life of the parent compound, preventing the formation of toxic metabolites, or shift the metabolism of BaP towards a more benign metabolite.

2. Materials and Methods

2.1 Fish Care

Adult killifish were collected from both a reference site at King's Creek, 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, Virginia (36°48'27.48"N, 76°17'35.77"W). Adult fish were depurated for 12

months in a recirculating system containing artificial seawater (ASW 25 ppt) prepared from Instant Ocean® (Mentor, OH). Fish were kept at 23–25° C on a photoperiod of 14:10 L:D, and fed daily a diet of TetraMin® Tropical Fish Food (Tetra Systems, Blacksburg VA, USA), and newly hatched brine shrimp (Artemia, Brine Shrimp Direct, Ogden, UT). Killifish embryos were obtained from *in vitro* fertilization of pooled oocytes mixed with pooled milt from multiple males. Embryos were examined 24 hours post fertilization (hpf) for viability and placed individually into 20 mL glass scintillation vials with 10 mL of treatment solution.

2.2 Chemicals and Exposure

Dimethyl sulfoxide (DMSO), BaP, FL, and ethoxyresorufin were purchased from Sigma-Aldrich (St. Louis, MO). Two co-exposure experiments were conducted with killifish embryos. In the first experiment embryos were exposed to a range of FL concentrations (0, 50, 100 and 500 µg/L) with or without 100 µg/L BaP. In the second experiment embryos were exposed to a range of BaP concentrations (0, 10, 50, 100, 200, and 400 µg/L) with or without 500 µg/L FL. Embryos from each population were exposed individually to the treatment solution or to the DMSO vehicle control from 24 to 120 hpf (n = 30). In all of the treatment groups DMSO concentration was maintained at less than 0.03%. At 120 hpf, embryos were removed from the dosing solution and placed into vials containing clean ASW. *In ovo* EROD (7-ethoxyresorufin-O-deethylase) was measured at 120 hpf and cardiac deformities were assessed treatment-blind by light microscopy 168 hpf. Embryos used for metabolic analysis were flash frozen 120 hpf in liquid nitrogen and stored at -80°C until time of extraction.

2.3 In Ovo EROD Assay

In ovo EROD assay was used to measure CYP1 activity in the developing embryo by the method outlined in Nacci et al (1998) and modified by Wassenberg and Di Giulio (2004a). Embryos were dosed individually from 24 to 120 hpf in 20 mL glass scintillation vials with 10 mL of treatment solution made with ASW (20 ppt) containing 21 µg/L ethoxyresorufin. At 120 hpf, embryos were placed in clean ASW and embryos were visualized by fluorescent microscopy (Zeiss Axioskop, 50x magnification using rhodamine red filter set). EROD induction was measured as intensity of resorufin fluorescence in the bi-lobed urinary bladder and quantified digitally by IP lab software (Scanalytics, Inc., Fairfax, VA). *In ovo* EROD values are expressed as a percentage of the mean fluorescence of DMSO exposed reference site embryos. Individuals with deformed bladders or with fluorescence in areas other than the bladder (such as the pericardial sac in some embryos with severe pericardial edema) were excluded from *in ovo* EROD measurement.

2.4 Deformity Assessment

Embryos were scored blind for heart elongation (tube heart), pericardial effusion, and hemorrhaging at 168 hpf. Heart deformities were found to be the most sensitive endpoint scored, so this endpoint was used for further analysis. Heart elongation severity was ranked as a 0, 1, or 2 representing no deformities, mild and severe deformities respectively as outlined in Matson et al (2008a). Results for each treatment were represented as an average of the individual scores.

2.5 Embryonic Extractions and Chemical Analysis

Ten embryos were pooled and homogenized in 15 µL methanol/mg tissue with a Polytron® for 30 seconds as outlined in Hawkins et al (2002). BaP and its metabolites were extracted with 600 µL methanol and passed through a 0.2 µm Acrodisc® nylon filter (PALL Life Sciences, Ann Arbor, MI). Samples were dried down under nitrogen and the residue was dissolved in 50 µL of HPLC grade acetonitrile. Sample extracts containing BaP and its metabolites were analyzed by injecting 1 µL onto a C-18 reverse phase ultra pressure liquid chromatography

(UPLC) column (ACQUITY UPLC™ BEH C18 1.7 μ m 2.1 $\times$ 50 mm). Separation of metabolites was achieved at a flow rate of 0.25 ml/min at 28°C using a 3-step gradient elution program as follows: 65:35 to 40:60 0.3% formic acid in water: acetonitrile in 6 minutes, to 0:100 in 9 minutes, and finally to 65:35 in 10 minutes (Zhu et al. 2008). Chromatograms were analyzed by mass spectrometry for the presence of the internal standard 6-OH chrysene, and for the presence of BaP and specific metabolites (BaP-7,8,9,10-tetrahydrotetrol, BaP-7,8-dihydrodiol, BaP-9,10-dihydrodiol, BaP-1,6-dione, BaP-3,6-dione, BaP-6,12-dione, BaP-9-OH and BaP-3-OH). The concentrations of the metabolites were determined by calculating the ratio of the metabolite to the concentration of the internal standard recovered.

2.6 Statistical Analyses

Data were analyzed using SPSS ver.15 (Chicago, IL). 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 metabolism data was determined to be normally distributed, and therefore was analyzed using an ANOVA and a Bonferroni-corrected post hoc comparison. Statistical significance was accepted at $p \leq 0.05$ for all tests.

3. Results

3.1 Dose-dependent EROD activity and cardiac deformities after co-exposure to BaP and FL

EROD activity was significantly affected by population ($p < 0.001$) and treatment ($p < 0.001$). In the KC embryos exposure to BaP increased EROD activity, and that activity was inhibited by each of the concentrations of FL examined (Figure 1A and Figure 2A). Relative to the KC controls, exposure to BaP had no significant effect on EROD activity in the ER embryos, either in the presence or absence of FL (Figure 1B and Figure 2B). The severity of cardiac deformities was significantly different between both populations ($p < 0.001$) and treatments ($p < 0.001$). Cardiac deformities greater than controls were observed in the KC embryos co-exposed to 500 μ g/L FL and each of BaP concentrations (10-400 μ g/L) (Figure 1A and Figure 2A). No significant deformities were observed in the KC embryos exposed to any concentration of BaP or FL alone or in the ER embryos for any of the treatments examined (Figure 1B and Figure 2B).

3.2 Recovery of BaP and BaP metabolites after co-exposure to BaP and FL

Chemical analysis revealed a significant interaction between population and treatment for the amount of BaP and BaP 9,10-dihydrodiol recovered from the embryos ($p < 0.05$) (Figure 3.4). Greater amounts of BaP and BaP 9,10-dihydrodiol were recovered from ER embryos when compared to KC embryos. Recovery of 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 in all of the treatments, therefore no conclusions could be made concerning their prevalence within the embryos. From the chemicals that were recovered, the great majority was identified as parent BaP in both the KC and the ER populations. FL demonstrated no observable effects on the recovery of BaP or any of the metabolites examined.

4. Discussion

We utilized the chemical CYP1A inhibitor FL and the refractive CYP1 phenotype of the ER killifish population to examine the effect of CYP1 inhibition on the biotransformation and teratogenicity of BaP. One hypothesis was that lack of CYP1 activity through either chemical inhibition or genetic adaptation would extend the half-life of the parent compound. This could

prevent toxicity by decreasing the formation of toxic metabolites, but could also allow for persistent activation of the AHR, resulting in toxicity similar to dioxin (Hahn 1998, Mandal 2005). The second hypothesis was that the inhibition of CYP1A could shift the biotransformation of BaP towards an alternate metabolic pathway, possibly through comparable enzymes such as CYP1B1 or CYP1C1 (Shimada and Fujii-Kuriyama 2004, Wang et al. 2006). This may result in a shift towards the formation of more toxic or more benign BaP metabolites. In this study we observed a significant interaction of BaP treatment and population in the amount of BaP recovered from the extractions. More parent BaP was recovered from the ER embryos than from the KC, suggesting that the ER fish may metabolize BaP at a slower rate compared to reference site fish. This difference could be a contributing factor in the protection of the ER embryos from the teratogenicity of BaP by either preventing the production of a reactive metabolite or by slowing the production until a stage when the embryos are less susceptible to cardiac deformities.

Both phase I and phase II metabolism are utilized for the detoxification and the elimination of BaP from the organisms; however, the intermediate products of phase I can result in toxicity that is greater than the parent compound. The primary phase I metabolites of BaP include dihydrodiols, phenols, quinones and epoxides, with the quinones and the epoxides being the most reactive (Miller and Ramos 2001). There was a significant interaction of BaP treatment and population regarding the recovery of the metabolite BaP-9,10-dihydrodiol, the pattern being that a greater amount of the metabolite was recovered from the ER embryos compared to those from the KC. These data indicate that the ER embryos are significantly different from the KC embryos in the way that they metabolize BaP. This shift in metabolism could contribute to the observed resistance to toxicity observed in the ER embryos and warrants further investigation. Studies examining the mutagenicity of BaP in TA98 and TA100 *Salmonella* strains showed that BaP-9,10-dihydrodiol is not mutagenic on its own (Levin et al. 1978). That study also showed that unlike the 7,8-dihydrodiol, the 9,10-dihydrodiol was not generally activated to a more mutagenic compound. Further transformation most often results in the addition of an epoxide at the 7,8 position resulting in the formation of the 9,10-diol,7,8-epoxide which is much less mutagenic than when the epoxide is located at the 9,10 position as occurs in the mutagenic metabolite BPDE (Levin et al. 1978, Stegeman and James 1985).

In a study performed by Zhu et al (2008), killifish adults were injected with BaP and the bile was analyzed for the presence of glucuronic acid and sulfate conjugated and free metabolites. In that study BaP-9,10-dihydrodiol was not detected; however, the metabolites 7,8-dihydrodiol, 1,6-dione, 3,6-dione and 3-OH were all recovered at elevated concentrations. One explanation for the differences in the metabolites recovered between these two studies may be that we were only able to examine the presence of free metabolites due to the limited amount of available tissue within the embryos. Additionally, these two studies may simply reflect differences in the metabolism of embryos versus adult fish. HPLC analysis of BaP metabolites formed by metabolites from killifish eleutheroembryos showed production of both the 7,8 and the 9,10-dihydrodiols (Binder et al. 1985). Petersen and Kristensen (1998) indicated that zebrafish (*Brachydanio rerio*) embryos had a lower rate of biotransformation and elimination of PAHs than their juvenile/adult counterparts. This difference in bioconcentration kinetics was considered the primary reason for the increased sensitivity to toxicity during early life stages.

Previous researchers have shown an ability of CYP1A inhibitors to alter the biotransformation of PAHs. Pre-exposure to the fungicide and CYP1A inhibitor clotrimazol increased the bioconcentration factor of BaP in gizzard shad (*Dorosoma cepedianum*) (Levine et al. 1997). Larval rainbow trout (*Oncorhynchus mykiss*) exposed to CYP1A inhibitor, α -naphthoflavone (ANF), exhibited a decrease in the concentration of polar hydroxylated metabolites and an increase in the recovery of less polar metabolites and parent retene (7-isopropyl-1-methyl

phenanthrene), a substituted PAH and weak inducer of CYP1 activity (Hodson et al. 2007). In the current study, there was no significant effect observed of FL on the recovery of parent BaP or any of the metabolites in either population. Although surprising, this result could be attributed to the low recovery of BaP metabolites in the study. All of the diones and the phenols were below detection limits; therefore, FL could have caused a shift in the metabolic profile of BaP that was not detectable in these experiments. Additionally, the increased deformities observed in the co-exposure could be due to increased biotransformation of FL in the presence of BaP. This was not studied in this experiment, but may be a focus of future work.

Palmqvist et al (2008) showed that *Capitella* sp. I, a marine polychaete worm, both accumulated and biotransformed FL over time to a greater degree than BaP after co-exposure even though the animals were exposed to a 1:1 molar ratio of the compounds. This study also showed that co-exposure to FL increased the recovery of water soluble metabolites. Therefore there is a possible role of FL in increasing phase I and/or phase II metabolism in the worms, resulting in the formation of more soluble metabolites. Although we did not see the same effect of FL on the alteration in BaP metabolism, a caveat to this study is that due to the fact that an AHR homologue has not been found in polychaetes, CYP induction may be regulated by a different mechanism and therefore metabolize PAHs differently in invertebrates (Palmqvist et al. 2008). In our experiments, the increase in CYP1 activity by BaP could have increased the metabolism of FL resulting in the increased toxicity of the co-exposure. However, this is unlikely considering that similar cardiac deformities were observed in both zebrafish and killifish embryos whose CYP1A activity was knocked down with morpholino technology and then subsequently exposed to the PAH-type AHR inducer β -naphthoflavone (Billiard et al. 2006, Matson et al. 2008a).

Due to the nature of PAH contamination in the environment and the current mode of assuming additivity in risk assessment, toxicologists have raised multiple concerns about the potential synergistic toxicity of PAH mixtures (Wassenberg and Di Giulio 2004a, Hodson et al. 2007, Timme-Laragy et al. 2007, Billiard et al. 2008). Embryos from trout, zebrafish and killifish all develop a synergistic increase in cardiac deformities after co-exposure to PAHs, to CYP1 inhibitors and AHR agonists (Wassenberg and Di Giulio 2004a, b, Wassenberg et al. 2005, Billiard et al. 2006, Hodson et al. 2007). This study increases these concerns by showing that this synergistic toxicity occurs with real world PAHs in a model estuarine fish. In the FL dose-response experiments, co-exposure with each of the concentrations of FL examined (50-500 $\mu\text{g/L}$) significantly inhibited the CYP1 activity of BaP in the KC embryos. However, significant cardiac deformities were only observed in the KC embryos co-exposed to BaP and the highest concentration of FL examined (500 $\mu\text{g/L}$), indicating a dose difference between the threshold for inhibition of CYP1 activity and the onset of cardiac toxicity. In the BaP dose response experiment, each dose examined significantly induced EROD induction in the KC embryos, but not 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 PAHs. Co-exposure to 500 $\mu\text{g/L}$ FL significantly reduced EROD induction and increased cardiac deformities in the KC embryos, but not the ER embryos, at each dose of BaP. These data support the role of FL as an inhibitor of CYP1 activity in reference site embryos and the importance of CYP1 activity in the protection from the teratogenicity of BaP. It also confirms the resistance of the killifish from the Elizabeth River to PAH toxicity and their refractory CYP1 phenotype as previously reported by Meyer et al (2002a).

In an attempt to explain this synergism, two alternate working hypotheses for this study are that CYP1 inhibition results in synergistic acute toxicity by shifting the metabolism of BaP or by preventing metabolism of the parent BaP, resulting in persistent activation of the AHR, similar to dioxin (Hahn 1998, Mandal 2005). Although we were surprised by the lack of

observable effect of FL on the metabolism of BaP, we acknowledge that the story is more complicated. From these data we are able to conclude that FL does not cause synergistic toxicity with BaP by extending the half-life of the parent compound. Admittedly, the limited amount of tissue that can be obtained from embryos may have reduced our ability to detect the impact of FL on a shift in the metabolic pattern of BaP. However, the teratogenic effects of these complex mixtures are of primary interest and therefore the study cannot be repeated in adults or juveniles. Future studies focused on elucidating the role of metabolism in the synergistic developmental toxicity of BaP and FL may have to utilize greater numbers of embryos or employ more sensitive methods, such as multi-photon laser scanning microscopy outlined by Hornung et al (2004).

Additionally, this study examines the role of chronic PAH exposure on the adaptation of populations of organisms. The ER embryonic, larval and adult killifish show significantly reduced levels of mRNA induction of multiple genes in the AHR pathway including AHR2, the AHR repressor (AHRR) and CYP1A (Meyer et al. 2003). These killifish also show lower levels of CYP1 enzymatic activity (Bello et al. 2001, Meyer and Di Giulio 2002, Meyer et al. 2002b, Wassenberg and Di Giulio 2004b). This adaptation is partially heritable through the F1 generation, and is thought to play a role in mediating their resistance to teratogenicity and lethality caused by PAHs (Meyer and Di Giulio 2002, Meyer et al. 2002b, Ownby et al. 2002, Meyer and Di Giulio 2003). In addition to their lack of CYP1A inducibility, the killifish from the Elizabeth River possess a variety of other biochemical differences that might also account for their resistance to toxicity. Some of these differences include increased resistance to the toxicity of t-butyl hydroperoxide (a model prooxidant), higher basal total oxyradical scavenging capacity (TOSC) values, increased concentrations of both total glutathione and glutathione disulfide, and increased protein levels of manganese superoxide dismutase (MnSOD) (Meyer and Di Giulio 2002, Meyer et al. 2002a, Bacanskas et al. 2004).

Our goal was to examine the ability of the ER embryos to biotransform PAHs in order to further examine the role of the refractory phenotype and to examine how this adaptive inhibition of CYP1 activity differs from chemical inhibition. Our hypotheses were that this lack of CYP1 activity would either extend the half-life of the parent compound, preventing the formation of toxic metabolites, or shift the metabolism of BaP towards a more benign metabolite. Our data suggest that both are true. The ER embryos have adapted in a manner that both extends the half-life of BaP and shifts the metabolism towards BaP-9,10 dihydrodiol, either by slowing down its elimination or by pushing BaP away from other metabolites, including BaP-7,8 dihydrodiol and the highly mutagenic BPDE. These results are supported by work done by Nacci et al. (2002b) in a resistant killifish population that reside in New Bedford Harbor (NBH). Similar to the ER population, the NBH killifish are recalcitrant to CYP1A induction and are resistant to the acute toxicity of dioxin-like compounds. After exposure to BaP, the NBH killifish had lower levels of EROD induction and formation of the BPDE-DNA adduct. This work suggests that the resistance of the NBH population may be mediated by a decrease in the formation of the toxic BPDE metabolite.

The fact that the metabolic profile of the ER embryos and the KC embryos co-exposed to FL were not the same confirms that the resistance of the ER embryos relies on other mechanisms in addition to decreased CYP1A activity. Recent research suggests a metabolic role for other enzymes in the CYP1 family (1B1 and 1C1); however, the ability of FL to inhibit their activity has not yet been elucidated (Harrigan et al. 2004, Wang et al. 2006). To date, FL has only been defined as a post translational inhibitor of CYP1A, while the refractory phenotype of the ER embryos is pre-translational in that none of the CYP1 enzymes are induced at the mRNA level (Willett et al. 1998, Meyer et al. 2003). It is therefore possible that the difference in metabolic profile and acute toxicity observed in the ER embryos is due to an alteration further upstream than P450s in the cellular pathway, such as with the AHR itself.

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A.

B.

Figure 1.

FL dose response of CYP1 enzymatic activity as measured by *in ovo* EROD assay and cardiac deformities (120 hpf). Killifish embryos were simultaneously exposed 24 - 120 hpf to waterborne concentrations of 21 µg/L ethoxyresorufin and either DMSO (vehicle control, 0.03% v/v), FL (50-500 µg/L), and/or 100 µg/L BaP. (A) The increasing deformity score represents increasing severity of cardiac deformities (CD) (0 = normal; 1 = mild deformities; 2 = severe deformities (see Figure 3.3)). Data are represented as the average deformity score $\pm$ SEM from three replicate experiments; $n=25$. In the KC population there was a significant interaction effect of BaP, FL and population ($p < 0.01$; non-parametric ANOVA). *In ovo* EROD data are represented as the average percentage of KC control induction $\pm$ SEM from three

replicate experiments; $n = 25$. In the KC population there was a significant interaction effect of FL, BaP, and population ($p < 0.01$; non-parametric ANOVA). (B) No significant increases in deformities were observed in the ER embryos for any of the treatments examined. Relative to the KC controls, exposure to FL had no significant effect on EROD induction in the ER embryos, either in the presence or absence of BaP. No significant increases in deformities were observed in the ER embryos for any of the treatments examined.

A.

B.

Figure 2.

BaP dose response of CYP1 enzymatic activity as measured by *in ovo* EROD assay and cardiac deformities (120 hpf). Killifish embryos were dosed by waterborne exposure 24-120 hpf to 21 µg/L ethoxyresorufin and either DMSO (vehicle control < 0.03% v/v) or BaP (10 - 400 µg/L) with or without co-exposure to 500 µg/L FL. (A) The increasing deformity score represents increasing severity of cardiac deformities (CD) (0 = normal; 1 = mild deformities; 2 = severe deformities (see Figure 3.3)). Data are represented as the average deformity score $\pm$ SEM from three replicate experiments; n=25. In the KC population there was a significant interaction effect of BaP, FL and population ($p < 0.01$; non-parametric ANOVA). *In ovo* EROD data are represented as the average percentage of KC control induction $\pm$ SEM from three replicate

experiments; $n = 25$. In the KC population there was a significant interaction effect of FL, BaP, and population ($p < 0.01$; non-parametric ANOVA). (B) Relative to the KC controls, exposure to FL had no significant effect on EROD induction in the ER embryos, either in the presence or absence of BaP. No significant increases in deformities were observed in the ER embryos for any of the treatments examined.

	0 $\mu\text{g/L}$ FL	500 $\mu\text{g/L}$ FL	
0 $\mu\text{g/L}$ BaP			0
10 $\mu\text{g/L}$ BaP			1
50 $\mu\text{g/L}$ BaP			
100 $\mu\text{g/L}$ BaP			
200 $\mu\text{g/L}$ BaP			2
400 $\mu\text{g/L}$ BaP			

Figure 3.

Cardiac deformities observed in KC embryos following exposure to DMSO vehicle control or BaP (10 - 400 $\mu\text{g/L}$) with or without co-exposure to 500 $\mu\text{g/L}$ FL (168 hpf)

A.

B.

Figure 4.

Parent BaP and BaP-9,10-dihydrodiol recovered and identified by UPLC/MS (120 hpf). Dashed lines represent detection limits (1 µg/L for BaP and 0.1 µg/L for BaP-9,10-dihydrodiol). There was a significant interaction of population and treatment on recovery of both BaP (A) and BaP-9,10-dihydrodiol (B) ($p < 0.05$; ANOVA). Significantly higher concentrations of BaP and BaP-9,10-dihydrodiol were recovered from ER embryos exposed to BaP compared to embryos from KC ($p < 0.05$). Data represented as an average mass of chemical recovered per embryo $\pm$ SEM from two replicate experiments; $n = 10$ pools of 10 embryos.