

The Role of Biotransformation on the Toxic Effects of Polycyclic Aromatic Hydrocarbons
in the Atlantic Killifish (*Fundulus heteroclitus*)

by

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Dissertation submitted in partial fulfillment of
the requirements for the degree of Doctor
of Philosophy in the Department of
Nicholas School of the Environment in the Graduate School
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ABSTRACT

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Abstract

Polycyclic aromatic hydrocarbons (PAHs) are prevalent contaminants in aquatic environments, and exposure can elicit a variety of toxic effects including teratogenicity, genotoxicity and carcinogenicity. Many PAHs are agonists for the aryl hydrocarbon receptor (AHR), which is involved in mediating their teratogenic and carcinogenic effects by unknown mechanisms of action. When activated, the AHR initiates the transcription of a variety of genes involved in both phase I and phase II metabolism, including the monooxygenase cytochrome P450s (CYP1A, CYP1B1, CYP1C1). The biotransformation of PAHs is necessary for their detoxification and excretion; however, oxidation by CYP1A results in the conversion of the parent PAH to reactive metabolic intermediates that can covalently bind to nucleic acids and proteins, or cause oxidative stress. There is a population of killifish (*Fundulus heteroclitus*) from a PAH contaminated site on the Elizabeth River (ER) in Portsmouth, Virginia whose embryos, larvae, and adults are refractory to the induction of CYP1A activity, protein or message when exposed to AHR agonists. The embryos and larvae of the ER population are resistant to acute PAH-induced teratogenicity and lethality. However, wild caught ER adults at the site have a high prevalence of hepatic and pancreatic neoplasms, suggesting that their resistance to intermediate and chronic PAH toxicity requires further investigation. The contaminated sediment at the ER is comprised of a complex mixture of PAHs with different mechanisms of action. Although there is limited

information concerning PAH mixtures, the current assumption for risk assessors is that their toxicity is additive. In studying the effects of the mixture of PAHs at the Elizabeth River, research has shown that co-exposure of reference site killifish embryos to PAH-type CYP1A inhibitors causes a synergistic increase in the embryotoxicity of PAH-type AHR agonists and PAH-contaminated sediment extract. This dissertation utilized the resistant Elizabeth River killifish, chemical inhibitors, and morpholino technology to determine how the chemical and molecular inhibition of CYP1 enzymes affects the biotransformation of PAHs and ultimately their teratogenicity, genotoxicity, and carcinogenicity. Toward these objectives, the mRNA inductions of the newly sequenced metabolic enzymes, CYP1B1 and CYP1C1, were characterized in embryos born to ER and reference site killifish after exposure to multiple AHR agonists. Embryos from both populations were also co-exposed to the PAHs benzo[a]pyrene (BaP; AHR agonist) and fluoranthene (FL; CYP1A inhibitor). Reference site embryos were injected with an antisense morpholino to knockdown translation of CYP1A and exposed to BaP. After chemical exposure, embryos were examined for CYP1 activity, teratogenesis, and BaP biotransformation. To examine the effects of early-life exposure on the chronic toxicity of PAHs, larval offspring of ER and reference site killifish were exposed to BaP, and examined for CYP1 activity, mitochondrial and nuclear DNA damage, and BaP biotransformation. Three and nine months after exposure, livers of exposed juveniles were analyzed histologically. The mRNA

inductions of the metabolic enzymes, *CYP1A*, *CYP1B1*, and *CYP1C1*, were significantly reduced in ER embryos exposed to AHR agonists compared to those from a reference site. CYP1 enzymatic activity was also reduced in these embryos, and they were resistant to the teratogenic effects of PAH both in single and combined exposures. These data suggest that the ER population have adapted an altered responsiveness of the AHR signaling pathway. Higher concentrations of parent BaP were recovered from the embryonic and larval offspring of the ER killifish. Additionally a higher concentration of the metabolite BaP-9,10-dihydrodiol was recovered from ER embryos, suggesting that the adaption alters PAH biotransformation and excretion in this population. ER larvae and juveniles were more resistant to PAH-induced genotoxicity and carcinogenicity compared to the reference site population, indicating that the adaptive resistance of the ER population to PAH-induced teratogenicity has also resulted in a resistance to both genotoxicity and carcinogenicity. Co-exposure of the reference site embryos to the PAH mixture and morpholino knockdown of *CYP1A* did increase teratogenicity; however, no interpretable alterations in BaP biotransformation were observed. Chemical and molecular alterations of metabolic enzymes can dramatically affect the toxic outcomes of exposure to PAHs. The study of biotransformation is an important step in understanding the mechanisms of PAH-induced toxicity, and evaluating the risks in aquatic ecosystems.

Dedication

This is dedicated to my amazing family and friends.

Thank you.

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List of Abbreviations

AHR- aryl hydrocarbon receptor
AWI- Atlantic Wood Industries
BaP- benzo[a]pyrene
BkF- benzo[k]fluoranthene
Ctrl- control
CYP- cytochrome P450
DLC- dioxin like compounds
DMSO- dimethyl sulfoxide
ER- Elizabeth River
EROD- ethoxyresorufin-*O*-deethylase
FCA- foci of cellular alteration
FL- fluoranthene
KC- King's Creek
LA-QPCR- long amplicon quantitative polymerase chain reaction
MO- morpholino
NBH- New Bedford Harbor
PAH- polycyclic aromatic hydrocarbon
PCB126- 3,3',4,4'-pentachlorobiphenyl
pHAH- planar halogenated aromatic hydrocarbons
TCDD- 2,3,7,8-tetrachlorodebenzo-*p*-dioxin
QPCR- quantitative real-time polymerase chain reaction

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1. Introduction

1.1 Dissertation Objectives

The research outlined in this dissertation uses the estuarine fish species *Fundulus heteroclitus* (killifish) to examine the interaction of the biotransformation and toxicity of the abundant class of environmental contaminants, polycyclic aromatic hydrocarbons (PAHs). PAHs pose a risk to both ecosystem and human health; therefore, it is imperative that we increase our knowledge of their chemical fate within organisms and the resultant toxic effects.

The first goal was to investigate the effects of PAHs at a population level by examining the role of biotransformation in mediating the resistance of the Elizabeth River (ER) killifish population, which inhabit a PAH contaminated estuary and have adapted to resist the acute and lethal effects of PAH exposure to embryos and larvae. In addition to understanding how pollutants affect individuals, the study of ecotoxicology also examines the consequences of chronic toxicant exposure on populations. Killifish have been used as a model of adaptive tolerance to pollution, and there are multiple case studies identifying populations that are resistant to pollutants including mercury, polychlorinated biphenyls, and PAHs (Nacci et al. 1999, Wirgin and Waldman 2004). Many of these adaptations involve alterations in metabolic enzymes, suggesting that changes in biotransformation may be involved in the resistance (Elskus et al. 1999, Bello et al. 2001).

The second goal was to explore the effects of PAHs as they occur in the environment, within complex mixtures. These mixtures often consist of PAHs with different mechanisms of action. 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. Co-exposure to PAHs with different modes of action can result in synergistic embryotoxicity (Billiard et al. 2008). In this work, I co-exposed killifish embryos from both the ER, and a reference site population on King's Creek (KC; Gloucester County, VA) to two environmentally relevant PAH with different modes of toxicity, examined the effect on biotransformation, and determined the resultant teratogenicity. Research investigating co-exposure to multiple contaminants with different modes of toxicity is necessary to more fully inform risk assessors and regulators with accurate information about toxicant exposures and potential outcomes (Donnelly et al. 2003).

The third goal of this work was to investigate the role of altered biotransformation of PAHs in mediating their genotoxic and carcinogenic effects in killifish. I exposed larvae born to parents captured from both the ER and the KC to PAHs and examined the differences in biotransformation, genotoxicity, and carcinogenesis. The early life stages of many organisms, including both humans and fish, are vulnerable to insult by xenobiotic compounds. This sensitivity not only has implications for teratogenicity, but can also result in injury to the organism that manifests in later life stages.

The results of the work provide important information pertaining to how alterations in PAH biotransformation can affect both acute and chronic toxicity. Although the conclusions drawn pertain directly to killifish, the findings have implications on PAH toxicity in other species, and therefore will contribute to a better understanding of ecosystem health.

1.2 Ecotoxicology

Ecotoxicology, or environmental toxicology, is defined as the study of the presence, fate and effects of natural and anthropogenic chemicals within an ecosystem. This diverse field of study consists of collaborations between chemistry, biochemistry, engineering, genetics, biology, and ecology. In addition to increasing the base of scientific knowledge, research within this field has contributed to risk assessment and regulation that aids in the protection of the environment (Nielsen 1993, Gastel 2001). The interaction of humans with the surrounding ecosystem has had both positive and negative effects on the health of both. While humans have acted as stewards and conservationists, many of our actions have resulted in unforeseen consequences on the environment. With the constant creation and production of anthropogenic chemicals, there is a continued need to examine the effects of these chemicals within the environment, and determine the best practices for protecting ecosystem health. There is also increasing awareness that human health is closely linked with the environment, therefore the more we understand about the ecosystem, the more we understand about ourselves.

Fish have been widely used to study ecotoxicology for multiple reasons. From a human health perspective, many of the cellular signaling pathways that are of concern in biomedical toxicology are highly conserved within vertebrate species, including fish (Keller and Murtha 2004, Shima and Mitani 2004, Burnett et al. 2007). Fish species that have transparent chorions and external development also allow for the exploration of the effects of toxicants on early-life stages, which is much more difficult in human and mammalian models. The study of toxicology in fish is also very important for determining and biomonitoring water quality guidelines. Maintaining healthy aquatic ecosystems provides a unique challenge because they accumulate large amounts of waste from multiple sources including direct input, runoff from soil and pavement, as well as atmospheric deposition (Van Metre and Mahler 2005). The complexity of aquatic food chains provides opportunity for bioaccumulation and biomagnification of pollutants (Petersen and Kristensen 1998). In addition to ingesting chemicals with their food sources, aquatic organisms are immersed in water which is an important route of exposure through the skin, gills, and permeable embryonic chorions for many species (Mackay and Milford 2008).

In addition to understanding the routes by which an organism can be exposed to a compound, researchers must also understand the fate of that compound within the organism. Biotransformation is a two phase process that utilizes enzymatic reactions to convert nonpolar lipophilic chemicals to water-soluble metabolites that can be easily excreted or eliminated (Parkinson 1996). Phase I reactions include oxidation, reduction and

hydrolysis, in which polar atoms are either exposed or added onto the compound. Phase II reactions consist of conjugating the compound with bulky polar endogenous compounds, or with compounds that sterically hinder further bioactivation. Although necessary for detoxification, the process of biotransformation can also produce intermediate metabolites that are more reactive and toxic than the parent compound (Miller and Ramos 2001, Schlenk et al. 2008).

1.3 Polycyclic Aromatic Hydrocarbons

PAHs are products of the incomplete combustion of organic materials, and are compounds of concern in both human and ecosystem health. Humans are exposed to PAHs primarily through food sources and cigarette smoke (Yan 1985, Hatterner-Frey and Travis 1991). The compounds enter into aquatic environments naturally through petroleum seeps, forest fires, and volcanoes. However, the greatest concern to ecosystem health are the anthropogenic sources including vehicle exhaust emissions, fossil fuel combustion, industrial processes, and oil contamination by disposal or spills (Walker et al. 2005). Increasing populations, land development and vehicle usage will result in a continued increase in the concentrations of PAHs that are entering our waterways via waste water, runoff, and deposition from the atmosphere (Bell et al. 2004, Van Metre and Mahler 2005).

There are currently 100 known PAHs and they have been found at 600 of 1,430 National Priority List sites. As a group they are ranked number eight on the 2007 Comprehensive Environmental Response, Compensation, and Liability Act (CERCLA)

priority list of hazardous substances (ATSDR 2007). PAHs as a class of contaminants vary on the common structure of planar hydrocarbon compounds comprised of at least two aromatic rings fused together. This structure makes PAHs very lipophilic with log K_{ow} values ranging from 2 to 7 (Verburgh et al. 1996). Exposure to PAHs can elicit a variety of toxic effects including phototoxicity, immunotoxicity, narcosis, teratogenesis, mutagenesis, and oxidative stress (Levin et al. 1978, Tuveson et al. 1987, Fay et al. 2000, Burdick et al. 2003, Bell et al. 2004, Carlson et al. 2004). Although the toxic effects of these compounds are well documented, there is still a need for more information about mechanisms by which PAHs elicit these effects.

1.4 Biotransformation

The process of biotransformation can be separated into phase I and phase II reactions. Phase I enhances the polarity of the parent compound through oxidation, reduction, and/or hydrolysis to allow for its detoxification and elimination. Phase II reactions conjugate either parent compounds or phase I metabolites to a variety of water-soluble endogenous compounds present in the cell including glutathione, glucuronic acid, amino acids, peptides, and sulfate (Goldstein and Faletto 1993, van der Oost et al. 2003). These modifications can enhance polarity to facilitate excretion, or they can provide steric hindrance to mask functional groups on the parent compound hindering its bioactivation. Although this process serves to detoxify and eliminate the parent compound, phase I reactions can create reactive intermediate metabolites, which have greater potential for

toxicity than the parent compound. The expression of the phase I and phase II metabolic enzymes can be regulated by multiple transcription factors including the aryl hydrocarbon receptor (AHR).

1.4.1 Aryl Hydrocarbon Receptor

Some PAHs are ligands for the AHR, which is a transcription factor in the basic helix-loop-helix/Per-ARNT-Sim (bHLH-PAS) family of proteins. The receptor is located in the cytoplasm where it is bound to chaperone proteins including two molecules of heat shock protein 90 (HSP90), the "X-associated protein" (XAP-2), and p23 (Carver et al. 1998, Powell et al. 1999, Hahn et al. 2006). The AHR is known to play a role in maintaining cellular homeostasis, as well as mediating the response to exogenous stressors (Wirgin and Waldman 2004). Research using *AHR* null mice provides a compelling argument that the receptor also plays an important role in development. Mice that develop without a functioning AHR exhibit a 40-50% rate of neonatal lethality, skin lesions, reduced liver size, inflammation of the bile ducts, and decreased accumulation of lymphocytes in the spleen and lymph nodes (Fernandez-Salguero et al. 1995, Schmidt et al. 1996, Lahvis et al. 2005).

In a search for endogenous AHR ligands, researchers have discovered that some metabolites of heme, tryptophan, arachidonic acid are all agonists for the receptor (Nguyen and Bradfield 2008). Indoles and flavanoids derived from fruits and vegetables also activate the receptor (Denison and Nagy 2003). However the most well characterized ligand for the AHR is 2,3,7,8-tetrachlorodebenzo-*p*-dioxin (TCDD)

(Schmidt and Bradfield 1996, Hahn 1998b, Nebert et al. 2000, Denison and Nagy 2003, Mandal 2005). The planar halogenated aromatic hydrocarbons (pHAHs), are referred to as dioxin-like compounds (DLCs), and share TCDD's strong binding affinity for the AHR. Although they are weaker ligands than dioxin, many PAHs, can also bind to the AHR and activate the signal transduction pathway (Figure 1.1) (Billiard et al. 2002, Denison and Nagy 2003, Billiard et al. 2004).

When ligands enter into the cell and bind to the AHR it undergoes a conformational change that exposes a nuclear localization signal which allows the AHR to migrate to the nucleus (Pollenz et al. 2006). Here it dimerizes with another bHLH-PAS protein, the aryl hydrocarbon receptor nuclear translocator (ARNT) (Dougherty and Pollenz 2008). The AHR-ARNT complex binds to a specific DNA enhancer sequence termed the xenobiotic response element (XRE) (Carney et al. 2004). The XRE is located in the 5' promoter region of numerous genes that encode for enzymes involved in both phase I and phase II metabolism including members of the cytochrome P450-1 family (Nebert et al. 2000).

1.4.2 Cytochrome P450

Cytochrome P450s (CYPs) are a superfamily of monooxygenases that are functional in the oxidative, reductive and peroxidative biotransformation of a variety of both endogenous (steroids, bile acids, fatty acids, prostaglandins) and exogenous compounds (natural plant products, drugs, and environmental pollutants) (Boelsterli 2003). The human genome is comprised of at least 57 functional CYP genes, while the fish species *Danio rerio*

(zebrafish) has at least 81 within their genome (Nelson 2003). The CYP class of enzymes is comprised of multiple families, each with different substrate specificities. These include the CYP2 gene family, which are involved in the metabolism of lauric acid, arachidonic acid, and sex steroid hormones (Waxman 1999, Lewis 2003). The CYP3 gene family has been associated with the metabolism of bile acids, retinoids, testosterone and benzo[a]pyrene (Aoyama et al. 1990, Gillam et al. 1993, Li et al. 1995). However, it is the CYP1 gene family that is most closely associated with the metabolism of PAHs.

Mammals have two CYP1A enzymes, CYP1A1 and CYP1A2, which have different functionalities and are distributed differently throughout the organism (Lewis and Lake 1996, Choudhary et al. 2005). Many fish species only have one CYP1A which is believed to be ancestral to both the mammalian CYP1A1 and CYP1A2 (Morrison et al. 1998). The activity of CYP1A can be easily measured through a kinetic assay, and its induction has been used as a biomarker of toxicant exposure since the 1970s (Hahn et al. 1998, Mandal 2005). In both mammals and teleosts, CYP1A is functional in the metabolism of a variety of endogenous compounds including arachidonic acid, bilirubin, and estradiol-17 β ; however, TCDD and DLCs are more potent known inducers of the enzyme (Hammond et al. 1997, Schlezinger et al. 1998, Zaccaro et al. 2001, Mandal 2005, Rifkind 2006, Volz et al. 2008).

Genes within the CYP1 family can be induced by both DLCs and PAHs through the AHR pathway; however the role of CYP1 enzymes in mediating toxicity is different between the compounds. TCDD and the DLCs are the most potent known inducers of

CYP1A; however, these compounds are poor agonists for CYP1A and are not easily biotransformed or excreted (Mandal 2005). 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 the formation of reactive intermediate metabolites (Parkinson 1996, Schlenk et al. 2008). Modulation of CYP1A activity has been associated with toxic effects of PAHs including, teratogenesis, immunosuppression, DNA adduct formation, and the development of carcinomas (Sjogren et al. 1996, VanVeld et al. 1997, Ploch et al. 1998, Carlson et al. 2004, Wassenberg and Di Giulio 2004a). Current data suggest that CYP1A mediated biotransformation and excretion of PAHs seems to play a protective role concerning the carcinogenicity and teratogenicity of 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 and CYP1C1 are also inducible by multiple AHR agonists and may also play a role in PAH biotransformation and toxicity (Wang et al. 2006, Timme-Laragy et al. 2007)

CYP1B1 was first identified in rodents and is functional in the metabolism of retinoic acid, estradiol-17 β , DLCs, and several PAHs (Hammond et al. 1997, Choudhary et al. 2004, Shimada and Fujii-Kuriyama 2004). 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). More recently, CYP1B1 has been identified in teleosts including scup (*Stenotomus chrysops*), plaice (*Pleuronectes platessa*), zebrafish, and killifish (*Fundulus heteroclitus*) (Godard et al. 2000, Jonsson et al. 2007b). Within fish CYP1B1 is inducible at both embryonic and adult stages in response to AHR agonists such as TCDD, 3-methylcholanthrene, and BaP (Willett et al. 2006, Jonsson et al. 2007b, Timme-Laragy et al. 2008, Yin et al. 2008). 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). Although CYP1C is 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).

1.5 *Fundulus heteroclitus* (killifish)

Fundulus heteroclitus, commonly referred to as killifish or mummichog, is a small estuarine fish species that is widely distributed along the US Atlantic coast and is a major fish species represented in intertidal systems (Bigelow and Schroeder 1953) (Yozzo *et al.*, 1994). The Atlantic killifish is an omnivorous species that consumes everything from detritus to small fish (Kneib and Stiven 1978). The general life expectancy of this species is 3-4 years and they reach sexual maturity with 1 year, spawning up to eight times during a single summer season (Wirgin and Waldman 2004). There are many characteristics of this fish species that make them both a unique and important model for laboratory and field

studies in toxicology. Adults of the species are easily maintained in a laboratory and can adapt to many environmental challenges including salinity, temperature, and levels of oxygen (Burnett et al. 2007). Killifish have large eggs (1-2 mm) with transparent chorions, which allows for observation during the early stages of development. The eggs hatch within 9-15 days of fertilization allowing for the study of killifish eleutheroembryos and larvae. A single female can lay 50 to several hundred eggs which can be externally fertilized to achieve synchronous fertilization of experimental subjects (Able and Hata 1984, Taylor 1986).

Many transcription factors involved in toxicant response have been identified and proven functional in killifish, including the AHR pathway (Karchner et al. 1999, Wirgin and Waldman 2004). Unlike mammals, killifish have two AHR genes, AHR1 and AHR2, which have different tissue expression profiles and most likely also different functions within the organism (Powell et al. 2000). In zebrafish AHR2 is the paralog involved in toxicant response, and it is possible that this is also true for killifish (Billiard et al. 2006, Jonsson et al. 2007a). Killifish also express the AHR regulated genes involved in phase I and phase II metabolism. Four CYP1 enzymes have been identified in fish, CYP1A, CYP1B1, CYP1C1, and CYP1C2 (Godard et al. 2005). All of these genes, except for CYP1C2, are currently identified in killifish (Wang et al. 2006).

1.5.1 Resistant Populations

Migration is limited in the killifish, with a home range of about 36m in the summer months, making them an excellent sentinel species for correlating toxic effects with local chemical pollution (Wirgin and Waldman 2004, Roark et al. 2005, Burnett et al. 2007). There are case studies of populations of killifish in contaminated waterways that have adapted to resist the toxic effects of multiple chemical contaminants (Wirgin and Waldman 2004). Killifish inhabiting Piles Creek, NJ have been chronically exposed to metal and organic contaminants including methyl mercury (Weis et al. 1987). Embryos of this population developed a genetic adaptation that produced chorions with reduced permeability and resulted in an increased rate of development. Embryos and larvae from the nearby Newark Bay are more resistant to the toxic effects of TCDD, and although the mechanism of resistance is not fully understood, this population lack responsiveness of CYP1A induction following exposure to AHR agonists (Elskus et al. 1999). In New Bedford Harbor (NBH), MA there is another population of killifish whose larvae and embryos are resistant to the teratogenic and lethal effects of DLCs, and adults at the site remain reproductively viable (Bello et al. 2001). The NBH population is also unresponsive to the induction of AHR regulated genes after exposure to agonists for the receptor (Nacci et al. 2002b). The Elizabeth River in Portsmouth, VA is home to another killifish population whose embryos and larvae are resistant to the toxic effects of acute exposure to PAHs, including lethality and teratogenicity (Armknrecht et al. 1998, Rose et al. 2000, Meyer et al. 2002b).

1.6 Elizabeth River

The Elizabeth River is a subestuary of the James River in Virginia and the southernmost tributary of the Chesapeake Bay (Bieri et al. 1986). The estuary has been widely studied due to the extent to which it has been damaged by human activities. The Southern Branch of the ER flows through a highly industrialized area that has included naval facilities, oil storage facilities, a fertilizer plant, and the Atlantic Wood Industries (AWI) (Huggett et al. 1992). From 1926 until 1992, the AWI was a wood treatment facility that primarily utilized pentachlorophenol (PCP) and creosote as preservatives (EPA 2007). In 1990 the area was classified as a Superfund site and listed on the National Priorities List (NPL) of hazardous waste sites primarily due to the extensive levels of PCP and creosote contamination (Bieri et al. 1986).

Creosote is a complex mixture consisting primarily of unsubstituted, heterocyclic, and phenolic PAHs. At the AWI site the mean concentration of PAHs in the sediment is 410 µg/g dry weight and consists primarily of fluoranthene, pyrene, chrysene, and benzo[a]pyrene (Bieri et al. 1986, Vogelbein et al. 2008). Sediment collected from the area surrounding AWI is acutely toxic and highly teratogenic to a variety of species of aquatic organisms including oysters (*Crassostrea virginica*), brackish water clams (*Rangia cuneata*), spot (*Leiostomus xanthurus*), hogchoker (*Trinectes maculatus*) and killifish (Hargis Jr et al. 1984, Bender et al. 1988, Huggett et al. 1992, Wassenberg and Di Giulio

2004b, Vogelbein et al. 2008). However, in spite of the toxicity of the sediment, there is a reproductively viable population of killifish that inhabits the site.

The ER embryonic, larval and adult killifish show significantly reduced levels of mRNA induction of multiple genes in the AHR pathway including AHR2, AHRR and CYP1A (Meyer et al. 2003a). These killifish also show lower levels of CYP1 enzymatic activity as measured by the ethoxyresorufin-*O*-deethylase (EROD) assay (Bello et al. 2001, Meyer and Di Giulio 2002, Meyer et al. 2002a, 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. 2002a, 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. 2002b, Bacanskas et al. 2004).

In spite of their resistance, the ER population does display multiple fitness costs including a compromised immune system and diminished tolerance to hypoxia (Meyer and Di Giulio 2003, Frederick et al. 2007). There has also been extensive research since

the early 1990s examining the wide range of pre-neoplastic and neoplastic lesions that have been observed in high prevalence levels in ER adults in the wild (Huggett et al. 1992, Fournie and Vogelbein 1994, Vogelbein and Unger 2006, Vogelbein et al. 2008). In a 1990 field study, ER killifish had a 93% incidence of liver lesions and a 33% frequency of liver cancer, while a reference site population had no detectable lesions (Vogelbein et al. 1990). Therefore, although killifish from the ER population are resistant to the acute and developmental toxicity of PAHs, their susceptibility to the chronic effects, including carcinogenicity, remains unclear.

1.7 PAH Mixtures

When analyzing the potential toxic effects at sites highly contaminated with PAHs, the general practice for risk assessors is to evaluate each of the individual PAHs within the contaminated sediment by their effects range low (ERL) and their effects range median (ERM), and then to evaluate the effects of the sum of the total PAHs together (Long et al. 1995, Barron et al. 2004, Walker et al. 2004). This technique does not take into account any potential interactions between compounds, and may be drastically underestimating the toxicity at PAH contaminated sites (Billiard et al. 2008). The Elizabeth River Superfund site is contaminated with a complex mixture of PAHs with different mechanisms of toxicity. Some of the PAHs at the site such as BaP and benzo[k]fluoranthene (BkF) are AHR agonists and CYP1 inducers (Barron et al. 2004). However, other PAHs in the ER sediment are fluoranthene (FL), carbazole and dibenzothiophene, which all act as inhibitors of the CYP1

enzymes (Willett et al. 2001b, Wassenberg et al. 2005). Previous research in our laboratory has shown that co-exposure of zebrafish and reference site killifish embryos to PAHs that are AHR agonists and to those that are CYP1A inhibitors, causes a synergistic increase in the frequency and severity of cardiac deformities (Wassenberg and Di Giulio 2004a, Wassenberg et al. 2005, Billiard et al. 2006). A similar synergistic increase in toxicity was observed after reference site embryos were co-exposed to sediment from the Elizabeth River and FL (Wassenberg and Di Giulio 2004b). One hypothesis to explain this synergistic developmental toxicity is that the chemical inhibition of CYP1 alters PAH biotransformation. This alteration could occur either by changing the relative proportions of reactive metabolites, or by increasing the half-life of the parent PAH compound allowing it to act more like a DLC (Billiard et al. 2008). To examine this question further, I exposed killifish embryos to two PAHs that are abundant at the ER: FL and BaP.

1.7.1 Fluoranthene

FL is an environmentally relevant PAH found at a variety of contaminated sites. At the Elizabeth River, FL comprises 26% of the total weight of PAHs measured at the site (Vogelbein et al. 2008). FL is a four-ring PAH that can elicit multiple toxic effects (Figure 1.2). FL is phototoxic, and forms reactive species in the presence of UV radiation that can cause oxidative stress in exposed organisms (Tuveson et al. 1987, Ahrens et al. 2002, Bell et al. 2004). Zebrafish embryos exposed to FL in the presence of hypoxia developed pericardial effusion and severe lordosis (Matson et al. 2008b). FL has low affinity for the

AHR and does not induce CYP1A (Barron et al. 2004). In an *in vitro* H4IIE bioassay, FL was characterized as a noncompetitive inhibitor of CYP1A activity, and has been demonstrated to inhibit the activities induced by other AHR agonists such as BaP and benzo(k)fluoranthene (BkF) both *in vitro* and *in vivo* (Willett et al. 1998, Willett et al. 2001b). Killifish embryos co-exposed to FL and PAH-model CYP1A inducer, β -naphthoflavone, developed a synergistic increase in cardiac deformities and pericardial effusion (Wassenberg and Di Giulio 2004a).

1.7.2 Benzo[a]pyrene

BaP is a compound of concern both in ecological and human health (Gautier et al. 1996). The dominant pathway for human exposure is the food chain, which accounts for about 97% of the daily intake of BaP (Hatterner-Frey and Travis 1991). BaP is a procarcinogen and has been implicated as a causative agent in cigarette induced lung cancer (Hoffmann et al. 1978). Exposure to BaP results in production of DNA-adduct and tumor formation in both rodent and fish laboratory models (Varanasi et al. 1986, Ploch et al. 1998, Miller and Ramos 2001, Uno et al. 2004). In addition to its carcinogenic effects, BaP can interact with PAHs that are CYP1A inhibitors to cause teratogenicity. Killifish embryos exposed to BaP and the CYP1A inhibitor α -naphthoflavone (ANF) had decreased levels of CYP1A activity and a synergistic increase in the occurrence of cardiac deformities as measured by heart elongation (Wassenberg and Di Giulio 2004a). Similarly, zebrafish embryos co-exposed to BaP and FL had a synergistic increase in pericardial effusion

(Matson et al. 2008b). At the Elizabeth River, BaP comprises 11% of the total weight of PAHs measured at the site and may contribute to both the carcinogenicity and teratogenicity of the sediment (Vogelbein et al. 2008).

BaP has been utilized as a model to better understand PAH biotransformation. The parent compound is considered to be benign, however BaP can be biotransformed to reactive metabolites that can interact with macromolecules and disturb cellular function (Gautier et al. 1996, Miller and Ramos 2001). BaP is a five-membered PAH that contains a two structural motifs of high electron density: the "k-region" (encompassing carbons 4 and 5), and the "bay region" (encompassing carbons 9-12) (Figure 1.3). The "bay region" is defined as a complex ring structure formed by a saturated angular benzene ring fused to an aromatic ring in the polycyclic hydrocarbon (Yan 1985). This angular ring is important to biotransformation because it forms an area of steric hindrance, where oxidation or radical formation can easily occur, but detoxification and conjugation are impeded (Miller and Ramos 2001).

Once in the cell BaP can be oxidized by CYP1A at multiple sites to form a variety of epoxides including the 2,3-, 4,5-, 7,8-, and 9,10- isomers (Figure 1.4). One possible pathway is the oxidation at C-7 and C-8 to form BaP-7,8-epoxide (7,8-ox), which is hydrolyzed by microsomal epoxide hydrolase (mEH) into BaP-7,8-dihydrodiol. This compound can then be reoxidized by CYP1A to form BaP-7,8-dihydrodiol-9,10-epoxide (BPDE) (Levin et al. 1978, Jerina and al. 1984). BPDE contains an epoxide ring in the "bay region" of the

compound making it recalcitrant to further mEH hydroxylation. This position also makes the epoxide much more susceptible to nucleophilic attack, allowing BPDE to react with DNA and cause mutagenicity and potential carcinogenicity (Varanasi et al. 1986, Ueng et al. 1994, Rose et al. 2001). The hydroxylation of BaP by CYP1A can also result in the formation of a variety of highly chemically active quinones, which 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 ($\cdot OH$) (Lemaire et al. 1994, Willett et al. 2000, Miller and Ramos 2001, Burdick et al. 2003, Hornung et al. 2007). Although the BaP-quinones have the potential to produce reactive oxygen species, there has not been much research examining the toxicity of these compounds.

1.8 Dissertation Outline

The resistant killifish from the Elizabeth River are inherently refractory to CYP1 induction and therefore provide an excellent tool for studying the mechanisms of toxicity for PAHs. By understanding how the embryos and larvae of this resistant population metabolize BaP, both alone and within a mixture, we will be able to better understand how they have developed their resistance to the PAH-contaminated sediment in the Elizabeth River.

The sediment at the Elizabeth Rive is contaminated with a complex mixture of PAHs with different modes of action. The discovery that PAH-type AHR agonists and CYP1A inhibitors cause synergistic developmental toxicity in killifish embryos has significant

implications on risk assessment and the way in which complex mixtures of PAHs should be analyzed and assessed. The research outlined in this dissertation will provide a deeper understanding of that interaction by determining the effects and metabolic profiles of killifish embryos after co-exposure to two PAHs of environmental concern, BaP and FL. It is generally accepted that the differences between the toxicity of PAHs and DLCs is a result of the strong affinity of PAHs for CYP1 enzymes resulting in their rapid metabolism. Alterations in CYP1 activity could increase the half-life of the PAHs, allowing them to chronically activate the AHR and cause a dioxin-like toxicity. Additionally, lack of CYP1 activity may lead to an increase in alternate metabolic pathways and change the prevalence and profile of PAH reactive metabolites.

In addition to the embryotoxic effects, some PAH metabolites are genotoxic and can produce carcinogenic effects. Wild-caught killifish from the ER do have a high prevalence of hepatic and pancreatic lesions; however, it is still unclear how the recalcitrant CYP1 phenotype of the ER larvae and juveniles affects their biotransformation of PAHs and ultimately their susceptibility to PAH-induced carcinogenesis.

The overall purpose of this study was to investigate the role of biotransformation on PAH-induced toxicity in two populations of killifish with different exposure histories. The three primary aims of this work were:

- 1) to further characterize the mechanisms of biotransformation

Approach: Investigate the induction of the CYP1 metabolic enzymes (CYP1A, CYP1B1 and CYP1C1) in response to AHR agonists in reference site and ER killifish embryos (Chapter 2)

2) to examine how the alteration of CYP1 enzymes affects the metabolic profile of BaP during embryo deformity manifestation.

Approach: Use ultra pressure liquid chromatography with mass spectrometry (UPLC-MS) analysis to identify and quantify BaP and select 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) (Figure 1.5). The metabolic profile of BaP was examined in killifish embryos with the following alterations to their CYP1 enzymatic activity:

- a) co-exposure to the chemical CYP1A inhibitor, FL (Chapter 3)
- b) in the ER embryos with a recalcitrant CYP1 phenotype (Chapter 3)
- c) after morpholino knockdown of CYP1A (Chapter 4)

3) to examine the influence of altered CYP1 phenotype of the ER embryos affects BaP biotransformation, genotoxicity, and carcinogenicity.

Approach: Investigate and contrast the effects of an early-life exposure to BaP on the chronic effects in ER and KC juveniles (Chapter 5)

These experiments show that, in addition to the refractory CYP1A phenotype of the Elizabeth River population, killifish embryos born to wild-caught ER parents also have a reduced mRNA induction of the newly characterized CYP1B1 and CYP1C1. This adaptation has resulted in an alteration in PAH biotransformation, as indicated by the recovery of higher concentrations of BaP and the benign metabolite BaP-9,10-dihydrodiol in ER embryos versus those from the KC. One of the deficits in our understanding of the ER killifish population is how their adaptation to the teratogenic effects of PAHs affects their susceptibility to the genotoxic and carcinogenic effects of the compounds. This work attempted to fill that gap by examining the effects of an early-life exposure to BaP on ER killifish in later life stages. This work demonstrates that the adaptation of the ER embryos and larvae has resulted in greater resistance to genotoxicity and carcinogenicity within the juveniles 9 months post-hatch.

Surprisingly, we did not detect any effect of FL co-exposure on the biotransformation of BaP. Additionally the effect of morpholino knockdown of CYP1A did not result in any interpretable differences in BaP biotransformation. However, both the co-exposure to FL and the injection with the CYP1A morpholino did cause a reduction in BaP induced CYP1 enzymatic activity, and a synergistic increase in BaP induced teratogenicity. It is possible that the method of UPLC-MS is not sensitive enough to detect differences in the metabolic profile within embryos. Further studies in adults and with more sensitive

methods of detection are needed to elucidate the role of altered biotransformation in mediating the embryotoxicity of PAHs.

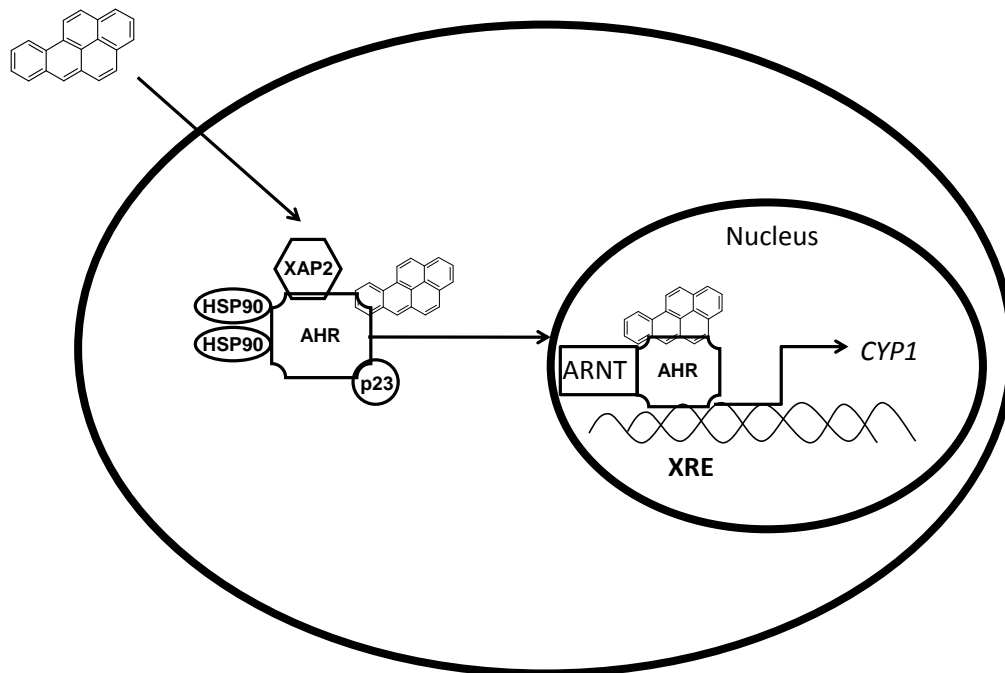
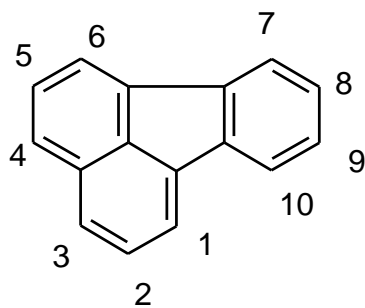


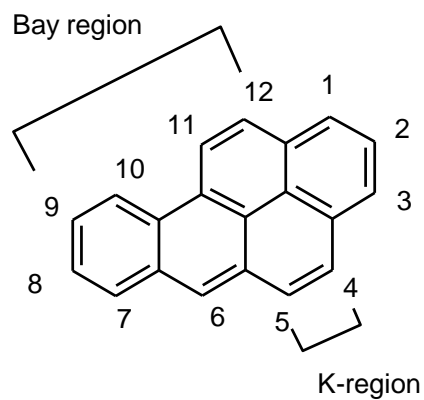
Figure 1.1: AHR pathway

In this example the ligand benzo[a]pyrene enters the cytoplasm of the cell and binds to the AHR complex. The AHR migrates to the nucleus where it dimerizes with ARNT. The AHR-ARNT complex binds to xenobiotic response elements and upregulates the induction of the CYP1 family of enzymes.



Fluoranthene

Figure 1.2: Structure of fluoranthene



Benzo[a]pyrene

Figure 1.3: "Bay region" and "K-region" sites on benzo[a]pyrene

Carbons 9-12 are considered to be the bay region of BaP with carbon 10 as the active center (α -carbon). The K-region (at carbons 4 and 5) is an area of high electron density and high metabolic activity (Miller and Ramos 2001).

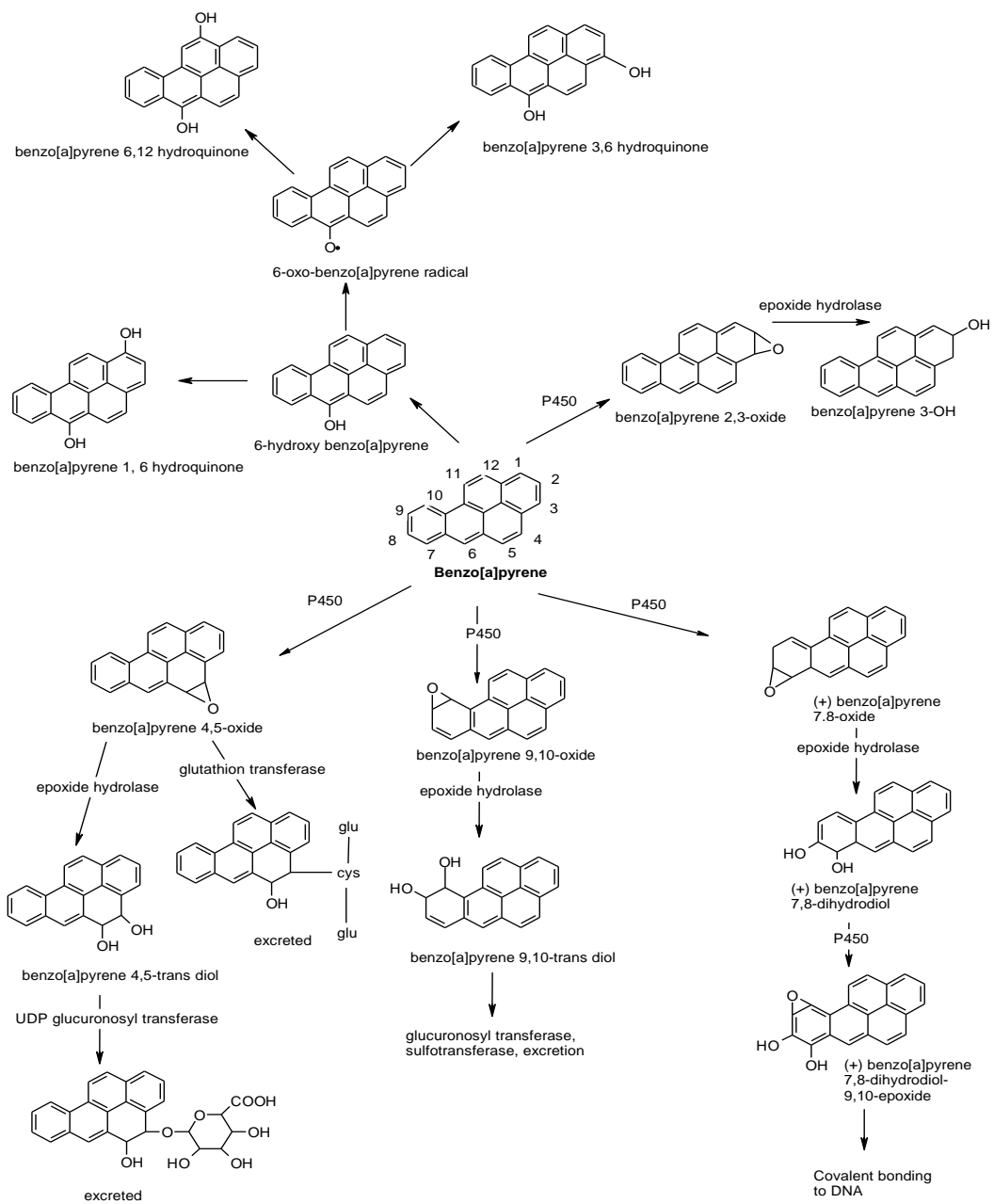


Figure 1.4: Summary of activation and detoxification pathways of BaP

(Miller and Ramos 2001)

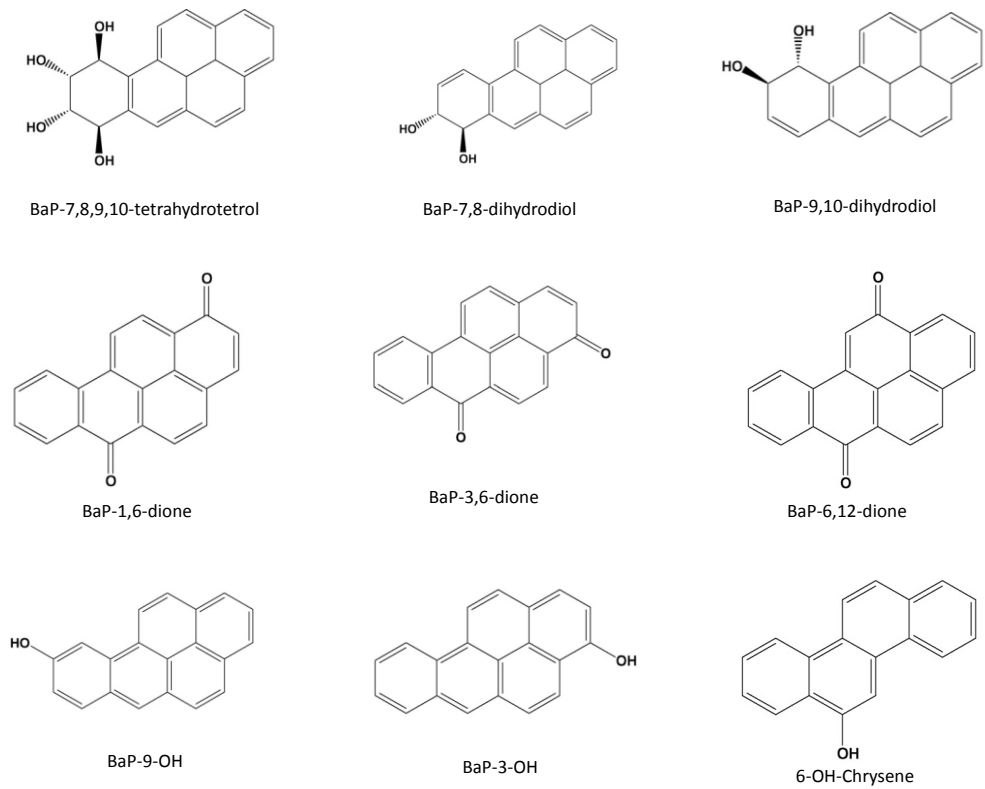


Figure 1.5: Structures of BaP metabolites examined for biotransformation experiments

(Zhu et al. 2008)

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

Portions of this work will be submitted for publication with Lauren P. Battle, Cole W. Matson, Chelsea D. Landon, and Richard T. Di Giulio as authors.

2.1 Introduction

Fundulus heteroclitus (killifish) are an adaptable species of fish found in estuaries along the Atlantic coast. There are many populations of fish within this species that have developed resistance to environmental contaminants. One such population is found at the Atlantic Wood Industries (AWI) Superfund site on the Elizabeth River (ER) in Portsmouth, VA. This site is highly contaminated with creosote, a complex mixture of polycyclic aromatic hydrocarbons (PAH). In response to chronic exposure to the sediment, the resident killifish population inhabiting the ER has adapted to the teratogenic effects of the PAH contamination. Many of the PAHs found at the AWI site activate the aryl hydrocarbon receptor (AHR), and are thought to mediate their toxic effects through this pathway. The activation of the AHR results in the induction of the metabolic enzymes within the cytochrome P450-1 family including CYP1A. Previous research has shown that the embryos, larvae and adults of the Elizabeth River (ER) killifish population have a recalcitrant CYP1A phenotype both at the level of mRNA induction and protein activity. This population is also highly resistant to the lethal embryotoxic effects of the sediment at the AWI site, as well as to a variety of PAHs and

dioxin-like compounds (DLCs). The mechanisms surrounding this observed resistance are still unclear, but the observed alteration of AHR pathway is likely involved. The recent discoveries of *CYP1B1* and *CYP1C1* in killifish have led us to ask questions about their inducibility in killifish in response to AHR agonism, and their level of inducibility within the ER population. It is also still unclear as to whether the observed recalcitrance primarily represents a suppression of normal CYP1 activity, or a shift in the initiation of the response. The purpose of this study was to more fully characterize the ER recalcitrant phenotype, and the role of AHR activation in mediating the observed resistance. We exposed embryos from the ER and from a reference site to two model PAHs, benzo[a]pyrene (BaP) and benzo[k]fluoranthene (BkF), and to the DLC, 3,3',4,4',5-pentachlorobiphenyl (PCB126). We compared their developmental and molecular responses by screening the embryos for CYP1 protein activity, cardiac deformities, and mRNA expression of *CYP1A*, *CYP1B1*, *CYP1C1*, and *AHR2*. The difference in threshold cycles (dC_Ts) of both *CYP1A* and *CYP1B1* in reference to the β-actin were 40 % higher in the KC DMSO control embryos compared to those from the ER ($p < 0.05$). This indicates a difference in mRNA expression levels between these two populations in response to the vehicle control (basal expression). *AHR2* and *CYP1C1* were not significantly different between the populations. Exposure of the KC embryos to BaP, BkF and PCB126 resulted in an induction of CYP1 activity and an increase in cardiac deformities ($p < 0.001$). Whereas, ER embryos induced CYP1 activity only in response to BkF

exposure ($p < 0.001$). However, this induction in ER embryos was significantly lower than that observed in KC fish at comparable concentrations ($p < 0.001$). ER embryos did not develop cardiac deformities in response to any of the chemicals that we tested. *CYP1A*, *CYP1B1* and *CYP1C1* were all significantly induced in the KC embryos after exposure to BaP, BkF and PCB126 ($p < 0.05$). Exposure to BaP and BkF in ER embryos resulted in a significant induction of *CYP1A* mRNA ($p < 0.01$); although the level of induction was significantly lower than what was observed in KC fish ($p < 0.01$). BaP exposure also resulted in a significant induction of *CYP1B1* at comparable levels in embryos from both populations ($p < 0.05$). ER embryos did not induce any of the *CYP1s* in response to PCB126, nor did they induce *CYP1C1* for any treatment group that we examined. Additionally *AHR2* was not significantly induced for any of the treatment groups. This study further characterizes the AHR response in killifish, and provides greater insight into the adapted ER phenotype.

2.2 Background

Fundulus heteroclitus (killifish) are teleosts that have been used for decades in ecological, biochemical, and toxicological research (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 east coast, and they have adapted to withstand the toxicity of multiple environmental

contaminants including metals, dioxin-like compounds (DLCs) and polycyclic aromatic hydrocarbons (PAHs). Two populations of killifish from Newark, NJ, one from Piles Creek and another population inhabiting Newark Bay, are resistant to the acute toxicity and teratogenicity of mercury and 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD, dioxin) respectively (Weis et al. 1987, Arzuaga and Elskus 2002). Killifish in a PCB contaminated site in New Bedford Harbor, MA are resistant to the embryotoxicity of DLCs and PAHs (Bello et al. 2001, Nacci et al. 2002b). There is also a population of killifish on the Elizabeth River (ER), VA whose offspring are resistant to the teratogenic effects of chemicals associated with the wood preservative creosote, which primarily consists of 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 (Cooper et al. 1999, Meyer et al. 2003b). However, the hallmark common to most of the adapted killifish populations is a recalcitrant CYP1A phenotype (Elskus et al. 1999, Bello et al. 2001, Meyer et al. 2002a, Wirgin and Waldman 2004).

The AHR is a ligand activated transcription factor in the basic helix-loop-helix Per-Arnt-Sim family of proteins (bHLH-PAS) (Schmidt and Bradfield 1996). Although there are natural ligands for the AHR such as idoles and heme metabolites, the most potent known ligand for the receptor is TCDD (Denison and Nagy 2003, Mandal 2005,

Nguyen and Bradfield 2008). Other xenobiotics that bind to the AHR include polychlorinated biphenyls such as PCB126, which is about 100 times less potent than TCDD, and PAHs which are about 1000 times less potent (Billiard et al. 2004, Nguyen and Bradfield 2008). Like many other fish species, killifish have two different AHR paralogs that have differential tissue expression and functionality, AHR1 and AHR2 (Powell et al. 2000, Hahn 2002). AHR1 is normally expressed in the brain, heart, and gonads of killifish and may play a role in development. In zebrafish (*Danio rerio*) AHR2 mediates the toxicity of DLCs and PAHs, and it is likely that this is also the case in killifish (Andreasen et al. 2002, Prasch et al. 2003, Billiard et al. 2006).

When DLCs and PAHs bind to the AHR, the receptor changes conformation, and migrates to the nucleus. In the nucleus, AHR binds with aryl hydrocarbon nuclear translocator (ARNT) (Song and Pollenz 2002, Pollenz et al. 2006). The AHR-ARNT complex is able to bind to target promoters called xenobiotic response elements (XREs) on a variety of responsive genes including the cytochrome P450-1 (CYP1s) family of metabolic enzymes (Schmidt and Bradfield 1996, Dougherty and Pollenz 2008). Recent studies show that fish express four genes in three CYP1 subfamilies (CYP1A, CYP1B1, CYP1C1 and CYP1C2 (Godard et al. 2000, Godard et al. 2005). Three of these are known to be expressed in killifish, CYP1A, CYP1B1 and CYP1C1(Wang et al. 2006).

CYPs are monooxygenases that aid in the biotransformation and excretion of many endogenous and exogenous compounds. CYP1A is widely used as a biomarker of

binding affinity for the AHR, and toxic potency of DLCs and PAHs (Billiard et al. 2002, Barron et al. 2004, Mandal 2005). Although TCDD and the DLCs are potent inducers of CYP1A, these compounds are poor agonists for CYP1A and are not easily biotransformed or excreted. The teratogenic and carcinogenic effects of DLCs are mediated by the AHR in both mice and in aquatic organisms (FernandezSalguero 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 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 (Parkinson 1996, 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 and CYP1C1 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).

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. 2002a, Wassenberg and Di Giulio 2004b, Vogelbein and Unger 2006). Molecular analysis of the AHR pathway, revealed that adult ER killifish dosed with AHR agonists lack mRNA inducibility of AHR2, CYP1A and the aryl hydrocarbon receptor repressor (AHRR) (Meyer et al. 2003a). 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 of the newly identified CYP1B1 and CYP1C1 have not yet been characterized in this population.

In this study we investigated the differential response of KC and ER killifish to two PAHs (BaP and BkF) and to a DLC (PCB126). 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. Finally, we provided further characterization of the altered AHR response in ER killifish, and extended our knowledge of the refractory

CYP1A phenotype observed in this population to other members of the CYP1 family of enzymes, CYP1B1 and CYP1C1.

2.3 Materials and Methods

2.3.1 Fish Care

Adult killifish were collected from both a reference site at King's Creek, 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, Virginia (36°48'27.48"N, 76°17'35.77"W). 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, USA), and newly hatched brine shrimp (*Artemia*, Brine Shrimp Direct, Ogden, UT). Killifish embryos were obtained from *in vitro* fertilization of pooled oocytes with pooled 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 24 hpf for normal development and placed individually into 20 mL glass scintillation vials with 10 mL of treatment solution.

2.3.2 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 1µg/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 RNA *Later* 144 hpf, flash frozen in liquid nitrogen and stored at -80°C until time of extraction.

2.3.3 *In Ovo* EROD Assay

The *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 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 embryo by fluorescent microscopy (Zeiss Axioskop, 50x magnification using rhodamine red filter set). EROD activity was measured as intensity of bladder fluorescence and quantified digitally by IPLab 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 effusion) were excluded from *in ovo* EROD measurement.

2.3.4 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). Further examination of these embryos confirmed that embryos scored as a 0 have a 92% hatch success, those scored as a 1 have a 58% hatch success, and embryos scored with a 2 all fail to hatch (data not shown).

2.3.5 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). The cDNA was synthesized using the Omniscript cDNA synthesis kit for Reverse Transcription (Qiagen, Valencia, CA) according to the manufacturer's instructions with 500 ng 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 determine that the target genes amplified at the same rate as the housekeeping gene, and to confirm maximal efficiency. Primer sequences are provided in Table 2.1.

QPCR was performed with a 25 μ l reaction containing 200 nM of each primer, 12.5 μ l 2x SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA), 9.5 μ l 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. Each of the samples was run in duplicate.

Data analysis was carried out using the software ABI PRISM 7000 Sequence Detection System, Version 1.1 (Applied Biosystems, Foster City, CA). The average fold induction was calculated by comparing the C_T (threshold cycle) of the target gene to that of β -actin (the reference gene). The gene expression of the reference gene remained consistent throughout each treatment. The fold induction was calculated according to the instructions provided by Applied Biosystems. The C_T of each of the technical replicates was averaged, and that average was used in the following formulas:

$$C_T (\text{target gene}) - C_T (\beta\text{-actin}) = \Delta C_T$$

$$\Delta C_T (\text{treatment}) - \Delta C_T (\text{control}) = \Delta\Delta C_T$$

$$2^{(-\Delta\Delta C_T)} = \text{fold change}$$

The fold change calculated for each of the biological replicate pools of 2 larvae was averaged across treatments. Six replicates were used to determine final the final fold change averages and standard error per treatment per time point. The 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.

2.3.6 Statistical Analyses

All of the data collected 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 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.

2.4 Results

2.4.1 Dose dependent EROD activity and cardiac toxicity

We exposed killifish embryos born to parents from the Elizabeth River Superfund site to multiple AHR agonists and compared their induction of EROD activity with that of embryos from a reference site on King's Creek. One goal of these experiments was to further characterize the CYP1 response of the ER population, and determine if their recalcitrance is characterized by a suppression of normal CYP1 activity or by a shift in the initiation of enzymatic response. Additionally we examined the dose dependent occurrence of cardiac deformities in the ER and KC populations, to further describe the teratogenic effects of these compounds in killifish and the differences between the two populations with respect to teratogenicity.

As previously reported, the KC embryos significantly induced EROD activity for all of the concentrations of BaP (10-400 µg/L) that were examined ($p < 0.001$) (Figure 2.1). This response was maximized at 10 µg/L and began to decrease at the 200 µg/L dose. The ER fish did not induce EROD activity at any of the concentrations of BaP that we examined, indicating that CYP1 activity is suppressed in the ER fish in response to BaP. KC embryos exposed to 400 µg/L suffered from cardiac deformities ($p < 0.001$), but ER fish did not manifest any teratogenic effects after exposure.

The PAH BkF significantly induced EROD activity in the KC embryos for all of the concentrations that were tested in this study (0.1-300 µg/L) ($p < 0.001$) (Figure 2.2).

CYP1 activity in these fish was maximized at 1 µg/L and began to decrease at the 300 µg/L dose. The ER embryos also induced EROD activity, with the initiation of the response occurring at 1 µg/L ($p < 0.001$). This activity was maximized at the 300 µg/L dose, but was significantly lower than the response observed in the KC embryos ($p < 0.001$). This response 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 µg/L developed cardiac deformities ($p < 0.001$); but no deformities were observed in the ER embryos.

PCB126 induced EROD activity in KC embryos at all of the concentrations that were tested (0.01-1 µg/L) ($p < 0.001$) (Figure 2.3). This induction was maximized at 0.1 µg/L and began to decrease at the 1 µg/L dose. The ER embryos did not significantly induce EROD activity in any of the PCB126 exposure groups. PCB126 was teratogenic at the 1 µg/L dose in KC embryos ($p < 0.001$); however, no teratogenic effects were seen in the ER embryos at any concentration.

2.4.2 Dose dependent mRNA induction

The recent identifications of CYP1B1 and CYP1C1 in fish have been important in gaining a greater understanding of AHR mediated toxicity. In this study we characterized the mRNA induction of *AHR2*, *CYP1A*, *CYP1B1*, and *CYP1C1* in response to the AHR agonists, PCB126, BkF and BaP. We also further examined the altered AHR phenotype of the ER population, by determining the response of three enzymes within the CYP1 family.

In the KC and ER DMSO control embryos, the ΔC_{Ts} (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 mRNA expression levels between these two populations in response to the vehicle control (basal expression). The fold induction of both *CYP1A* and *CYP1B1* were 40 % higher in the KC DMSO control embryos compared to those from the ER (Figure 2.4). *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 (Figure 2.5).

In response to BaP exposure, ER fish had a significantly lower induction of *CYP1A* and *CYP1C1* compared to KC embryos ($p < 0.001$) (Figure 2.6). 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 ($p < 0.01$), they did not induce *CYP1C1* 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 induce *CYP1B1* or *CYP1C1*, and had reduced *CYP1A* induction compared to the KC population ($p < 0.001$) (Figure 2.7). KC embryos significantly induced *CYP1A* 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 ER embryos did induce *CYP1A*, 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 ($p < 0.001$) (Figure 2.8). There was no induction of any of the metabolic enzymes examined in ER embryos in response to PCB126 exposure.

2.5 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. ER embryos did induce *CYP1A* mRNA, 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 700% increase in enzyme activity above control as measured by the *in ovo* EROD assay. Although significant, this induction was still 2700% lower than the maximum induction observed in KC embryos. Embryos born to parents of the ER population are also completely resistant to the teratogenic effects of both DLCs and PAHs, specifically PCB126, BkF, and BaP.

The initial work in the ER killifish population hypothesized that the resistance of these fish was associated primarily with the recalcitrant CYP1A phenotype (Elskus et al. 1999, Meyer et al. 2002a, Timme-Laragy et al. 2005). However, later research 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). 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 and killifish embryos (Billiard et al. 2006, Matson et al. 2008a). These data suggest that the CYP1A mediated biotransformation and/or elimination of PAHs is protective from teratogenesis. However, in zebrafish, morpholino knock-down of the AHR2 resulted in protection from PAH induced toxicity (Billiard et al. 2006). 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 while mRNA induction and subsequent enzymatic inhibition of CYP1A is detrimental in PAH exposures, preventing the induction of CYP1 enzymes at the level of the mRNA may actually protect embryos from teratogenicity.

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 CYP1 enzymes. 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.

One possibility is that the ER fish have adapted such that their AHR is non-responsive. Killifish have two AHR paralogs (AHR1 and AHR2), which are differentially expressed in adult tissues, AHR1 is primarily expressed in the brain heart and gonads, while AHR2 is equally abundant in many tissues (Karchner et al. 1999). Powel et al (2000) compared tissue-specific AHR expression in reference site killifish with resistant killifish from New Bedford Harbor (NBH), and found that NBH adults expressed AHR1 mRNA in multiple tissues including the gills, gut, kidney and spleen. This altered expression pattern of AHR1 was not observed in the laboratory reared offspring of the NBH population, and was not inducible in reference site fish dosed with TCDD. It is possible that the ubiquitous expression of AHR1 is involved in the mechanism of

resistance for the adapted populations. AHR2 and AHR repressor (AHRR) were also found to be highly polymorphic in seven populations of killifish with histories of environmental contamination (Hahn et al. 2005, Hahn et al. 2006). Based on zebrafish studies, AHR2 is thought to mediate the toxicity of DLCs and PAHs; therefore it is possible that variations within this gene play a role in the mechanism of resistance observed in the ER embryos (Billiard et al. 2006, Jonsson et al. 2007a).

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. 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. These data indicate a disconnect between the induction of mRNA and the translation to functional 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 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 had higher

enzymatic activity. Again this demonstrates 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 TCDD (Tanguay et al. 1999). Research in zebrafish also shows that the *CYP1s* play a physiological role in development and their expression levels vary throughout embryonic development (Jonsson et al. 2007a). Timme-Laragy et al (2007) co-exposed zebrafish embryos 24 hpf to the model PAHs β -naphthoflavone (BNF), and α -naphthoflavone (ANF). In that study the PAH-induced expression levels of *AHR2*, *CYP1A*, *CYP1B1* and *CYP1C1* were significantly different 48 and 96 hpf. 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. It is possible that in killifish, 144 hpf is too late to observe any expression differences of the AHR.

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. This data indicates that the ER adaptation can be characterized by a suppression of normal induction of AHR regulated genes. The only enzyme that we did not observe this with was 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 seem 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 did have a 40% higher level of *CYP1B1* expression compared to ER embryos, indicating that equal fold inductions between the two populations does not translate to equal amounts 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* has not yet been identified in this species. Although the functionality of *CYP1C1* is not yet

understood, researchers have induced its mRNA expression in zebrafish with PCB-126, 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 we examined. As the functionality of *CYP1C1* in fish becomes better understood, it may provide greater insight into the mechanism of resistance in ER embryos.

This study provides additional information about the mechanism of resistance observed in the embryonic offspring of ER killifish exposed to DLCs and PAHs. 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. These data suggest that the ER adapted phenotype can be described as a suppression of CYP1 induction, and future studies in this population should focus on signaling events upstream of the cytochrome p450s.

Gene	GenBank ID	Forward Primer (5'-3') Reverse Primer (5'-3')
<i>B-ACTIN</i>	AY735154	ACCACACATTTCTCATACTCGGG CGCCTCCTTCATCGTTCAGTTT
<i>CYP1A</i>	AF026800	AAGAATGGAGGACACTGGATGACC AGATTACAGGACAACACGACAGCG
<i>CYP1B1</i>	AF235140	AAAGTGGAGGAGCACAGGCAGA ATGAAGGCATCCAGGTAAGGCAT
<i>CYP1C1</i>	DQ133571	TCTGGACGCCTTCATCTACGA GTGACGTCCGATGTGGTTGA
<i>AHR2</i>	U29679	AGGGTCAAGAGCTACTTTAAAGCTTC TTAGCACAGCCCTGAGAGAATTCTGC

Table 2.1: cDNA target genes, GenBank identification, and primers used for QPCR

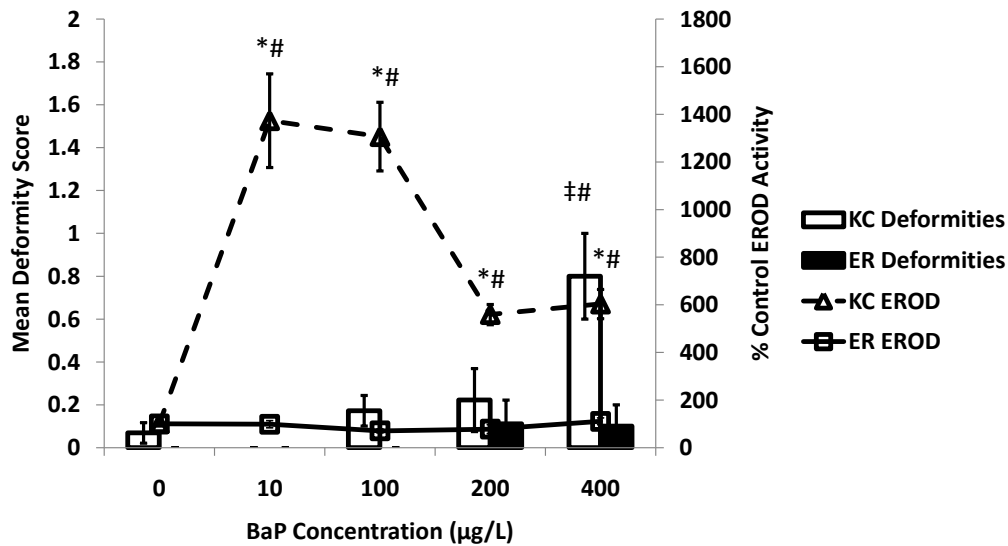


Figure 2.1: BaP dose response curve of CYP1 enzymatic activity as measured by the *in ovo* EROD assay (96 hpf) and cardiac deformities (144 hpf)

Main effects and the interaction of population and BaP treatment on EROD induction were significant ($p < 0.001$; non-parametric ANOVA). There was a significant increase in EROD activity compared to controls in KC embryos exposed to each concentration of BaP examined ($p < 0.001$; Dunnett's *post hoc* test). There was no significant increase in EROD activity in BaP dosed ER embryos. Main effects and interaction of population and BaP treatment on cardiac deformities were significant ($p < 0.001$; non-parametric ANOVA). There was a significant increase of cardiac deformities in KC embryos exposed to 400 µg/L BaP compared to control ($p < 0.001$; Dunnett's *post hoc* test). There was no significant increase of cardiac deformities in ER embryos dosed with BaP. 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.

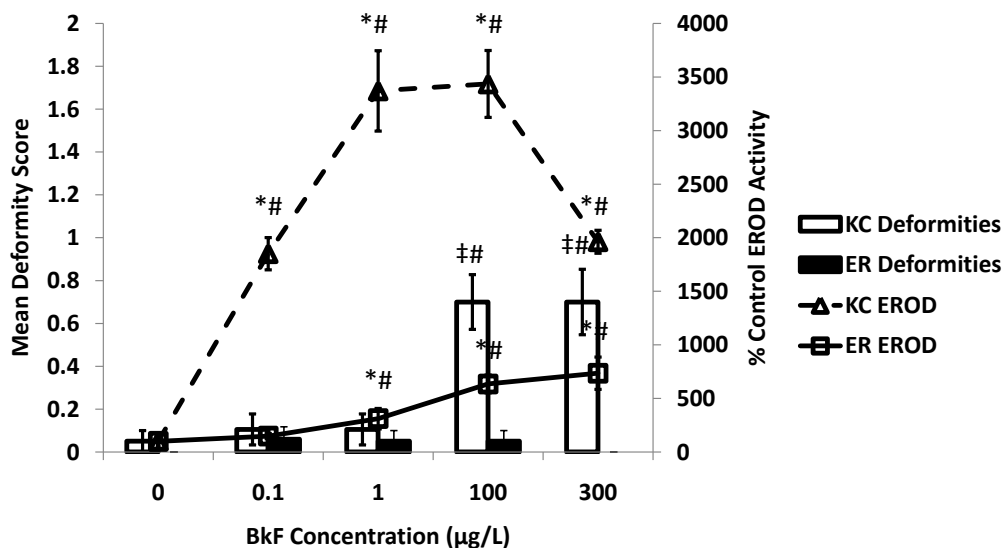


Figure 2.2: BkF dose response curve of CYP1 enzymatic activity as measured by the *in ovo* EROD assay (96 hpf) and cardiac deformities (144 hpf)

Main effects and the interaction of population and BkF treatment on EROD induction were significant ($p < 0.001$; non-parametric ANOVA). There was a significant increase in EROD activity compared to controls in KC embryos exposed to each concentration of BkF examined ($p < 0.001$; Dunnett's *post hoc* test). There was a significant increase in EROD activity compared to controls in ER embryos exposed to 1, 100, and 300 µg/L BkF ($p < 0.001$; Dunnett's *post hoc* test). Main effects and interaction of population and BkF treatment on cardiac deformities were significant ($p < 0.001$; non-parametric ANOVA). There was a significant increase of cardiac deformities in KC embryos exposed to 100 and 300 µg/L BkF compared to control ($p < 0.001$; Dunnett's *post hoc* test). There was no significant increase of cardiac deformities in ER embryos dosed with BkF. 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.

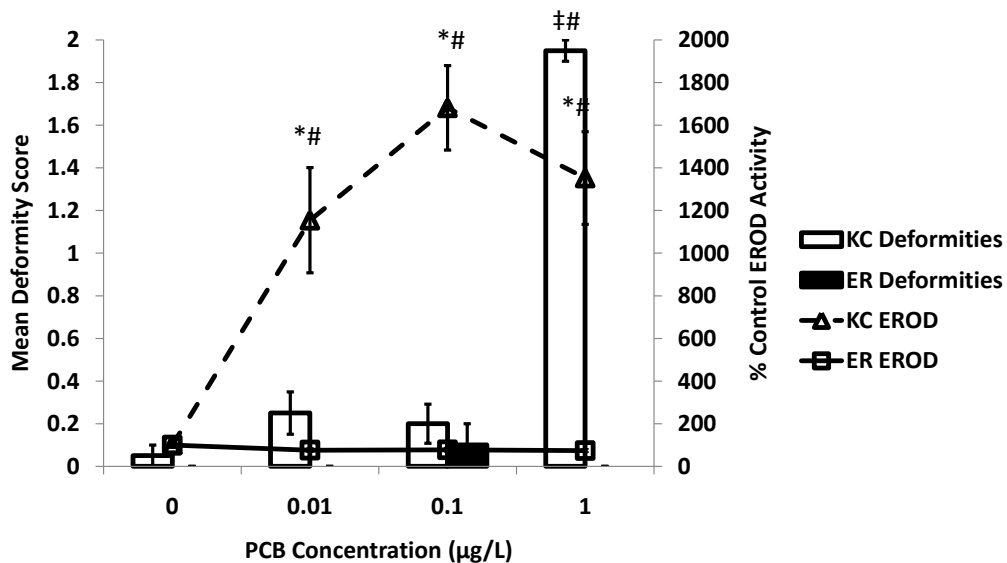


Figure 2.3: PCB126 dose response curve of CYP1 enzymatic activity as measured by the *in ovo* EROD assay (96 hpf) and cardiac deformities (144 hpf)

Main effects and the interaction of population and PCB126 treatment on EROD induction were significant ($p < 0.001$; non-parametric ANOVA). There was a significant increase in EROD activity compared to controls in KC embryos exposed to each concentration of PCB126 examined ($p < 0.001$; Dunnett's *post hoc* test). There was no significant increase in EROD activity in PCB126 dosed ER embryos. Main effects and interaction of population and PCB126 treatment on cardiac deformities were significant ($p < 0.001$; non-parametric ANOVA). There was a significant increase of cardiac deformities in KC embryos exposed to 1 µg/L PCB126 compared to control ($p < 0.001$; Dunnett's *post hoc* test). There was no significant increase of cardiac deformities in ER embryos dosed with 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.

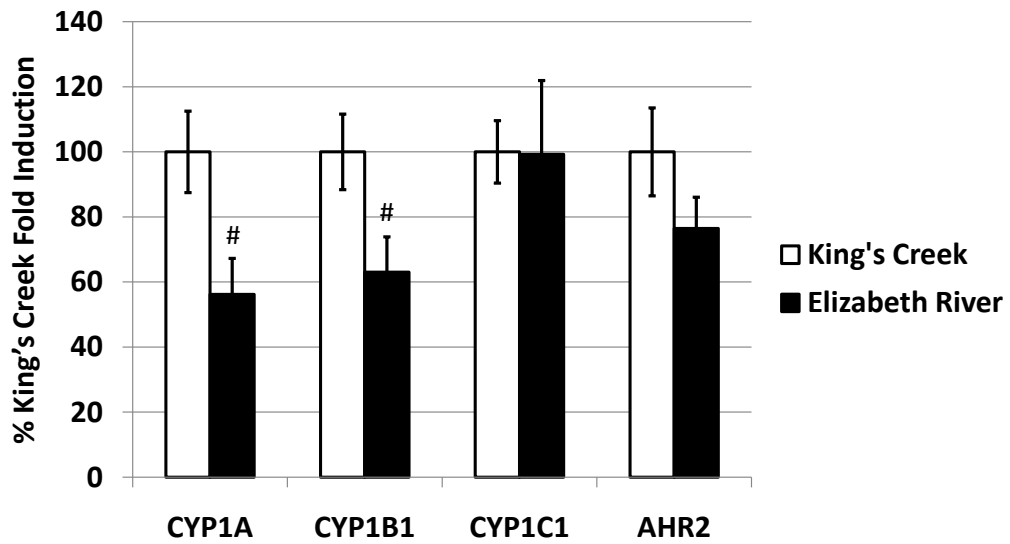


Figure 2.4: *CYP1A*, *CYP1B1*, *CYP1C1*, and *AHR2* mRNA induction in KC and ER embryos exposed to the DMSO vehicle control

Statistical analysis was performed using an ANOVA and Dunnett's *post hoc* test to determine treatments that differed from controls. The fold inductions of *CYP1A* and *CYP1B1* were significantly lower in the ER embryos compared to those from the KC ($p < 0.05$). The fold inductions of *CYP1C1* and *AHR2* were not significantly different between the populations. Data represented as average fold induction \pm SEM; $n \geq 6$ pools of 2 embryos. "#" indicates a significant difference between populations.

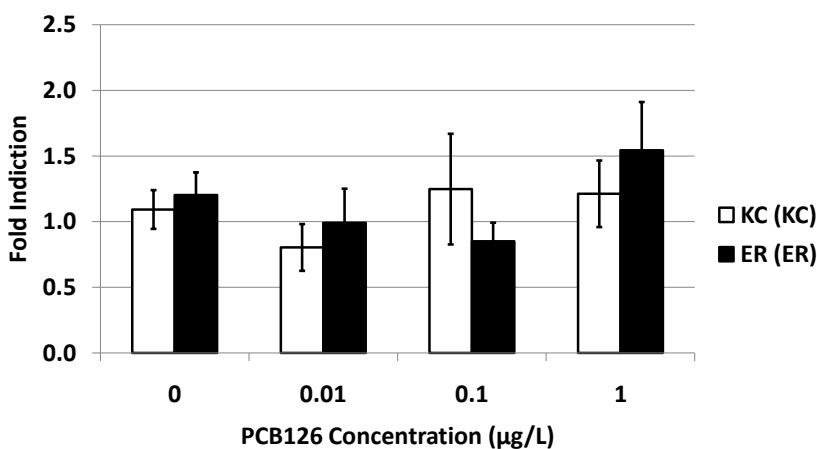
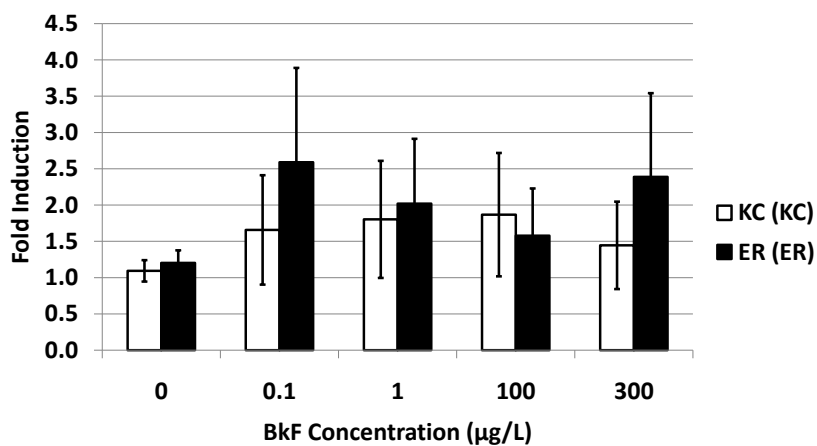
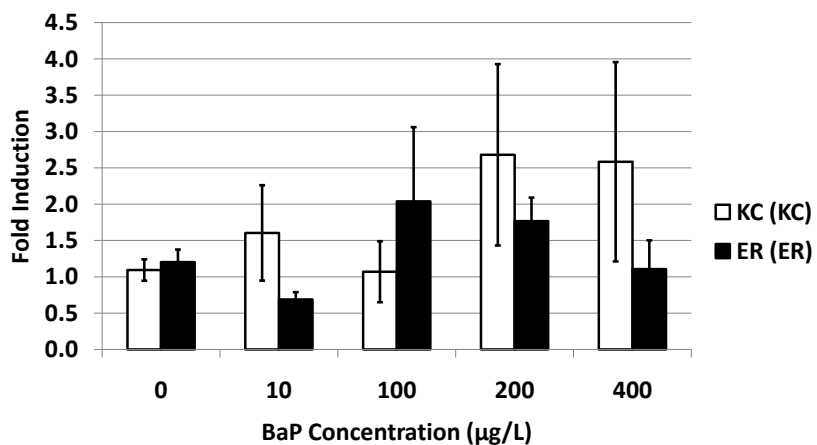


Figure 2.5: *AHR2* mRNA induction in KC and ER embryos exposed to BaP, BkF and PCB126

Figure 2.5: 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.

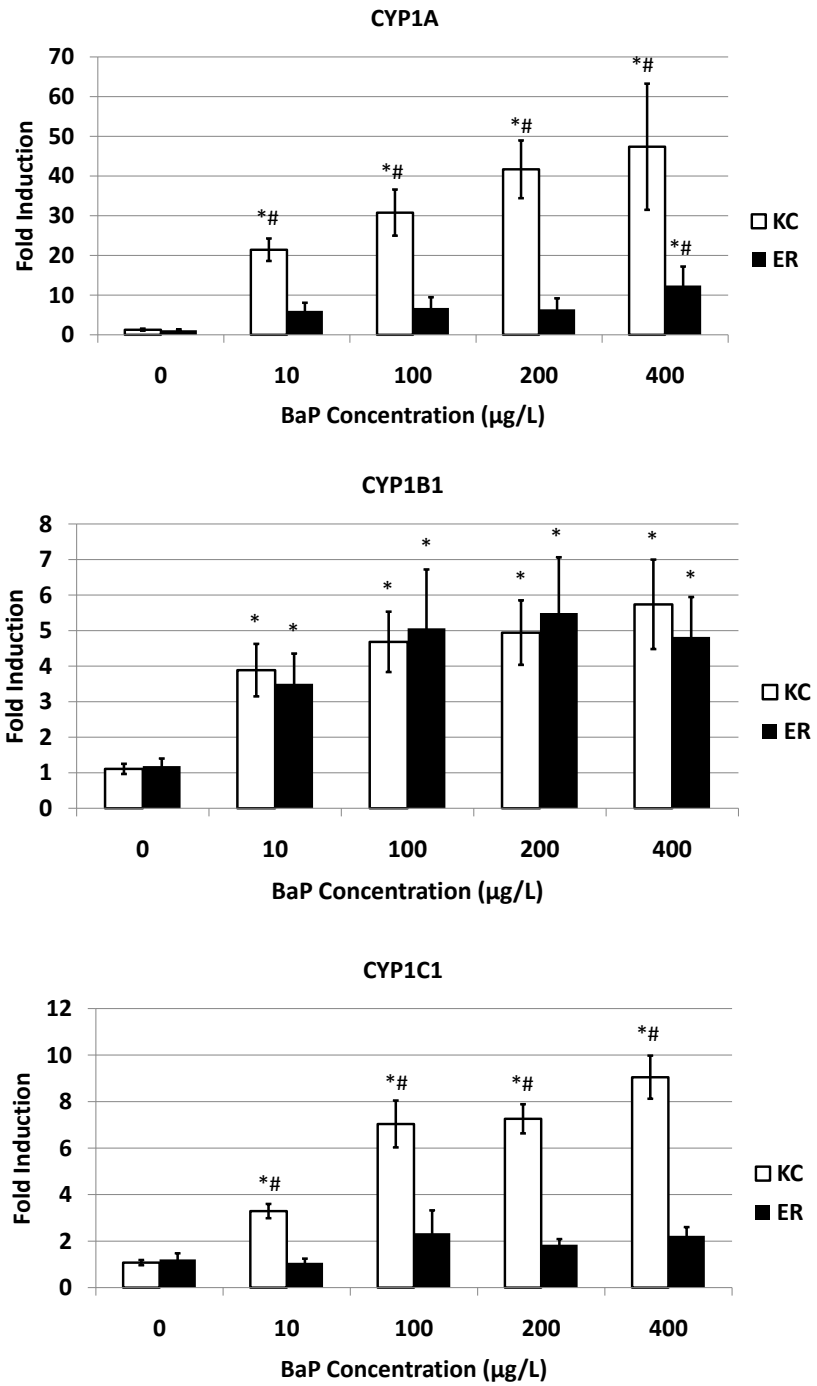


Figure 2.6: mRNA induction of the metabolic enzymes CYP1A, CYP1B1, and CYP1C1 in KC and ER embryos exposed to BaP

Figure 2.6: 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 $\mu\text{g/L}$ BaP ($p \leq 0.01$). *CYP1B1* was induced at levels above control in KC and ER embryos dosed with 10, 100, 200, and 400 $\mu\text{g/L}$ BaP ($p < 0.05$). *CYP1A* was induced in ER embryos exposed to 400 $\mu\text{g/L}$ 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.

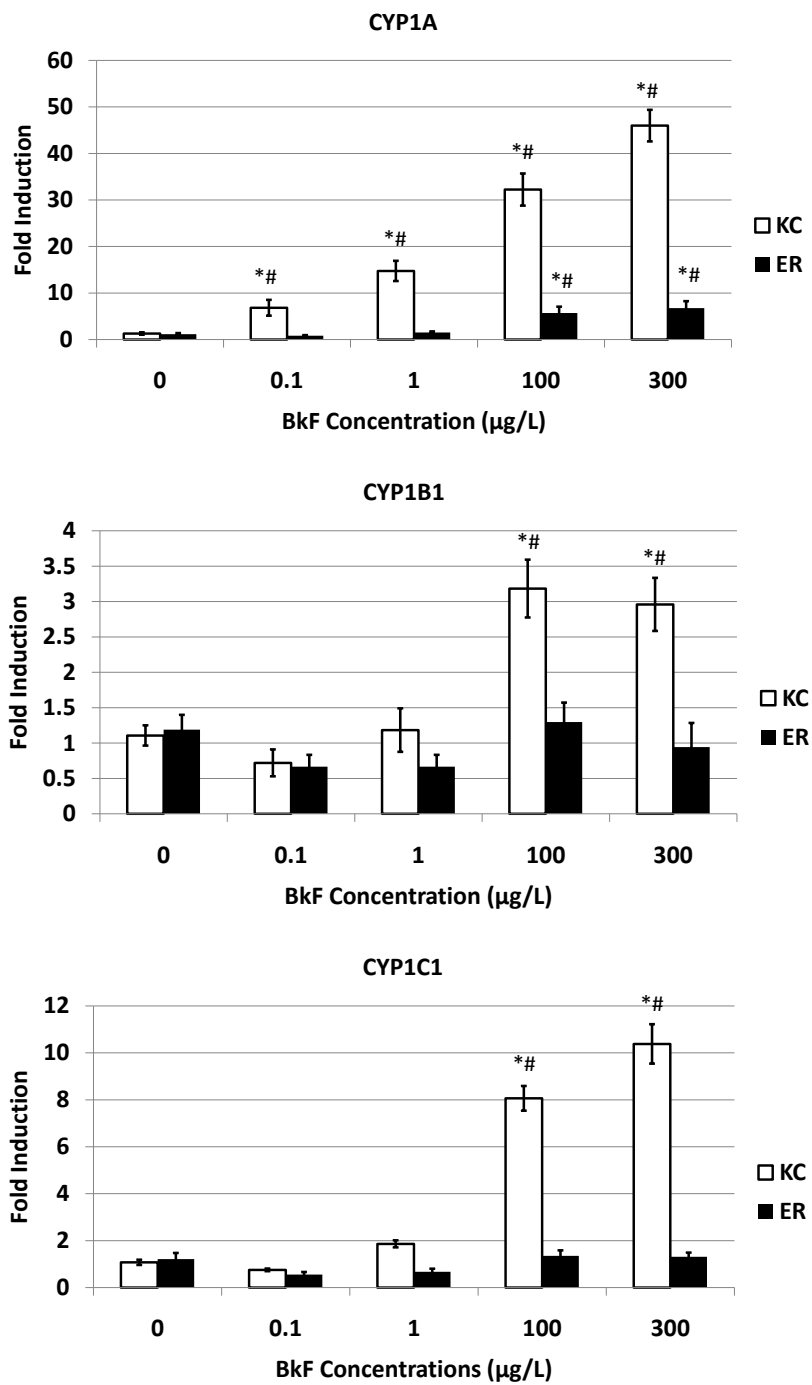


Figure 2.7: mRNA induction of the metabolic enzymes CYP1A, CYP1B1, and CYP1C1 in KC and ER embryos exposed to BkF.

Figure 2.7: 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 $\mu\text{g/L}$ BkF ($p < 0.001$) and in ER embryos dosed with 100 and 300 $\mu\text{g/L}$ BkF ($p < 0.001$). *CYP1B1* and *CYP1C1* were induced at levels above control in KC embryos dosed with 100 and 300 $\mu\text{g/L}$ 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.

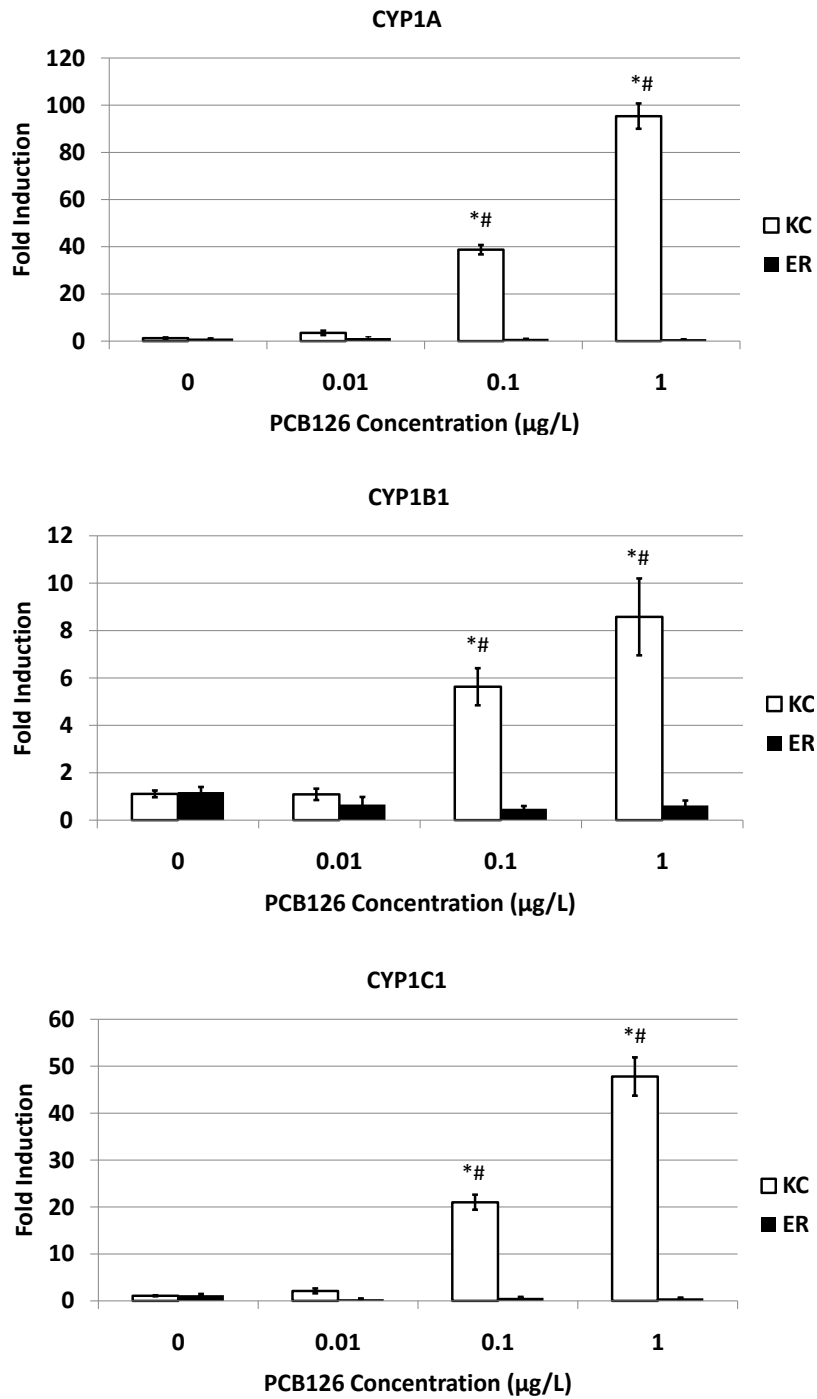


Figure 2.8: mRNA induction of the metabolic enzymes CYP1A, CYP1B1, and CYP1C1 in KC and ER embryos exposed to PCB126

Figure 2.8: 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 $\mu\text{g/L}$ 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.

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

This chapter has been submitted for publication to the journal of Aquatic Toxicology under the same title, by Lauren P. Battle, Shiqian Zhu, Kristine L. Willett, and Richard T. Di Giulio.

3.1 Introduction

Creosote-contaminated sediment from a Superfund site on the Elizabeth River (ER), in Portsmouth, VA is teratogenic to killifish (*Fundulus heteroclitus*) from reference sites. However, killifish from the site are resistant to this teratogenicity. Mechanisms underlying the resistance are not well understood; however, ER killifish are refractory to induction of cytochrome P4501A (CYP1A) mRNA, protein and activity. Fluoranthene (FL), a CYP1A inhibitor, and benzo[a]pyrene (BaP), an agonist for the aryl hydrocarbon receptor (AHR), are dominant polycyclic aromatic hydrocarbons (PAHs) at the site, and co-exposure causes synergistic embryotoxicity in reference site embryos. The purpose of this work is to determine how co-exposure to fluoranthene and subsequent CYP1A inhibition affects the metabolism of BaP and ultimately, teratogenicity in killifish. We also examined how the ER resistant phenotype affects biotransformation of BaP and teratogenicity with and without FL, compared to killifish from the reference site (King's Creek (KC), VA). Embryos were dosed from 24 to 120 hours post fertilization (hpf) with 0 - 500 µg/L FL with or without 100

$\mu\text{g/L}$ BaP. Maximum CYP1 enzymatic inhibition and cardiac deformities were observed after co-exposure to 500 $\mu\text{g/L}$ FL, therefore that dose was used for the chemical analysis. To examine the dose response effects of BaP embryos were dosed as previously described with 0 - 400 $\mu\text{g/L}$ BaP with or without 500 $\mu\text{g/L}$ FL, 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 $\mu\text{g/L}$ 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.

3.2 Background

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 increase 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.

The Elizabeth River (ER) is a sub-estuary of the James River, which is the southernmost tributary of the Chesapeake Bay. This river was home to the Atlantic Wood Industries wood treatment facility. 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 $\mu\text{g/g}$ dry weight in the sediment (Bieri et al. 1986, Vogelbein et al. 2008). Although the sediment is both acutely lethal and teratogenic to a variety of aquatic organisms, there is a population of *Fundulus heteroclitus* (killifish), which are 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.

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).

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, 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.

Due to the nature of their production, 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 AHR agonists and CYP1 inhibitors. 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). Willett et al. (2001a) found that killifish adults co-exposed to BaP and FL had 55% lower CYP1 activity than those exposed to BaP alone. Therefore 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.

3.3 Materials and Methods

3.3.1 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 maintained 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. Eggs at 2 hpf were treated with 0.3% hydrogen peroxide (H₂O₂) to prevent infection and rinsed three times with clean ASW (20 ppt). Eggs were examined 24 hours post fertilization (hpf) for viability and placed individually into 20 mL glass scintillation vials with 10 mL of treatment solution.

3.3.2 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 as previously described 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.

3.3.3 *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. While the embryos were in the dosing solution, resorufin, the fluorescent product of CYP1A metabolism of ethoxyresorufin, accumulated in the bilobed urinary bladder. 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 activity was measured as intensity of bladder fluorescence 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.

3.3.4 Deformity Assessment

Embryos were scored blind for heart elongation (tube heart), pericardial effusion, tail shortening, 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.

3.3.5 Embryonic Extractions and Chemical Analysis

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

(Figure 1.5). The concentrations of the metabolites were determined by calculating the ratio of the metabolite to the concentration of the internal standard recovered.

3.3.6 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.

3.4 Results

3.4.1 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 ($p < 0.001$) (Figure 3.1a and Figure 3.2a.). In the KC embryos exposure to BaP increased EROD activity, and that activity was inhibited by each of the concentrations of FL examined. 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. The severity of cardiac deformities was significantly different between both populations ($p < 0.001$) and treatments ($p < 0.001$) (Figure 3.1b and Figure 3.2b). Cardiac deformities greater than controls were observed in the KC embryos co-exposed to 500 $\mu\text{g/L}$ FL and each of BaP

concentrations (10-400 µg/L) (Figure 3.2b). 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.

3.4.2 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.

3.5 Discussion

In this study, we utilized the refractive CYP1 phenotype of the ER killifish population and the chemical CYP1A inhibitor FL to examine the effect of CYP1 inhibition on the biotransformation and teratogenicity of BaP. There was 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 at concentrations of environmental relevance, 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 activity in the KC embryos, but not in the ER embryos. 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 synergistic PAH toxicity and their refractory CYP1 phenotype as previously reported by Meyer et al (2002b).

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 1998a, 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. 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. A possible explanation for this altered metabolism is that the ER killifish exhibit elevated levels of lymphoid cyclooxygenase-2 (COX-2). Elevated levels of COX-2 are associated with a state of macrophage activation, but may also alter metabolism through cooxidation-peroxidase pathways (Frederick et al. 2007).

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. 2003a). 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.

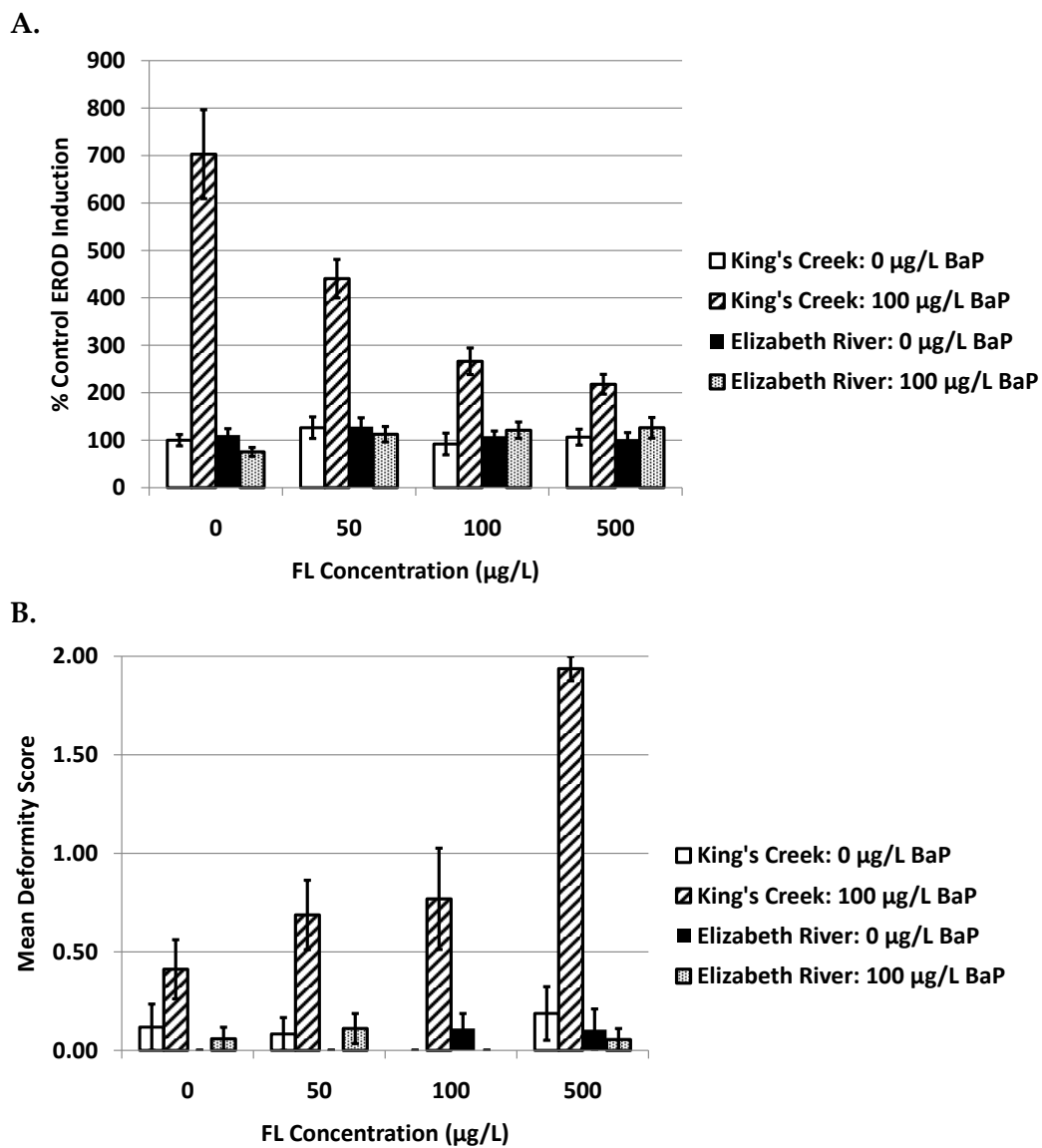


Figure 3.1: FL dose response of CYP1 enzymatic activity as measured by *in ovo* EROD assay and cardiac deformities (120 hpf)

Figure 3.1: Killifish embryos were simultaneously exposed 24 - 120 hpf to waterborne concentrations of 21 $\mu\text{g/L}$ ethoxyresorufin and either DMSO (vehicle control, 0.03% v/v), FL (50-500 $\mu\text{g/L}$), and/or 100 $\mu\text{g/L}$ BaP. (A) There was a significant interaction effect of FL, BaP, and population ($p < 0.01$; non-parametric ANOVA). Data are represented as the average percentage of KC control induction \pm SEM from three replicate experiments; $n = 25$. (B) The increasing deformity score represents increasing severity of deformities (0 = normal; 1 = mild deformities; 2 = severe deformities (see Figure 3.3)). There was a significant interaction effect of BaP, FL and population ($p < 0.01$; non-parametric ANOVA). Data are represented as the average deformity score \pm SEM from three replicate experiments; $n=25$

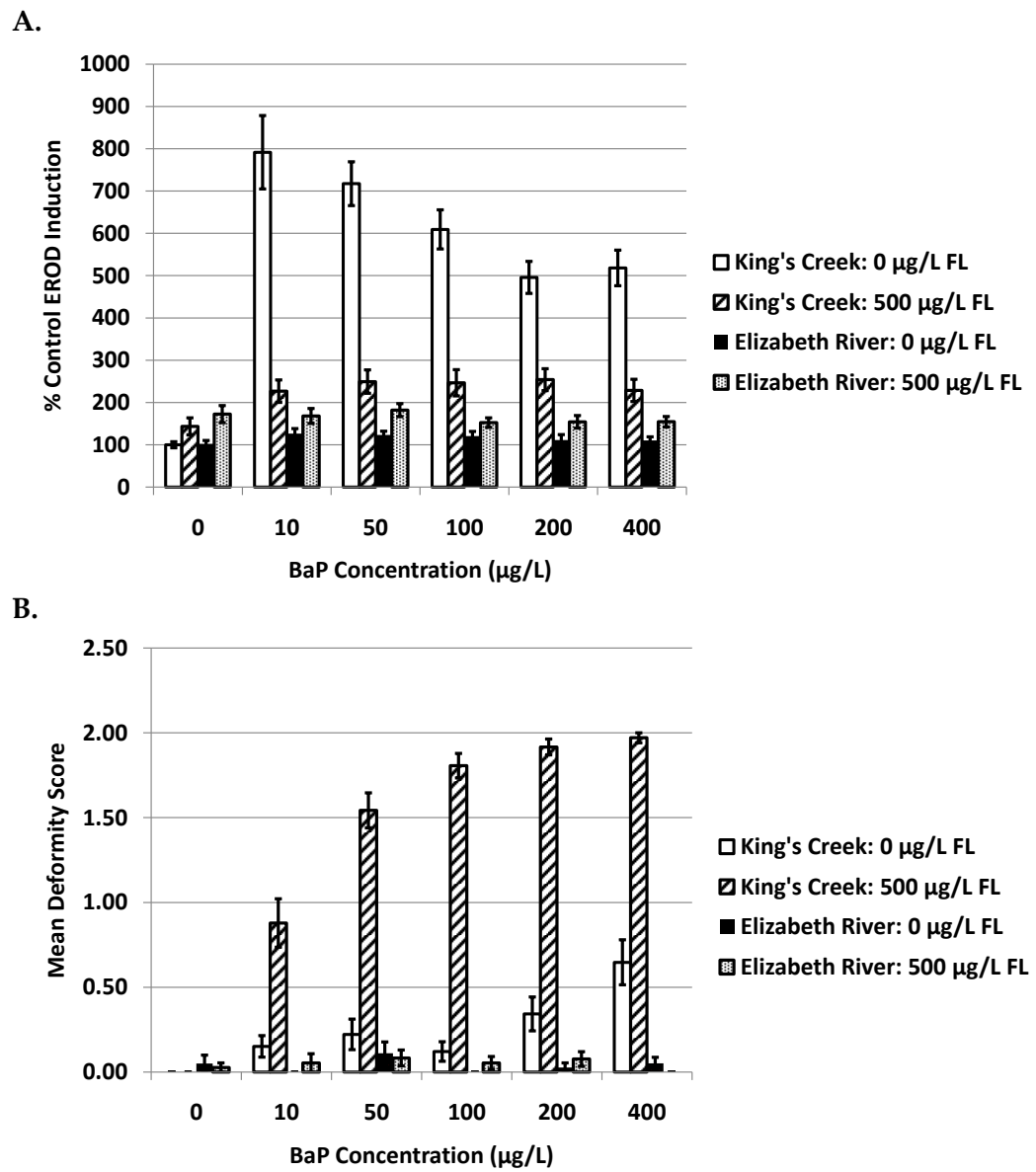


Figure 3.2: BaP dose response of CYP1 enzymatic activity as measured by *in ovo* EROD assay and cardiac deformities (120 hpf)

Figure 3.2: Killifish embryos were dosed by waterborne exposure 24-120 hpf to 21 $\mu\text{g/L}$ ethoxyresorufin and either DMSO (vehicle control < 0.03% v/v) or BaP (10 - 400 $\mu\text{g/L}$) with or without co-exposure to 500 $\mu\text{g/L}$ FL. (A) There was a significant interaction effect of BaP, FL, and population ($p < 0.01$; non-parametric ANOVA). Data are represented as the average percentage of KC control induction \pm SEM from three replicate experiments; $n = 25$. (B) The increasing deformity score represents increasing severity of deformities (0 = normal; 1 = mild deformities; 2 = severe deformities (see Figure 13)). There was a significant interaction effect of BaP, FL and population ($p < 0.01$; Bonferroni corrected non-parametric ANOVA). Data are represented as the average deformity score \pm SEM from three replicate experiments; $n = 25$

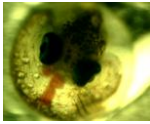
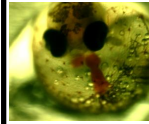
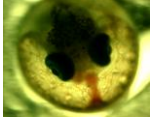
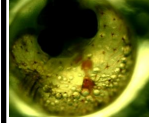
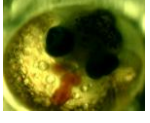

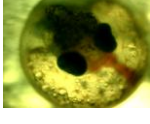
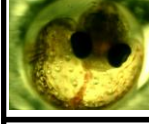



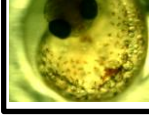
	0 $\mu\text{g/L}$ FL	500 $\mu\text{g/L}$ FL	
0 $\mu\text{g/L}$ BaP			0
10 $\mu\text{g/L}$ BaP			1
50 $\mu\text{g/L}$ BaP			
100 $\mu\text{g/L}$ BaP			
200 $\mu\text{g/L}$ BaP			2
400 $\mu\text{g/L}$ BaP			

Figure 3.3: 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)

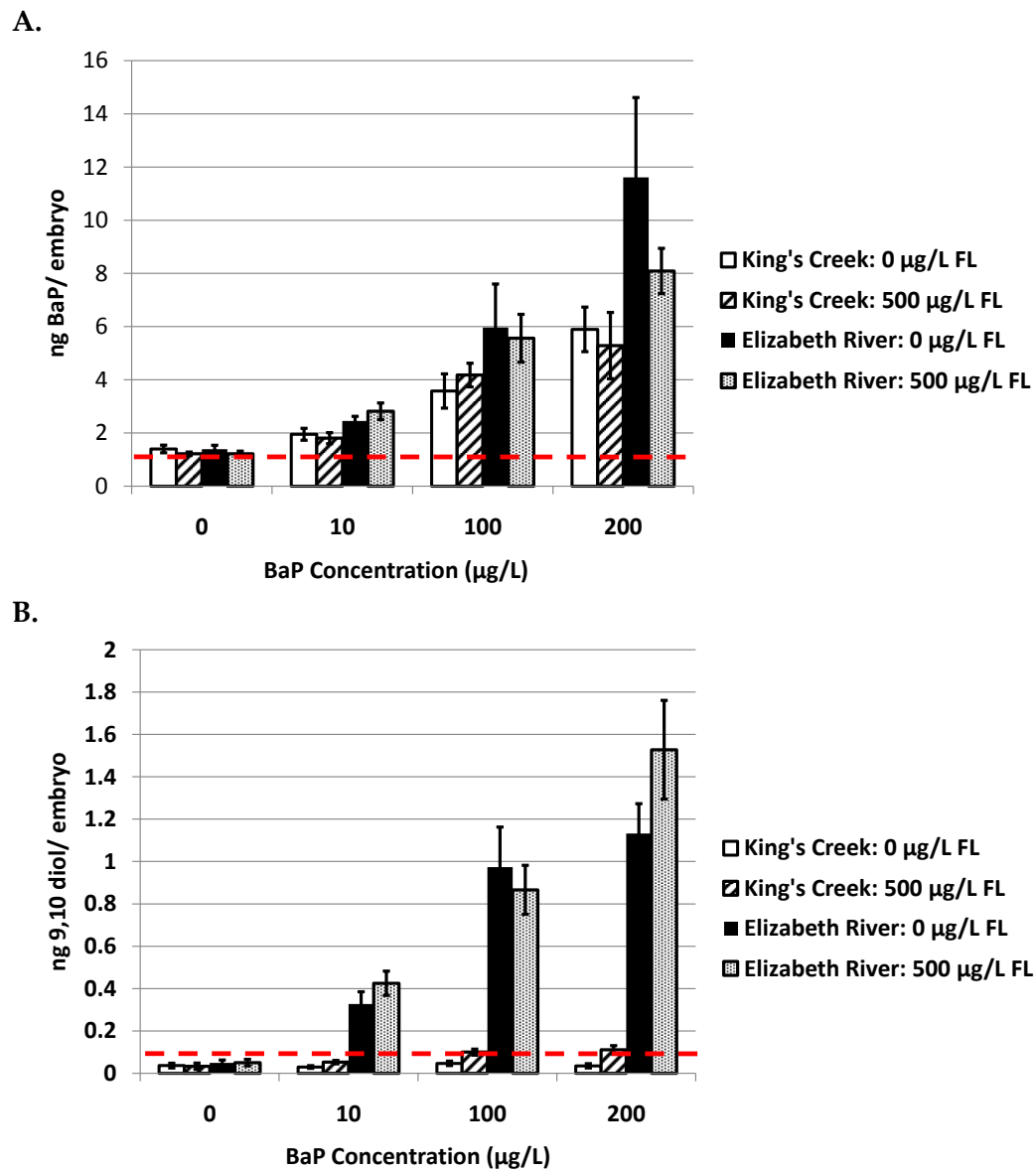


Figure 3.4: Parent BaP and BaP-9,10 dihydrodiol recovered and identified by UPLC/MS (120 hpf)

Dashed lines represent detection limits. 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.

4. Morpholino knock-down of CYP1A alters embryotoxicity and PAH metabolism in *Fundulus heteroclitus*

This work was the product of a collaborative effort with Bryan W. Clark, Cole W. Matson, Shiqian Zhu, and Kristine L. Willett.

4.1 Introduction

Previous research has shown an important role of biotransformation of benzo[a]pyrene (BaP) in mediating its carcinogenic effects, but less work has been done to determine the role of BaP metabolism in causing its teratogenic effects. BaP is a polycyclic aromatic hydrocarbon (PAH) and an agonist of the aryl hydrocarbon receptor (AHR). The AHR regulated enzyme, cytochrome P450-1A (CYP1A) is thought to play an important role in the biotransformation and toxicity of BaP. Reducing CYP1A activity in embryos, using both chemical inhibition and morpholino knockdown, results in a significant decrease in PAH-induced CYP1A activity and causes an increase in the incidence of cardiac teratogenesis (Wassenberg and Di Giulio 2004a, Billiard et al. 2006, Matson et al. 2008a). The goal of this study was to use morpholino knockdown to further determine the role of CYP1A in mediating the biotransformation of BaP in *Fundulus heteroclitus* (killifish) embryos, and to elucidate the possible role of that biotransformation on cardiac teratogenesis. We confirmed that, compared to non-injected embryos and those injected with a control morpholino, CYP1A morpholino injection caused a significant decrease in CYP1A enzymatic activity and a significant

increase in the occurrence of cardiac deformities. We did not observe any differences between non-injected and morpholino-injected embryos in the amount of BaP recovered six days after exposure. Significantly lower concentrations of BaP-7,8,9,10-tetrahydroterol were recovered from embryos injected with the control and the CYP1A morpholino, suggesting that there may be a morpholino effect on biotransformation that will have to be addressed in future studies. None of the other BaP metabolites examined were recovered at high enough concentrations to determine the effect of CYP1A knockdown on their formation. These data confirm the role of CYP1A in the embryotoxic effects of BaP, but are inconclusive concerning the role of biotransformation in mediating these effects.

4.2 Background

PAHs are the byproducts of combustion of organic materials. There are natural sources of these compounds, including volcanoes, oil seeps and forest fires; however the anthropogenic sources of PAHs are of the greatest concern (Van Metre et al. 2000, Hwang and Foster 2006, Chalmers et al. 2007). PAHs are lipophilic making them highly bioavailable to organisms, including those in aquatic ecosystems (Lin et al. 1996, Deshpande et al. 2002). Exposure of multiple fish species, including zebrafish (*Danio rerio*), rainbow trout (*Oncorhynchus mykiss*) and killifish, to PAHs during early life stages results in cardiac dysfunction, similar to the effects observed by dioxin-like compounds (DLCs) (Billiard et al. 1999, Incardona et al. 2004, Wassenberg and Di Giulio 2004a, b).

Although the mechanisms responsible for PAH-induced cardiac teratogenesis are still being elucidated, many of these compounds are agonists for the aryl hydrocarbon receptor (AHR). It is hypothesized that this interaction plays an important role in embryotoxicity (Billiard et al. 2002, 2006, Incardona et al. 2006).

Benzo[a]pyrene (BaP) is one of the most studied PAHs primarily due to its carcinogenic effects in both humans and other vertebrates. BaP is a ligand for the AHR, which when activated, regulates the expression of multiple genes including the metabolic enzymes CYP1A, CYP1B1 and CYP1C1 (Godard et al. 2000, Shimada and Fujii-Kuriyama 2004, Wang et al. 2006, Yin et al. 2008). BaP a prototypical PAH consisting of a five-membered ring that can be oxidized by CYP1 enzyme to form reactive metabolites, including epoxides, phenols, dihydrodiols and quinones (Miller and Ramos 2001). The epoxides can be further oxidized to form dihydrodiol-epoxides that can covalently bond to DNA resulting in the formation of bulky adducts, as is the case with the highly mutagenic BaP-7,8-dihydrodiol-9,10-epoxide (BPDE) (Dunn et al. 1987, van Gijssel et al. 2004). The quinones can also alter cellular function through the production of reactive oxygen species that can damage lipids, proteins and DNA (Burdick et al. 2003). The intermediate reactive metabolites of BaP could be playing a role in the embryotoxic effects through oxidative stress or disturbing cellular signaling. Altering the activity of metabolic enzymes responsible for the biotransformation of BaP may alter the reactive metabolites that are formed and the change the toxicity outcomes.

Although the functionality of CYP1B1 and 1C1 in fish are still being elucidated, CYP1A has been found to be important in protecting against PAH-induced embryo toxicity in both rodent and aquatic models. Compared to wild type, four week old CYP1A1 (-/-) mice orally exposed to BaP cleared the parent compound at 4-fold slower rate and had a 100% decrease in survival 30 days after exposure (Uno et al. 2004). Wassenberg et al (2004a) also showed that co-exposure to BaP and the CYP1A inhibitor α -naphthoflavone, synergistically increased the occurrence of cardiac deformities in killifish embryos. CYP1A plays an important role in the detoxification of PAHs, and it is likely that the biotransformation process is involved in the observed teratogenicity of BaP (Hawkins et al. 2002, Hodson et al. 2007).

The use of antisense morpholino (MO) technology has been a critical advance in elucidating the mechanisms of developmental toxicity. Traditionally used in zebrafish and frogs (*Xenopus laevis* and *X. tropicalis*), this technology has recently been adapted in our laboratory for use in the estuarine fish, *Fundulus heteroclitus* (killifish) (Matson et al. 2008a). Killifish have emerged as an invaluable scientific model, and are particularly useful in examining how both individuals and populations interact with their environment and respond to contamination (Bigelow and Schroeder 1953, Burnett et al. 2007). Previous research has shown that MO knockdown of CYP1A in both zebrafish and killifish results in a significant increase in cardiac deformities observed with PAH-type AHR agonists (Billiard et al. 2006, Matson et al. 2008a). What remains unclear is the

mechanism causing this enhanced teratogenesis. The CYP1A-MO provides a powerful technique for elucidating the effects of CYP1A on the biotransformation of BaP and gain greater insight into BaP induced developmental toxicity.

4.3 Materials and Methods

4.3.1 Animals

Adult killifish were collected from King's Creek in Gloucester County, Virginia, (37°17'52.4"N, 76°25'31.4"W), and maintained on a photoperiod of 14:10 L:D at 23-25°C. Fish were housed in a recirculating system containing artificial seawater (ASW; 25 ppt) prepared from Instant Ocean® (Foster & Smith, Rhinelander, WI). They were fed daily with a mixture of TetraMin® Tropical Flakes (Tetra, Blacksburg, VA) and newly hatched *Artemia* (Brine Shrimp Direct, Ogden, UT). In order to breed the fish, light pressure was applied to the abdomens of reproductively mature females to collect eggs, and sperm was milked from adult males. Eggs were fertilized *in vitro* and washed 30 minutes post fertilization with 0.3% hydrogen peroxide. In order to remove debris from the exterior of the chorion, eggs were "rolled" on durable wet paper towels as previously described by both Marty et al (1990) and Matson et al (2008a).

4.3.2 Morpholino microinjection

Embryos were injected at the 2-4 cell stage (1-2 hours post fertilization (hpf)) with 2-5 nL of 250 µM morpholino antisense oligo stock as described in Matson et al (2008a). Morpholinos were designed and manufactured by Gene Tools (Philomath, OR) using

the *Fundulus heteroclitus* cytochrome P4501A (CYP1A) (GenBank, AF026800) as a template. The sequence for the CYP1A-MO used is 5'-ATGCCCATGATGACAACCTTTTCTCTG-3' (the start codon is underlined) (Matson et al. 2008a). The morpholino injected control (Ctrl-MO) was performed using the Gene Tools' standard control morpholino (5'-CCTCTTACCTCAGTTACAATTTATA-3'). Sham injections were performed by piercing the embryo without the injection of a morpholino.

4.3.3 Chemical Exposure

Ethoxyresorufin, dimethyl sulfoxide (DMSO) and benzo[a]pyrene (BaP) were purchased from Sigma-Aldrich (St. Louis, Mo). Twenty-four hpf embryos were dosed individually in 20-mL glass scintillation vials (VWR) with 10 mL of treatment solution made with ASW (20 ppt) containing 21 µg/L ethoxyresorufin for performing the EROD (7-ethoxyresorufin-O-deethylase) assay as described below. Embryos were individually exposed to 200 µg/L BaP or to the vehicle control DMSO at 24 hpf for five days. In all treatment groups DMSO concentration was maintained at less than 0.03% by volume. Five days after dosing, embryos were placed into vials containing clean ASW. *In ovo* EROD was measured 120 hpf and cardiac deformities were assessed blind at 168 hpf as described below. Embryos used for metabolic analysis were flash frozen in liquid nitrogen 168 hpf and stored at -80°C until processed for chemical extraction.

4.3.4 *In ovo* EROD assay

The *in ovo* EROD assay was used to measure CYP1 activity in the developing embryo using the protocol outlined in Nacci et al. (1998) and modified by Wassenberg and Di Giulio (2004b). During the five-day dosing period, resorufin, the fluorescent product of CYP1A metabolism of ethoxyresorufin, accumulated in the bi-lobed urinary bladder. At 120 hpf embryos were placed in clean ASW and embryo bladders were visualized by fluorescent microscopy. EROD activity was measured as fluorescence intensity within the bladder and was quantified digitally with IP Lab software (Scanalytics, Inc., Fairfax, VA).

4.3.5 Deformity analysis

Embryos were examined blind by light microscopy for heart elongation (tube heart) and pericardial effusion 168 hpf. Heart elongation severity was ranked as a 0, 1, or 2 representing normal, mild, and severe deformities respectively as outlined in Matson et al (2008a). Representative images for each treatment are shown in Figure 4.3. Further examination of these embryos confirmed that embryos scored as a 0 have a 92% hatch success, those scored as a 1 have a 58% hatch success, and embryos scored with a 2 all fail to hatch (data not shown).

4.3.6 Embryonic extractions and chemical analysis

Embryos were pooled in groups of 10 and homogenized for 30 seconds in 15 μ l methanol/mg tissue (Hawkins et al. 2002). The resulting homogenate was then extracted

with 600 µl methanol and passed through a 0.2-µM nylon filter (Acrodisc®; PALL Life Sciences, Ann Arbor, MI). The samples were dried under nitrogen and the residue was dissolved in 50 µl of HPLC grade acetonitrile. One µl of the extract was injected onto a C-18 reverse phase ultra pressure liquid chromatography (UPLC) column (ACQUITY UPLC™ BEH C18 1.7 µm 2.1 × 50 mm). Metabolites were separated and analyzed using a three-step gradient elution program (65:35 to 40:60 0.3% formic acid in water: acetonitrile in six minutes, to 0:100 in 9 minutes and 65:35 in 10 minutes) at a flow rate of 0.25 ml/min at 28°C (Zhu et al. 2008). The chromatograms were analyzed by mass spectrometry and the concentrations of the metabolites were calculated by determining the ratio of the metabolite to the concentration of the internal standard, 6-OH chrysene. The samples were analyzed for the presence of parent BaP and the following metabolites: BaP-7,8,9,10-tetrahydrodiol, BaP-7,8-dihydrodiol, BaP-9,10-dihydrodiol, BaP-1,6-dione, BaP-3,6-dione, BaP-6,12-dione, BaP-9OH, and BaP-3-OH (Figure 1.5).

4.3.7 Statistical analysis

All data collected were analyzed using SPSS ver.15 (SPSS Inc, Chicago, IL). The EROD data and the deformity data were both determined not to be normally distributed according to the Kolmogorov-Smirnov test. For analysis data were rank transformed and examined using a non-parametric Analysis of Variance (ANOVA) to test for significant differences among treatments. Metabolic data was normally distributed and therefore analyzed using an ANOVA. Pairwise comparisons were analyzed using a

Bonferroni-corrected post hoc comparison. Statistical significance was accepted at $p \leq 0.05$ for all tests.

4.4 Results

In the non-injected and Ctrl-MO injected embryos, the exposure to 200 $\mu\text{g/L}$ BaP increased EROD activity, with no significant difference between the two groups. The induction of EROD activity was significantly reduced by 60% in BaP-exposed embryos injected with the CYP1A-MO ($p < 0.05$) (Figure 4.1). Injection with the CYP1A-MO resulted in a significant increase in the severity of cardiac deformities observed after BaP exposure compared to non-injected and Ctrl-MO injected embryos ($p < 0.05$) (Figure 4.2). There was no significant increase in cardiac deformities for non-injected or Ctrl-MO embryos dosed with BaP.

Chemical analyses revealed no significant differences among non-injected, sham-injected and morpholino-injected embryos in the amount of parent BaP recovered from the embryos (Figure 4.4). Greater amounts of the metabolite BaP-7,8,9,10-tetrahydrotetrol (BaP-tetrol) were recovered from non-injected embryos when compared to those injected with both the Ctrl-MO and the CYP1A-MO ($p < 0.05$) (Figure 4.4). There was no significant difference in the amount of BaP-tetrol recovered between the sham-injected embryos and any of the other injection groups. None of the other metabolites examined were found in concentrations above the detection limits and therefore no conclusions can be drawn about the effect of morpholino injection on their production.

4.5 Discussion

In this work, we utilized the CYP1A-MO to elucidate the effects of CYP1A inhibition on the biotransformation of BaP and to gain greater insight into BaP-induced developmental toxicity. We did not observe an effect of CYP1A knockdown on the amount of parent BaP recovered. One reason for this could be that when the activity of CYP1A is reduced, CYP1B1 and/or CYP1C1 metabolize enough BaP to compensate. Both CYP1B1 and CYP1C1 have been shown to be inducible by AHR agonists, and CYP1B1 does have the ability to metabolize BaP (Shimada and Fujii-Kuriyama 2004, Wang et al. 2006, Timme-Laragy et al. 2007). The activity of CYP1C1 in this regard is currently unknown.

We did observe a significant decrease in the recovery of BaP-tetrol after injection with the CYP1A-MO. BaP-tetrol is a degradation product of the highly carcinogenic metabolite BPDE (Miller and Ramos 2001). The CYP1A oxidation of BaP to BaP-7,8-epoxide and the subsequent oxidation to BPDE is necessary for the formation of BaP-tetrol; therefore, it is intuitive that less of this compound would be recovered in embryos with decreased CYP1A expression. However, because we saw similar effects with the Ctrl-MO it may be that this decrease is an artifact and not biologically relevant. One possible reason for this observed decrease in BaP-tetrol for both sets of morpholino-injected embryos could be an effect of a structure inherent within the morpholinos such as the carboxyfluorescein modification on the 3' end. It is possible that this modification

is biologically active and is causing a metabolic alteration. It is also possible that the stress of the injection process affected BaP biotransformation; however, this is unlikely because the sham injected embryos showed no significant differences in the amount of BaP-tetrol that was recovered. Another possible explanation is that the calculated concentration may be inaccurate due to the fact that the amount of BaP-tetrol that was recovered was close to the detection limits of the UPLC/MS.

The lack of detection of any other BaP metabolites may also be a cause for concern. Killifish adults that were injected with BaP showed significant recovery of BaP-7,8-dihydrodiol, BaP-1,6-dione, BaP-3,6-dione, and BaP-3-OH in the bile (Zhu et al. 2008). In these adult killifish exposures, BaP-tetrol was not recovered at concentrations above detection limits. These inconsistencies could be explained by differences in dosing regime or differences in the metabolism between adult and embryonic fish. Zebrafish embryos have been shown to eliminate and biotransform PAHs at a slower rate than their juvenile and adult counterparts, which may explain their increased sensitivity to the toxic effects of these compounds (Petersen and Kristensen 1998). Another possible reason for the lack of metabolite detection is the small amount of tissue within the embryos and our inability to examine the presence of conjugated metabolites. Future studies examining biotransformation within early life stages may need to employ more sensitive methods of detection. Multi-photon laser scanning microscopy, as outlined in Hornung et al (2004), allows for the tissue specific detection of multiple metabolites in

fish throughout early development, and thus would provide a more sensitive detection method.

Hodson et al. (2007) showed that CYP1A inhibition with the model PAH α -naphthoflavone (ANF) at low levels (32-100 $\mu\text{g/L}$) resulted in an increase in lethality and blue sac disease caused by retene (7-isopropyl-1-methyl phenanthrene) in larval rainbow trout (*Oncorhynchus mykiss*). These concentrations of ANF also shifted the metabolism of retene away from the polar hydroxylated metabolites and towards both less polar metabolites and parent retene. At higher concentrations of ANF (320 $\mu\text{g/L}$), the toxic effects of retene were reduced and parent retene was the predominant form in the tissue. These data suggest that the toxic effects in larvae were caused by a metabolite of retene and not the parent compound. It is possible that the increase in cardiac deformities observed in CYP1A-MO injected embryos was due to a shift towards the formation of alternate metabolites by CYP1B1 and CYP1C1 that we were not able to detect in this study. CYP1B1 can play an important role in the production of reactive PAH metabolites (Harrigan et al. 2004, Shimada and Fujii-Kuriyama 2004). CYP1B1(-/-) mice showed protection against the carcinogenic effects of 7,12-dimethyl-benz[a]anthracene and dibenzo[a,l]pyrene indicating that CYP1B1 may preferentially form reactive metabolites (Buters et al. 1999). Wang et al (2006) reported that in killifish CYP1C1 is inducible by BaP and has broad tissue distribution in both adults and embryos; therefore, it is possible that it may also play an important role in biotransformation.

Biotransformation contributes to the toxicity of multiple compounds, including aflatoxin B, carbamates, and PAHs (Schlenk et al. 2008). Killifish populations that have adapted to resist the teratogenic effects of PAHs, have reduced mRNA induction and activity of the CYP1 enzymes (Bello et al. 2001, Meyer et al. 2003a, Wirgin and Waldman 2004). This suggests that the ability of these embryos to alter biotransformation may protect them from PAH-induced embryotoxicity. The data presented in this study concerning EROD activity and the incidence of cardiac deformities confirms that CYP1A inhibition through both chemical interference and morpholino knock-down results in a decrease in EROD activity and an increase in PAH-induced cardiac abnormalities in fish embryos (Wassenberg and Di Giulio 2004a, Wassenberg et al. 2005, Billiard et al. 2006, Hodson et al. 2007, Matson et al. 2008a).

One potentially interesting finding is that the morpholino knockdown of CYP1A only resulted in a 60% reduction in EROD activity. The residual 40% may be explained by the fact that injection of the CYP1A-MO knocked down CYP1A protein expression, but did not completely eliminate it. Additionally, CYP1B1 and CYP1C1 are inducible by BaP, and an increase in their expression could also contribute to increased EROD activity (Harrigan et al. 2004, Wang et al. 2006). Other families of CYP enzymes including CYP2K and CYP3A can also be induced by PAH, and may contribute to EROD activity in the absence of CYP1A (Schlenk et al. 2008).

In the ambient environment, organisms are exposed to complex mixtures of compounds with different mechanisms of toxicity. Even within PAHs, some are potent AHR agonists and CYP1 inducers, while others act as inhibitors of CYP1A enzymatic activity. Co-exposure to these PAHs with different modes of action results in synergistic embryonic and larval toxicity, possibly mediated by a biotransformation related-mechanism (Wassenberg et al. 2005, Hodson et al. 2007, Billiard et al. 2008). As environmental toxicologists continue to examine the role of pollution in the environment, the advancement of morpholino technology provides the unique ability to examine the importance of specific proteins on development and metabolism. Now that this technology has been harnessed for use in the ecologically relevant aquatic model of the killifish, environmental toxicologists will be able to examine the mechanisms of toxicity in even greater detail. Future studies should utilize morpholinos against other genes in the AHR pathway including, but not limited to, CYP1B1 and CYP1C1, as well as against AHR2 itself.

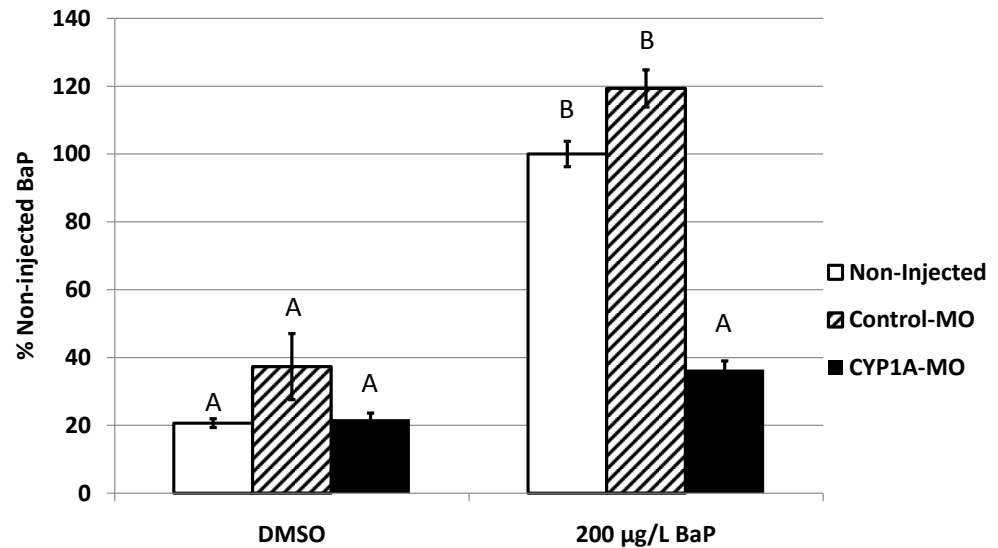


Figure 4.1: Induction of CYP1 enzymatic activity as measured by the *in ovo* EROD assay in non-injected and morpholino-injected killifish embryos after exposure to either the DMSO vehicle control or to 200 µg/L BaP (120 hpf)

CYP1A-MO injection resulted in a significant reduction in BaP induced EROD activity compared to non-injected and Ctrl-MO embryos ($p \leq 0.05$; Bonferroni corrected non-parametric ANOVA). Data are represented as average percent activity relative to non-injected BaP exposed embryos \pm SEM from three replicate experiments; $n = 70$. Letters represent groups that are significantly different from each other.

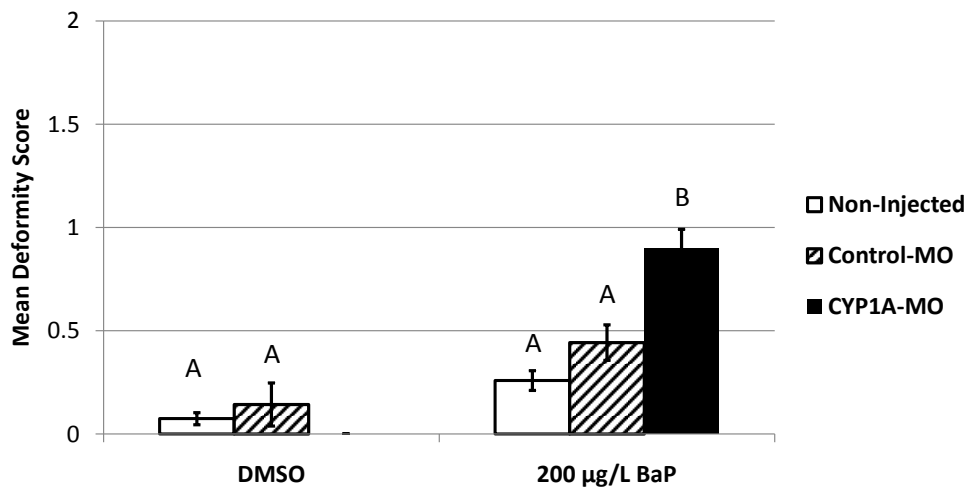
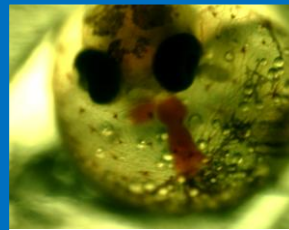


Figure 4.2: Average cardiac deformity scores for non-injected and morpholino injected killifish embryos exposed to either the DMSO vehicle control or 200 µg/L BaP (168 hpf)

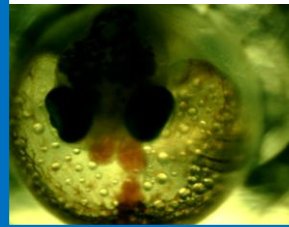
Embryos were examined given a score based on the increasing severity of cardiac deformities (0 = normal; 1 = mild deformities; 2 = severe deformities). CYP1A-MO resulted in a significant increase in BaP induced cardiac deformities compared to non-injected and Ctrl-MO embryos ($p < 0.05$; Bonferroni corrected non-parametric ANOVA). Data represented as average deformity score \pm SEM from three replicate experiments; $n = 60$. Letters represent groups that are significantly different from each other.

200 $\mu\text{g/L}$ BaP

Non-injected

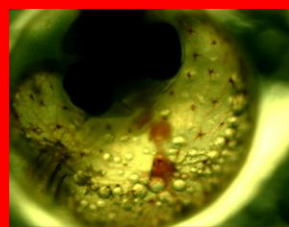


Control - MO



0

CYP1A - MO



1

Figure 4.3: Cardiac deformities observed in both non-injected and morpholino-injected killifish embryos following exposure to 200 $\mu\text{g/L}$ BaP (168 hpf)

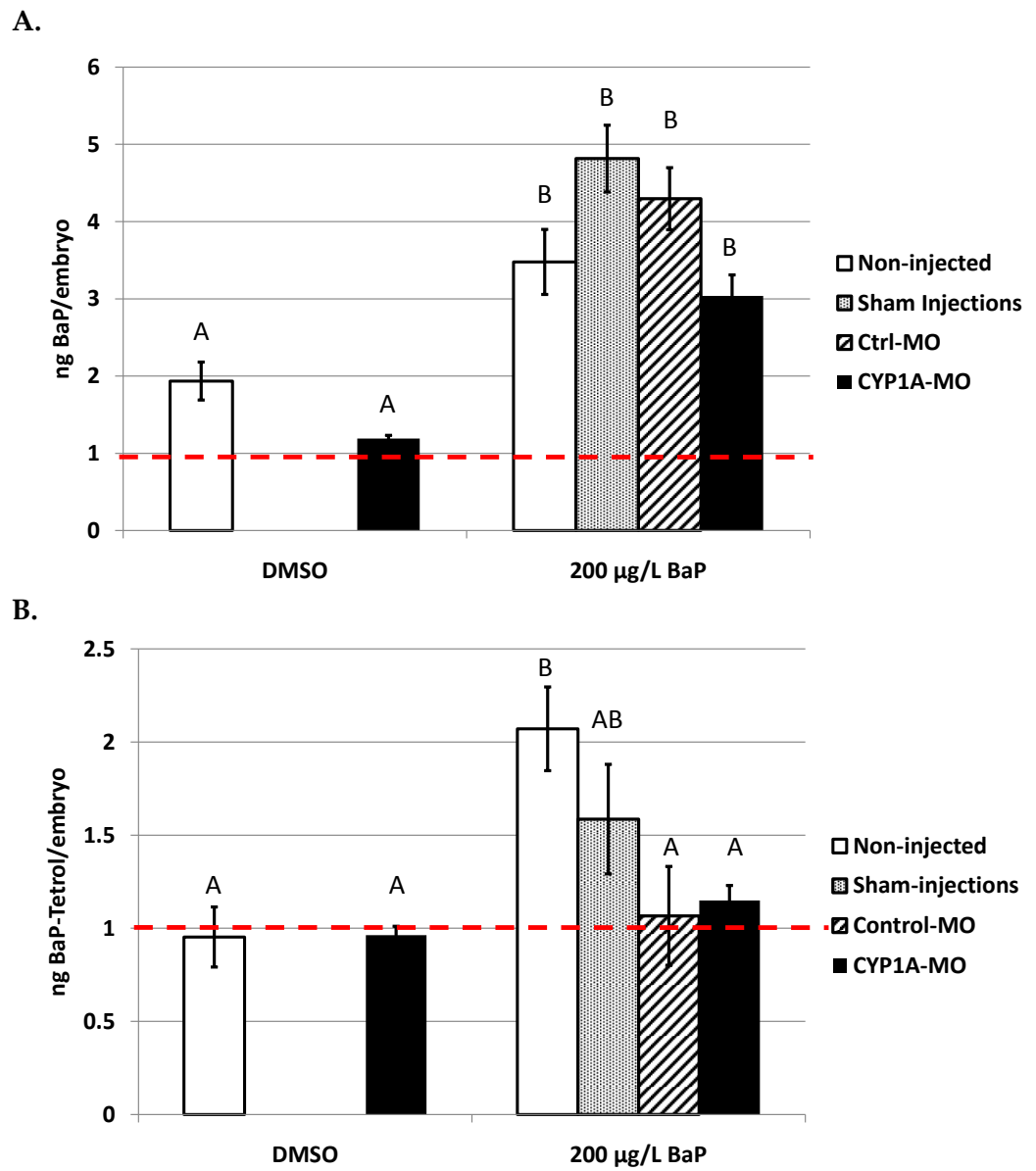


Figure 4.4: Parent BaP and BaP-7,8,9,10-tetrahydrodiol recovered and identified by UPLC/MS (168 hpf)

Figure 4.4: Dashed lines represent detection limits. A) There were no differences observed in the concentration of parent BaP recovered between non-injected, sham injected, Ctrl-MO, and CYP1A-MO embryos. B) Ctrl-MO and CYP1A-MO resulted in a significant decrease in the amount of BaP-7,8,9,10-tetrahydrodiol recovered from embryos exposed to BaP compared to those that were non-injected ($p \leq 0.05$; Bonferroni corrected ANOVA). Sham injected embryos showed no significant observable differences in the amount of BaP-tetrol recovered relative to the other treatment groups. Data represented as an average mass of chemical recovered per embryo \pm SEM from two replicate experiments; $n = 10$ pools of 10 embryos. Letters represent groups that are significantly different from each other.

5. Comparative chronic liver toxicity of benzo[a]pyrene in two populations of *Fundulus heteroclitus* with different exposure histories.

Portions of this work will be submitted for publication with Lauren P. Battle, Dawoon Jung, Kara Koehn, Shiqian Zhu, Kristine L. Willett, David E. Hinton, and Richard T. Di Giulio as authors.

5.1 Introduction

In this study larval killifish (*Fundulus heteroclitus*) born to parents caught from a Superfund site on the Elizabeth River (ER) in Portsmouth, VA were compared to naïve larvae from a reference site to determine their differential susceptibility to chronic liver toxicity including the carcinogenic effects of benzo[a]pyrene (BaP). The Atlantic Wood Industries Superfund site on the ER is contaminated with polycyclic aromatic hydrocarbons (PAH) derived from the wood preservative, creosote. There is a population of killifish inhabiting the site whose embryos and larvae are refractory to the induction of cytochrome P450-1 (CYP1) activity, protein, or message when exposed to agonists for the aryl hydrocarbon receptor (AHR), and are resistant to PAH induced lethality and teratogenicity. Adult killifish collected from the ER show a greater prevalence of hepatic and pancreatic tumors when compared to killifish collected from a reference site, suggesting that their resistance to intermediate and chronic PAH toxicity requires further investigation. Killifish from both the ER and from a reference site on King's Creek (KC) were given two 24 hour

exposures of either DMSO (vehicle control), or BaP (10 - 400 µg/L). The first exposure was conducted on larvae at 4 days after hatching, and the second exposure was administered 7 days later. After the first exposure, a subset of larvae was analyzed for the presence of parent BaP and select metabolites 24, 48 and 96 hours following exposure. Immediately after the second exposure, a subset of larvae was analyzed for CYP1A activity and both nuclear and mitochondrial DNA damage. Three and nine months after the second exposure, livers were analyzed histologically. KC larvae showed significant induction of CYP1A activity at each concentration of BaP ($p < 0.001$), while the ER larvae showed no change in CYP1A activity for any of the treatment groups. Over 96 hours there was a significant difference in the amount of parent BaP recovered from the ER and KC larvae ($p < 0.001$), with the KC fish showing a greater reduction in the concentration of BaP over time. These data suggest that the ER larvae had a slower rate of biotransformation, excretion, or both. Mitochondrial and nuclear DNA lesion frequency increased significantly in BaP exposed KC larvae ($p < 0.001$). Although ER larvae exhibited a higher basal level of DNA damage than the KC larvae, DNA lesion frequency did not increase after BaP exposure. No differences in weight or mortality were observed in either population for any of the treatment groups throughout the nine month period. Nine months following exposure, 2 out of the 25 (8%) KC juveniles examined developed foci of cellular alteration (FCA) in response to 200 µg/L BaP. Following the 400 µg/L exposure, 9 out of the 30 (30%) KC juveniles

examined developed eosinophilic and basophilic FCA ($p < 0.05$). Three of these individuals also developed hepatocellular carcinoma. Only 2 out of 30 (6%) ER juveniles developed FCA after exposure to 400 $\mu\text{g/L}$ BaP; however no fish with hepatocellular carcinomas were found in this population. These data suggest that although the ER fish do develop hepatic neoplasms in the wild, they are less susceptible to the genotoxic and carcinogenic effects of laboratory exposure to BaP.

5.2 Background

The Atlantic Wood Industries (AWI) in Virginia was a wood treatment facility from 1926 until 1992. While in operation, AWI spanned about 48 acres on the waterfront of the Elizabeth River (ER) estuary in Portsmouth, VA (EPA 2007). In 1990 the area was classified as a Superfund site and listed on the National Priorities List (NPL) of hazardous waste sites due to extensive pollution of the wood preservatives pentachlorophenol (PCP) and creosote (Bieri et al. 1986). Sediment from the site is acutely toxic and highly teratogenic to a variety of species of aquatic organisms including oysters (*Crassostrea virginica*), brackish water clams (*Rangia cuneata*), spot (*Leiostomus xanthurus*), hogchoker (*Trinectes maculatus*) and killifish (Hargis Jr et al. 1984, Bender et al. 1988, Huggett et al. 1992, Wassenberg and Di Giulio 2004b, Vogelbein et al. 2008). However, there is a population of reproductively active killifish that inhabit the contaminated estuary. Embryos and larvae born to ER wild caught parents are resistant to the acute toxicity and developmental abnormalities that occur in embryos of reference

site adults exposed in the laboratory to the creosote contaminated sediment (Meyer et al. 2002a, Wassenberg and Di Giulio 2004b).

Creosote is a complex mixture of unsubstituted heterocyclic and phenolic polycyclic aromatic hydrocarbons (PAHs), which are byproducts of organic combustion. PAHs are teratogenic, immunotoxic, and narcotic; however it is their carcinogenic effects that are the most well characterized (Yan 1985, Di Toro and McGrath 2000, Carlson et al. 2004, Billiard et al. 2008). At the AWI site the mean concentration of PAHs in the sediment is 410 µg/g dry weight and consists primarily of fluoranthene, pyrene, chrysene, and benzo[a]pyrene (Bieri et al. 1986, Vogelbein et al. 2008). Many of the PAHs at the ER are agonists for the aryl hydrocarbon receptor (AHR), and there are abundant data indicating that they elicit their toxic effects in part through this pathway (Billiard et al. 2002, Denison and Nagy 2003, Billiard et al. 2006, 2006). The biotransformation of PAHs plays an important role both in their activation and elimination, and often occurs through AHR mediated induction of the CYP1 family of metabolic enzymes (Hawkins et al. 2002, Shimada and Fujii-Kuriyama 2004, Hodson et al. 2007).

The ER embryonic, larval and adult killifish show significantly reduced inducibility of the CYP1 enzymes both at the level of mRNA induction and protein activity as characterized in Chapter 4 and described by Meyer et al (2003a). 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. 2002a, Ownby et al. 2002, Meyer et al. 2005). However, ER killifish larvae are more susceptible to hypoxia and phototoxicity, and adults caught in the wild have a high incidence of hepatic and extra-hepatic lesions, suggesting that there may be tradeoffs and fitness costs of the adaptation, as well as differing lifetime exposure patterns associated with residence at this site (Vogelbein et al. 1990, Meyer and Di Giulio 2003). Therefore, killifish from the ER population are resistant to the acute and developmental toxicity of PAHs, but their susceptibility to the chronic effects, including carcinogenicity, remains unclear.

BaP is a genotoxic PAH abundant at the AWI Superfund site, contributing about 12% of the total dry weight of PAHs in the sediment (Vogelbein et al. 2008). It has a five ring structure containing two regions of high metabolic activity, the "k-region" (carbons 4 and 5) and the "bay region" (carbons 9-10) (Figure 1.3) (Yan 1985). The "bay region" is of particular importance because in addition to providing high electron density, this region also forms an area of steric hindrance. The result is high levels of oxidation or radical formation, but low levels of detoxification and conjugation (Miller and Ramos 2001). BaP can be oxidized at multiple sites on the molecule to form phenols, quinones and epoxides. BaP-phenols and quinones can undergo redox cycling and produce reactive oxygen species that can damage DNA, lipids and proteins resulting in toxicity. The epoxide metabolites of BaP can be further oxidized, resulting in the formation BaP-dihydrodiol-epoxides. BaP-7,8-epoxide can be hydrolyzed by microsomal epoxides

hydrolase (mEH), and then reoxidized by the CYP1 enzymes in the "bay region" to form BaP-7,8-dihydrodio-9,10-epoxide (BPDE), a highly reactive metabolite that forms bulky adducts at the N² position of guanine within DNA (Miller and Ramos 2001). These adducts are associated with the mutagenicity and carcinogenicity of BaP (James et al. 1991, Rojas et al. 2004) In addition to causing nuclear DNA damage, BaP and its metabolites can also enter the mitochondria where they can damage the mitochondrial genome and disrupt aerobic respiration (Allen and Coombs 1980, Backer and Weinstein 1980).

While research has shown that CYP1A (-/-) and CYP1B1 (-/-) knock out mice do show reduced formation of reactive metabolites, the ultimate effects of these alterations on toxicity are not clear (Buters et al. 1999, Shimada and Fujii-Kuriyama 2004). Uno et al (2004) showed that CYP1A (-/-) mice exposed to BaP exhibited a dramatic increase in DNA adduct formation compared to wild type, suggesting the CYP1A is protective by aiding in the excretion of BaP. However, CYP1B1 (-/-) mice developed 60% fewer lymphomas than wild type mice in response to the PAH, dimethylbenz[a]anthracene, indicating that CYP1B1 mediates PAH toxicity through reactive metabolite production (Buters et al. 1999). These data confirm the need for more research to examine the role of the CYP1 enzymes in PAH excretion, biotransformation, and ultimately toxicity, particularly over the chronic timeframe needed for development of tumors and related endpoints.

In this study we conducted experiments to test the hypothesis that while embryo and larval ER killifish are more resistant to acute PAH toxicity, the adaptation and altered responsiveness of the AHR pathway may have resulted in an altered sensitivity to the genotoxic and carcinogenic effects of PAH exposure. To examine this question, we dosed F1 larval killifish born to wild caught parents from the ER and from a reference site on King's Creek (KC) in VA with a 2-hit exposure to BaP (10 - 400 µg/L). The first hit was administered to larvae 4 days post hatch (dph) for 24 hours, and the second hit was administered seven days later for an additional 24 hours. This 2-hit dosing regime is adapted from the mouse skin tumor promotion model, and is based on research that indicates repetitive exposure to mutagenic agents results in the increased accumulation of genetic alterations necessary for tumor development (Owens et al. 1999). It has proven effective in inducing carcinogenesis in both mammalian and aquatic laboratory studies (Dragan et al. 1993, Tugiyono and Gagnon 2002). After exposing the larvae to BaP, we examined inducibility of CYP1 activity, the time-course of BaP biotransformation and excretion, and DNA damage both in the nuclei and in the mitochondria. Three and nine months post exposure, we examined the histology of the juveniles to determine chronic hepatotoxicity which included steatosis, eosinophilic and basophilic foci of cellular alteration (FCA), and hepatocellular neoplasms including adenomas and early carcinomas. The findings of this study will be used to further characterize the costs and benefits of the ER adaptation to PAH induced toxicity.

5.3 Materials and Methods

5.3.1 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). Fish were kept at 23 - 25°C in 25 ppt artificial seawater (ASW). They were maintained on a photoperiod of 14:10 L:D, and were fed 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 stripped from female fish that was incubated with pooled milt from multiple males. Two hours post fertilization (hpf) eggs were treated with 0.3% hydrogen peroxide (H₂O₂) to prevent fungal infection and rinsed three times with clean ASW (20 ppt). Eggs were allowed to develop in petri dishes on damp filter paper and kept at 23-25° C for 14 days. At 14 days post fertilization (dpf) the embryos were hatched by filling the petri dishes with 20 ppt ASW and placed on a shaker for 30 minutes. Viable larvae were then placed individually into glass scintillation vials and fed a daily diet of newly hatched brine shrimp. Animal care and maintenance protocols were in accordance with regulations mandated by the Duke University Institutional Animal Care and Use Committee (DUIACUC).

5.3.2 Larval Microsome Preparation

Larval killifish from both the ER and KC populations were used to prepare microsomes for *in vitro* analysis of CYP1A activity. Dimethyl sulfoxide (DMSO), BaP, and ethoxyresorufin were purchased from Sigma-Aldrich (St. Louis, Mo). Larvae were dosed individually by waterborne exposure 4 days post hatch (dph) to either DMSO or BaP (10, 100, 200 µg/L) for a 24 hour period. Larvae were placed in clean ASW for seven days and then re-exposed to the dosing solution for 24 hours. DMSO concentrations were maintained below 0.1% for each treatment. Four days after the second exposure larvae were pooled in groups of 10 and homogenized in cold buffer (0.25 M sucrose, 0.1 M tris-HCl, 1 mM EDTA, pH 7.4), and resultant homogenates were centrifuged at 10,000 × g for 20 minutes at 4°C to isolate microsomes. Resultant supernatants were flash frozen in liquid nitrogen and stored at -80°C. The Bio-Rad Protein Assay kit (Hercules, CA) was used to determine the protein concentrations of the microsomal preparations.

5.3.3 *In Vitro* Ethoxyresorufin-O-deethylase (EROD) Assay

CYP1 activity was measured by the *in vitro* EROD assay in larval microsomes as described by Willett et al. (1997) with the following modifications. Bovine serum albumin (BSA) or 200 µg microsomal protein and cofactor buffer (0.1 M HEPES, 100 µM NADH, 115 µM NADPH and 5 mM magnesium sulfate, pH=8) were loaded into a 96-well plate and incubated at room temperature for 5 minutes. The addition of 2.5 µM ethoxyresorufin started the reaction. The microsomal enzymatic activity was calculated

by determining the production rate of resorufin, the fluorescent byproduct of CYP1 metabolism of ethoxyresorufin. Fluorescence was measured at 535/590 excitation/emission each minute for a total of 20 minutes on a FLUOstar OPTIMA microplate reader (BMG Labtech, Offenburg Germany). EROD activity was reported as the average pmol resorufin/mg protein/min.

5.3.4 Larval Extractions and Chemical Analysis

ER and KC larvae were individually dosed 4 dph to either DMSO or BaP (100 µg/L) for 24 hours (n = 40). Killifish were frozen in liquid nitrogen 24, 48, and 96 hours after exposure and stored at -80°C until the time of extraction. Larvae in groups of 10 were placed in 15 µl methanol/mg tissue and homogenized according to the protocol described in Hawkins et al (2002). The resulting homogenate was extracted with 600 µl methanol and filtered through a 0.2 µM nylon Acrodisc® (PALL Life Sciences, Ann Arbor, MI). The methanol was removed by drying the samples under nitrogen. The resulting residue was dissolved in 50 µl of acetonitrile. Samples were injected in two, 1 µl replicates onto a C-18 reverse phase ultra pressure liquid chromatography (UPLC) column (ACQUITY UPLC™ BEH C18 1.7 µm 2.1 × 50 mm). A 3-step gradient elution program was used to separate the metabolites (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) at a flow rate of 0.25 ml/min at 28°C as outlined in Zhu et al (2008). Samples were analyzed by mass spectrometry for the presence of the internal standard 6-OH chrysene and the

following metabolites: BaP, BaP-3-OH, BaP-9-OH, BaP-1,6-dione, BaP-3,6-dione, BaP-6,12-dione, BaP-7,8-dihydrodiol, BaP-9,10-dihydrodiol, and BaP-7,8,9,10-tetrahydrotetrol (Figure 1.5). Results are reported as average metabolite concentration calculated by determining the ratio of the metabolite to the concentration of the internal standard recovered.

5.3.5 Long Amplicon Quantitative PCR

Larvae were dosed 4 dph as previously described to either DMSO or BaP (100 or 200 µg/L) for two consecutive 24 hour periods seven days apart. Four days after the second exposure, larvae were flash frozen in 20% glycerol and stored at -80°C. Larvae were homogenized in pools of 10, and DNA was extracted using the Genomic-tip 20/G kit (Qiagen Inc., Valencia, CA, USA). Long amplicon quantitative polymerase chain reaction (LA-QPCR) was performed according to a protocol outlined in Jung et al (2008). We amplified 10 ng of DNA from each sample with *rTth* polymerase (Applied Biosystems, Foster City, CA, USA). Primers for the large and small nuclear targets were designed for the cystic fibrosis transmembrane conductance regulator gene (CFTR) (Jung et al. 2008). The primer sequences for the large mitochondrial target were obtained from (Kim et al. 2004). Primers and amplicon sizes are described in Table 2.

DNA was amplified and the resulting concentrations were converted to relative lesion frequencies per 10kB DNA, based on alterations in amplification efficiency (Ayala-Torres et al. 2000). With each PCR reaction, we included 5 ng of one of the

control DNAs to monitor amplification quality. Only PCR products in which the amplification of 5 ng DNA was 40-60% of the control DNA (10 ng), indicating that the PCR reaction was quantitative, were used in the analysis.

5.3.6 Histology

Larvae were dosed 4 dph as previously described to either DMSO or BaP (50,100, 200, or 400 µg/L) for two consecutive 24 hour periods seven days apart (n = 60). Subsets of 30 juvenile killifish from each population were weighed and sacrificed 3 and 9 months after exposure. Juveniles were killed by an overdose of MS-222 (100 ppm). Tails were surgically removed, and a midventral incision through the abdominal body wall was extended from the anal pore to the level of the pectoral fins. Thereafter, individuals were immediately placed in 10 volumes of 10% neutral buffered formalin, decalcified using Decalcifying Solution (Richard-Allan Scientific, Kalamazoo, MI) dehydrated in a graded ethanol series, cleared in xylene, and embedded in paraffin. Tissue blocks were sectioned at 5 µm, mounted on glass histoslides, and stained with Harris' hemotoxylin and eosin. A total of 25-30 fish from each treatment were evaluated histologically. For each individual fish we evaluated 3 sections 10 µm apart. All histoslides were surveyed and imaged with a Nikon Eclipse E600 light microscope equipped with a Nikon DXM 1200 digital camera and EclipseNet imaging software (Nikon, Melville, NY). Analysis was focused on the liver, but the intestine and neighboring mesentery with exocrine pancreas was also included within the sections. After the initial read by two separate

individuals, concurrence on lesions was established, and the number of positive individuals was determined per treatment.

Lesions included: eosinophilic and basophilic FCA, hepatocellular adenomas and early hepatocellular carcinomas as defined by Vogelbein et al (1990). FCA were defined as foci of altered hemotoxylin and eosin staining and were basophilic or eosinophilic when compared to the surrounding, non-involved parenchyma. Foci stained differently but otherwise blended with architecture of surrounding hepatocytes that showed normal staining patterns. Eosinophilic foci were characterized by eosinophilic cytoplasm with a ground glass consistency. The border between the focus and the normal parenchyma was irregular, and the nuclei of focal regions were slightly enlarged. Basophilic foci were characterized by a hyperbasophilic cytoplasm and moderate amounts of lipid within hepatocytes. Hepatocellular adenomas were classified as hypertrophied hepatocytes and hepatocyte tubules with an eosinophilic ground-glass cytoplasm and altered cellular architecture. Margins of adenomas were distinct. The nonneoplastic hepatocytes at the sharp lesion border contained large quantities of lipid. Hepatocellular carcinomas exhibited disorganized cellular structure, and irregular borders with neoplastic cells invading the surrounding parenchyma. The cytoplasm within these tumors was eosinophilic and fibrillar in nature.

5.3.7 Statistical Analyses

All of the data collected were analyzed using SPSS ver.15 (Chicago, IL). Data sets were analyzed by the Kolmogrov-Smirnov test to determine if they were normally distributed. Normally distributed data was analyzed using an analysis of variance (ANOVA). Pairwise comparisons were analyzed for statistical significance using a Bonferroni-corrected post hoc comparison. The lesion data was analyzed using a global Chi square analysis with manual post hoc testing between comparable treatment groups. Statistical significance was accepted at $p \leq 0.05$ for all tests.

5.4 Results

5.4.1 *In vitro* EROD Activity

Previous studies have shown that ER embryos, larvae and adults are refractive to the induction of CYP1A enzymatic activity measured by *in ovo* and *in vitro* EROD activity after exposure to AHR agonists (Bello et al. 2001, Meyer and Di Giulio 2002, Meyer et al. 2002a, Meyer et al. 2003a, Wassenberg and Di Giulio 2004b). This study repeated those experiments and confirmed that in laboratory reared larval killifish from parents of the KC population there was significant induction of CYP1A enzymatic activity 4 days after repeated 24 hour exposures to 10-200 $\mu\text{g/L}$ BaP ($p < 0.001$) (Figure 5.2). ER killifish showed no significant induction of CYP1A activity after BaP exposure.

5.4.2 Time-Dependent Recovery of BaP

Chemical analysis revealed a significant interaction between population, treatment, and time for the amount of BaP recovered from the larvae ($p < 0.05$) (Figure 5.3). No detectable levels of BaP were recovered from KC or ER DMSO vehicle control larvae (data not shown). There was a significant decrease over time in the amount of BaP recovered from the KC larvae exposed to 100 $\mu\text{g/L}$ BaP ($p < 0.05$). There was no significant change in the amount of BaP recovered over time in the exposed ER larvae. Recovery of all of the other 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-9OH and BaP-3-OH) remained below detection limits and therefore no conclusions could be made concerning their production in the larvae.

5.4.3 DNA Damage

Using LA-QPCR we detected a significant increase in lesion frequency in both the nuclear and mitochondrial DNA in KC larvae 4 days after repeated 24 hour exposure to 200 $\mu\text{g/L}$ BaP ($p < 0.001$) (Figure 5.4a). This increased DNA damage was significantly higher in the mitochondrial than in the nuclear DNA ($p < 0.001$). There was no significant increase in DNA damage in the KC larvae after exposure to 100 $\mu\text{g/L}$ BaP. The ER larvae had a higher basal level of mitochondrial and nuclear lesion frequency; however no significant change in DNA damage was observed after exposure to either 100 or 200 $\mu\text{g/L}$ BaP (Figure 5.4b).

5.4.4 Mortality, Weight and Histopathology

Mortality throughout the nine month experiment was maintained below 20% for all treatment groups and there were no significant differences in mortality among treatment groups (data not shown). Similarly, no significant differences in body weight between populations or treatments were observed at either 3 or 9 months post exposure (Table 5.2).

No FCA or hepatic neoplasms were observed in KC or ER larvae exposed to DMSO, and there were no lesions found in any juveniles 3 months after exposure to BaP (50, 100, 200, and 400 µg/L) (data not shown). Nine months after dosing, 2 out of the 25 KC juveniles exposed to 200 µg/L BaP developed eosinophilic FCA denoting an 8% increase in lesion frequency, however this was not significantly different from DMSO control (Figure 5.5). 9 out of the 30 KC juveniles exposed to 400 µg/L BaP developed an increase in FCA, 2 of which also developed early hepatocellular adenomas, and 1 hepatocellular carcinoma. These observations denoted a 30% increase in lesion frequency that was significantly different from DMSO control ($p = 0.26$). Only 2 out of the 30 ER juveniles dosed with 400 µg/L BaP developed eosinophilic FCA resulting in a 6% increase in lesion frequency; however this increase was not significantly different from control. No hepatocellular adenomas or carcinomas were found in the ER juveniles that we examined. There was also a significant difference in the percent lesion incidence between the ER and the KC juveniles exposed to 400 µg/L BaP ($p = 0.18$)

5.5 Discussion

The genotoxic effects of the carcinogenic PAH, BaP, were compared in offspring of two populations of Atlantic killifish: one from a reference site and one from an area along the Elizabeth River that is highly contaminated with PAHs. ER embryos, larvae and adults have been shown to be recalcitrant to CYP1 induction by AHR agonists and to be resistant to acute PAH toxicity. However, this population does display multiple fitness costs including a compromised immune system, diminished tolerance to hypoxia, and susceptibility as adults to hepatocellular and pancreatic carcinomas (Vogelbein et al. 1990, Fournie and Vogelbein 1994, Vogelbein et al. 1999, Meyer and Di Giulio 2003, Frederick et al. 2007). These findings led us to ask the question as to whether the offspring of the ER killifish population are more or less resistant than reference site fish to the chronic toxicity of PAHs.

In this study we compared the responses to the genotoxic and chronic effects of a repeated dose of BaP in F1 larvae from PAH adapted fish from the Elizabeth River, and a reference site population collected from the nearby King's Creek. While BaP exposure caused a significant induction of EROD activity in KC larvae, ER killifish did not significantly induce CYP1 activity in response to any of the BaP concentrations that we examined. I have shown that the refractory phenotype of the ER larvae extends beyond CYP1A, and is also characterized by decreased mRNA induction of CYP1B1 and CYP1C1 (Chapter 4). The differences between the KC and ER killifish in the induction

and activity of the CYP1 metabolic enzymes suggests that there are differences between these two populations in their ability to biotransform BaP either by activation or elimination of the parent compound.

Although we were not able to detect any of the metabolites of BaP in either population, there was a significantly lower amount of the parent compound recovered from exposed ER juveniles compared to those from the KC. This indicates that these fish may metabolize BaP at a slower rate than KC fish. Perhaps analysis of bile from larger juveniles would serve to provide additional information as outlined in the adult studies of English Sole (*Parophyrus vetulus*) from Puget Sound in Washington (Malins et al. 1988). It is possible that the ER fish have shifted their metabolism towards a more benign metabolite that we were not able to detect. A significantly higher concentration of BaP-9,10-dihydrodiol was recovered from ER embryos exposed to BaP when compared to embryos from the KC (Chapter 2). 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). It is possible the ER killifish have shifted away from the production of other toxic metabolites, including BaP-7,8-dihydrodiol, which is a precursor for the highly carcinogenic BPDE. This theory is supported by the fact that there was not an increase in lesion frequency in the ER juveniles after exposure to BaP.

The induction of CYP1A enzymatic activity and the decreased amount of BaP recovered from the KC larvae suggest that there was metabolic activity occurring and

that BaP was being either biotransformed or excreted. Reactive metabolites of BaP can result in DNA damage through the formation of bulky adducts and through reactive oxygen species (Varanasi et al. 1986, Burdick et al. 2003). In these experiments, LA-QPCR was used to detect both mitochondrial and nuclear DNA damage in the KC and ER fish after exposure to BaP. This assay allows for the examination of general DNA injury including the formation of bulky adducts and oxidative damage, both of which may be playing a role in BaP-induced carcinogenicity. However, this assay does not distinguish between forms of DNA damage. In the KC killifish we saw an increased level of DNA damage after exposure to 200 µg/L BaP. We also saw a higher level of damage in mitochondrial DNA relative to nuclear DNA. This confirms what has been previously found in mammalian cell culture studies which show that the mitochondrial genome is more susceptible to PAH induced damage (Allen and Coombs 1980, Backer and Weinstein 1980). We observed that the ER killifish had a higher level of basal mitochondrial and nuclear DNA damage compared to larvae from the KC population. However, the ER larvae showed no increase in either mitochondrial or nuclear DNA damage after BaP exposure. Nacci et al (Nacci et al. 2002b) showed similar results in a resistant killifish population 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 DNA adduct formation, indicating that they may also be resistant to the carcinogenic effects of PAHs.

Epizootics of liver neoplasms in marine and fresh water fish have been associated with carcinogenic PAHs in the sediments of many contaminated waterways, and tumor incidences in wild populations have been used to monitor ecosystem health (Dawe et al. 1964). There are strong, site-specific associations between environmental contamination and liver cancer. Brown Bullhead catfish (*Ameiurus nebulosus*) collected from the Black River in Ohio displayed a 39% prevalence of liver neoplasms associated with a coking facility that released high levels of PAHs (Baumann and Harshbarger 1998). Lesions have also been observed in English Sole (*Parophrys vetulus*) from the PAH polluted waterways of the Puget Sound (Myers et al. 1990). Killifish have been classified as one of the best model systems to examine the relationship between exposure to toxicants and carcinogenesis in wild populations of fish (Burnett et al. 2007). One of the reasons for this is that there has been extensive research since the early 1990s examining the relationship between PAH exposure and the development of a variety of liver lesions and cellular alterations. These investigations have focused on the Elizabeth River population, where a wide range of pre-neoplastic and neoplastic lesions have been observed in high prevalence levels (Vogelbein et al. 1990, Huggett et al. 1992, Fournie and Vogelbein 1994, Vogelbein and Unger 2006, Vogelbein et al. 2008). Moreover, ER killifish are one of the only fish populations to develop hepatoblastomas, a primitive

liver cell neoplasm that is similar in appearance to the developing liver of an embryo or fetus (Vogelbein et al. 1999). Vogelbein et al (1990) reported that ER killifish had a 93% incidence of liver lesions and a 33% frequency of liver cancer, while a reference site population had no detectable lesions.

The majority of the research on chemical carcinogenesis in fish is still based on the paradigm that carcinogenesis is the result of a multistage process involving initiation, promotion and progression (Rotchell et al. 2008). Mammalian and medical literature has shifted away from this terminology, and primarily refers to carcinogenic agents as either genotoxic or nongenotoxic. However, the majority of the research in fish is still based on the original multistage process. Preneoplastic liver lesions, such as foci of cellular alteration and nuclear pleomorphism, have been used and considered as early pathological stages for the onset of hepatic neoplasms (Hinton et al. 1988, Hinton and Lauren 1990, Myers et al. 1990, Hinton et al. 1992, Myers et al. 1992, Simpson 1992). In the wild it is difficult to determine the cause-and-effect relationship between preneoplastic lesions and pollutant exposure due to possible synergistic or antagonistic interactions between chemicals, and the ability of many fish to migrate into and out of regions with high levels of chemical contamination. Laboratory studies, in which both biochemical and cytological endpoints are used, provide an opportunity to examine the etiology of chemical exposure on liver alterations within species or populations of fish with different chemical sensitivities.

In this study, offspring of KC killifish exposed to 400 µg/L BaP had a significantly higher incidence of altered eosinophilic and basophilic foci, hepatocellular adenomas, and hepatocellular carcinomas. Lesions were observed in this population beginning at the 200 µg/L dose but frequencies were not statistically different from control. Although two fish with altered foci were observed in the ER killifish, no hepatocellular adenomas or carcinomas were observed suggesting that ER larvae are resistant to the carcinogenic effects of BaP. Additionally these lesions were only observed at the highest dose of BaP (400 µg/L) and no altered foci were observed in any of the other BaP treatments (50-200 µg/L). Interestingly no biliary cell involvement was seen in hepatic tumors of this study, which may be of use in the future studies that focus on intermediate life stages between embryo, larvae and adults.

The onset of liver toxicity occurred at a higher concentration in the ER fish and was not as severe as that observed in the KC population. One reason why the offspring of ER killifish may be more resistant to the onset of the carcinogenic effects of BaP is their lack of the inducibility of their CYP1 enzymes and altered responsiveness of the AHR. The inhibition of chemical carcinogenesis has been studied extensively in rainbow trout (*Oncorhynchus mykiss*) in response to the carcinogens aflatoxin B1 (AFB₁) and N-nitrosodiethylamine (DEN) (Hendricks et al. 1977, Shelton et al. 1984, Fong et al. 1988). Trout that were fed AFB₁ simultaneously with β-naphthoflavone showed enhanced bile elimination of AFB₁, significantly reduced rates of DNA adduct formation, and a

decrease in liver tumor incidence (Takahashi et al. 1996). This suggests that the protection from carcinogenesis was the result of inhibiting the early events of uptake, distribution and/or activation by phase I metabolism. Blocking enzymatic activity by knocking down or knocking out the AHR is protective against PAH-induced teratogenicity and carcinogenicity (Billiard et al. 2006, Incardona et al. 2006). Shimizu et al (2000) found that unlike wild-type mice, AHR (-/-) animals did not develop subcutaneous or skin tumors in response to BaP exposure. Talaska et al (2006) showed that AHR (-/-) mice had a 90% reduction of skin BaP adduct levels compared to wild-type.

Even though wild-caught adult ER fish do have a high frequency of hepatic lesions and neoplasms, this study suggests that they are more resistant than reference site population to PAH induced carcinogenicity. One of the reasons for this observation is that the complex mixture of PAHs at the Elizabeth River may be a more potent carcinogen than BaP alone. According to principles outlined by Peters et al. (1987), one approach to establish an association between environmental contamination and neoplasia in fish is to confirm the field data by long-term experiments. One way of confirming this would be to expose reference site fish to extracts of contaminated sediment and determine if they developed an elevated prevalence of tumors similar to that observed with the population inhabiting the contaminated area. Rainbow trout sac fry that were microinjected with PAH-contaminated sediment from Hamilton Harbor

developed gross hepatic neoplasms (7%) that were histologically confirmed to be hepatocellular carcinomas twelve months after exposure (Metcalf et al. 1990). Black et al (1985) induced an 80% tumor frequency in Swiss mice when exposed to PAH-contaminated sediment from the Black River in Ohio, compared to 64% frequency in mice exposed to 200 µg/ml BaP. Future studies of this site should include a chronic exposure of KC and ER laboratory reared F1 larvae to the Elizabeth River sediment, and an examination of the resulting hepatotoxicity.

In conclusion, these experiments have shown that in addition to their resistance to the acute toxicity of PAH, ER killifish are more resistant than reference site populations to the carcinogenic effects of BaP. These experiments contribute to a greater understanding of the effects of early-life exposure to PAHs on the chronic and carcinogenic effects of these compounds. Additionally, reduced inducibility of the CYP1 enzymes observed in the ER killifish resulted in a reduction in the rate of BaP biotransformation and/or excretion in the ER population. Unfortunately, we were not able to identify any of the BaP metabolites formed in either population. One reason for this may be the small amount of tissue available within the larvae. Future studies examining BaP metabolites in exposed adult killifish from both populations may provide more insight about biotransformation in the ER population and the role that it may be playing in their toxic response.

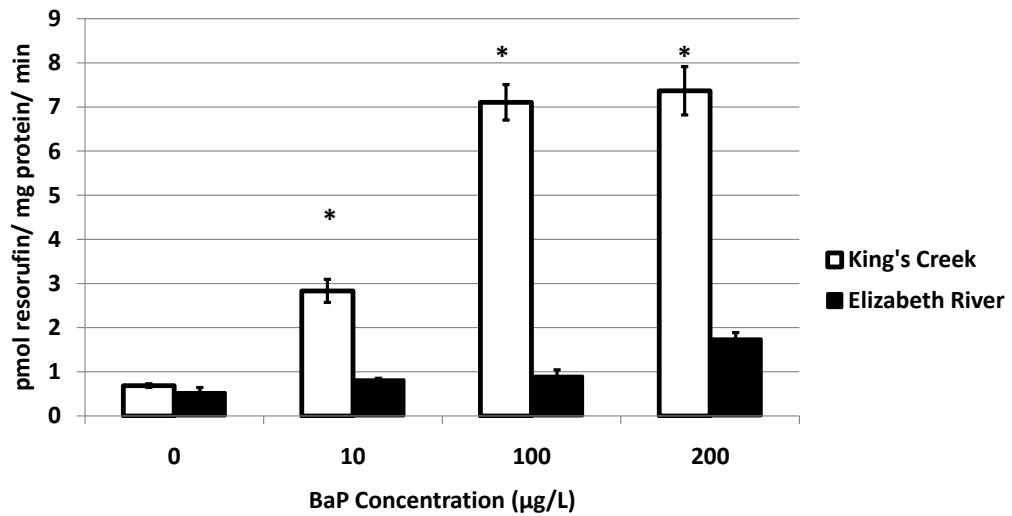


Figure 5.1: Induction of CYP1 activity as measured by the *in vitro* EROD assay in King's Creek and Elizabeth River killifish larvae after laboratory exposure to either the DMSO vehicle control or BaP (10 - 200 µg/L)

CYP1 enzymatic activity was measured by in larvae 4 days post repeated 24 hour BaP exposures. There was significant induction of CYP1 enzymatic activity in the KC larvae at all of the concentrations of BaP examined ($p \leq 0.001$; Bonferroni corrected ANOVA). There was no significant induction of activity in the ER larvae at any concentration of BaP. Data are represented as average pmol resorufin produced per mg protein per minute \pm SEM; $n = 4$ pools of 10 larvae. "*" signifies treatments that are significantly different from control.

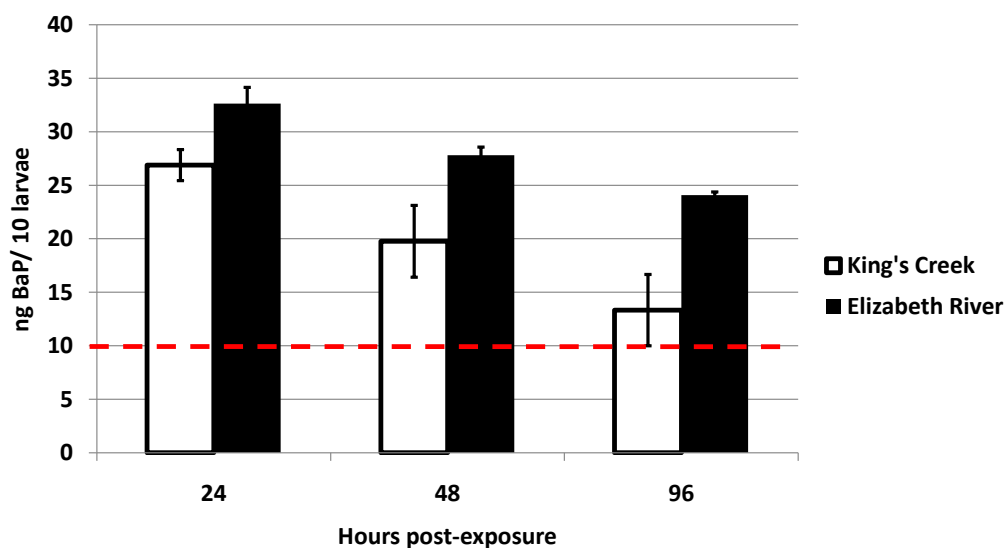


Figure 5.2: Parent BaP recovered and identified by UPLC/MS 24, 48, and 96 hours after a 24 hour exposure to 100 µg/L BaP

Dashed lines represent detection limits. There was a significant effect of population and treatment on the recovery of BaP ($p < 0.05$; ANOVA). The interaction of population and treatment was not significant ($p = 0.06$). No BaP was detected in the larvae exposed to the vehicle control, DMSO. The data are represented as average nanograms (ng) of BaP recovered per 10 larvae \pm SEM; $n = 5$ pools of 10 larvae.

Target	Forward Primer (5'-3') Reverse Primer (3'-5')
Large nuclear target 11459 bp	CAGCCGCCCGCAAATTCTCA CAGAATGCGGGCCTTGCTGA
Small nuclear target 234 bp	GCCGCTGCCTTCATTGCTGT ATGAGCTGGGTGTGCGCTGA
Long mitochondrial target 9416 bp	TTGCACCAAGAGTTTTTGGTTCCTAAGACC GATGTTGGATCAGGACATCCCAATGGTGCA
Small mitochondrial target 264 bp	ATCTGCATGGCCAACGCCTA GGCGGTGCCAGTTTCCTTTT

Table 5.1: Primers used for *Fundulus heteroclitus* LA-QPCR assay

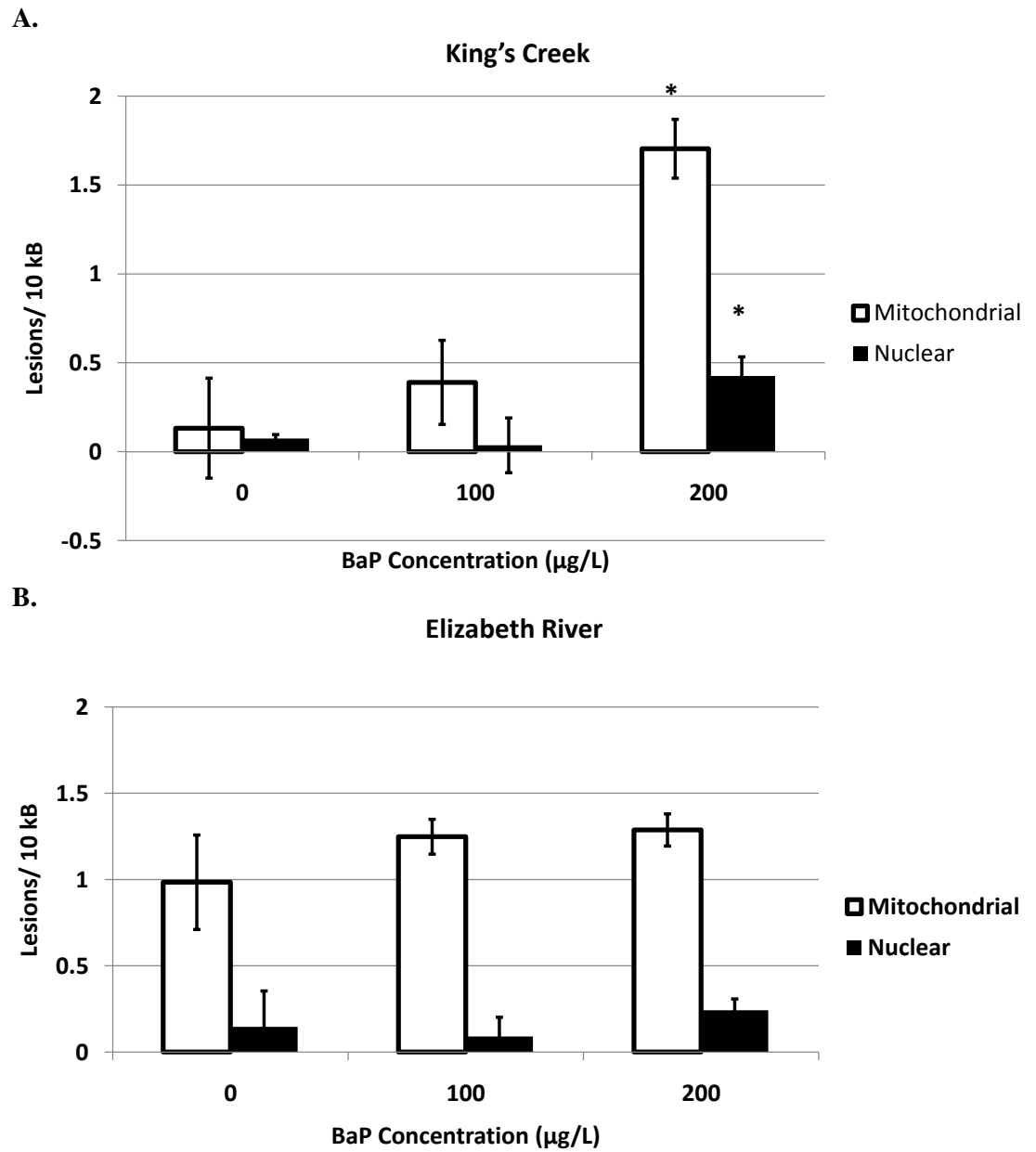


Figure 5.3: Mitochondrial and nuclear DNA damage in killifish larvae 4 days after repeated 24 hour exposures to DMSO vehicle control or BaP (100 or 200 µg/L)

Figure 5.3: A) The KC larvae showed a significant increase in relative lesion frequency in both the mitochondrial and nuclear DNA after exposure to 200 $\mu\text{g/L}$ BaP ($p \leq 0.001$; Bonferroni corrected ANOVA). B) The ER larvae showed no significant increase in either mitochondrial or nuclear DNA lesion frequency for any of the concentrations of BaP examined. ER DMSO controls showed a significantly higher level of mitochondrial DNA lesion frequency compared to KC larvae ($p < 0.05$). Data are presented as average lesion frequency \pm SEM; $n = 4$ pools of two larvae. "*" signify treatments that are significantly different from control.

	BaP ($\mu\text{g/L}$)	DMSO	50	100	200	400
Weight (mg) 3 months post exposure	KC	82.6 \pm 4.3	79.8 \pm 4.3	89.8 \pm 4.9	90.2 \pm 5.0	84.6 \pm 4.6
	ER	88.3 \pm 8.5	83.0 \pm 4.0	69.9 \pm 4.7	115.4 \pm 41.8	70.3 \pm 2.4
Weight (mg) 9 months post exposure	KC	357.4 \pm 29.7	356.3 \pm 23.4	367.6 \pm 34.2	356.7 \pm 28.8	345.6 \pm 25.8
	ER	437.3 \pm 51.3	302.1 \pm 28.2	343.5 \pm 33.2	338.9 \pm 69.4	392.3 \pm 58.5

Table 5.2: Weight data for killifish juveniles 3 months and 9 months post repeated 24 hour exposure to either the DMSO vehicle control or BaP (50- 400 $\mu\text{g/L}$)

No significant differences in weight were observed at either time point, in either population, at any of the doses of BaP examined. Weights are represented as the average \pm SEM; $n \geq 20$ juveniles

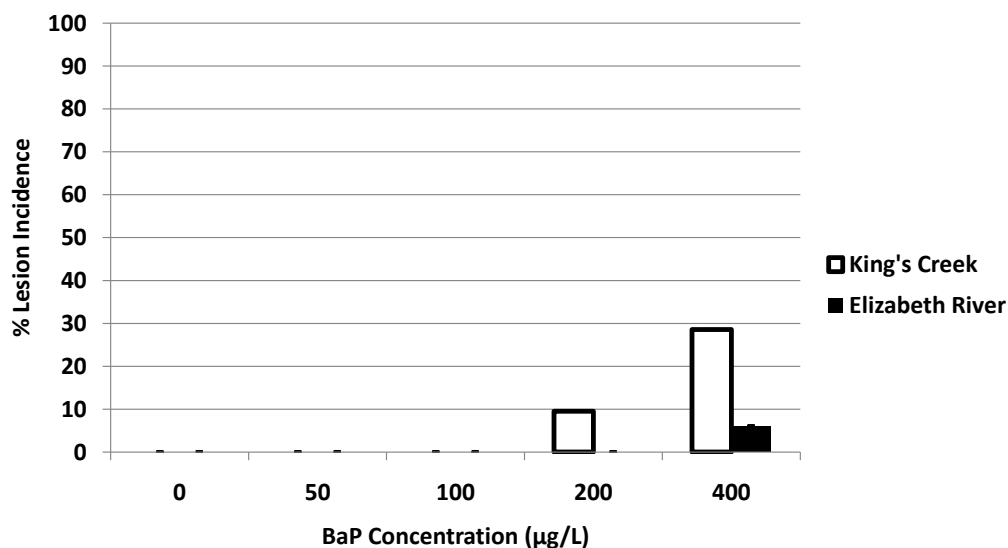


Figure 5.4: Prevalence of hepatic lesions in KC and ER juvenile killifish 9 months post exposure to BaP (50-400 µg/L)

Hepatocellular lesions were diagnosed as foci of cellular alteration, hepatocellular adenomas and hepatocellular carcinomas as described by Baumann et al. (1990). There was a significant increase in the incidence of hepatic lesions in KC juveniles exposed BaP ($p < 0.05$). There was a 10% increase in hepatic lesions in KC fish exposed to 200 µg/L, and a 30% increase after exposure to 400 µg/L. ER fish showed no incidence of hepatic lesions after exposure to 200 µg/L. They developed 6% more hepatic lesions after exposure to 400 µg/L. Lesion incidence after the 400 µg/L exposure was 20% higher KC juveniles than in those from the ER ($p < 0.05$). Data are represented as percent lesion incidence; $n \geq 20$ juveniles.

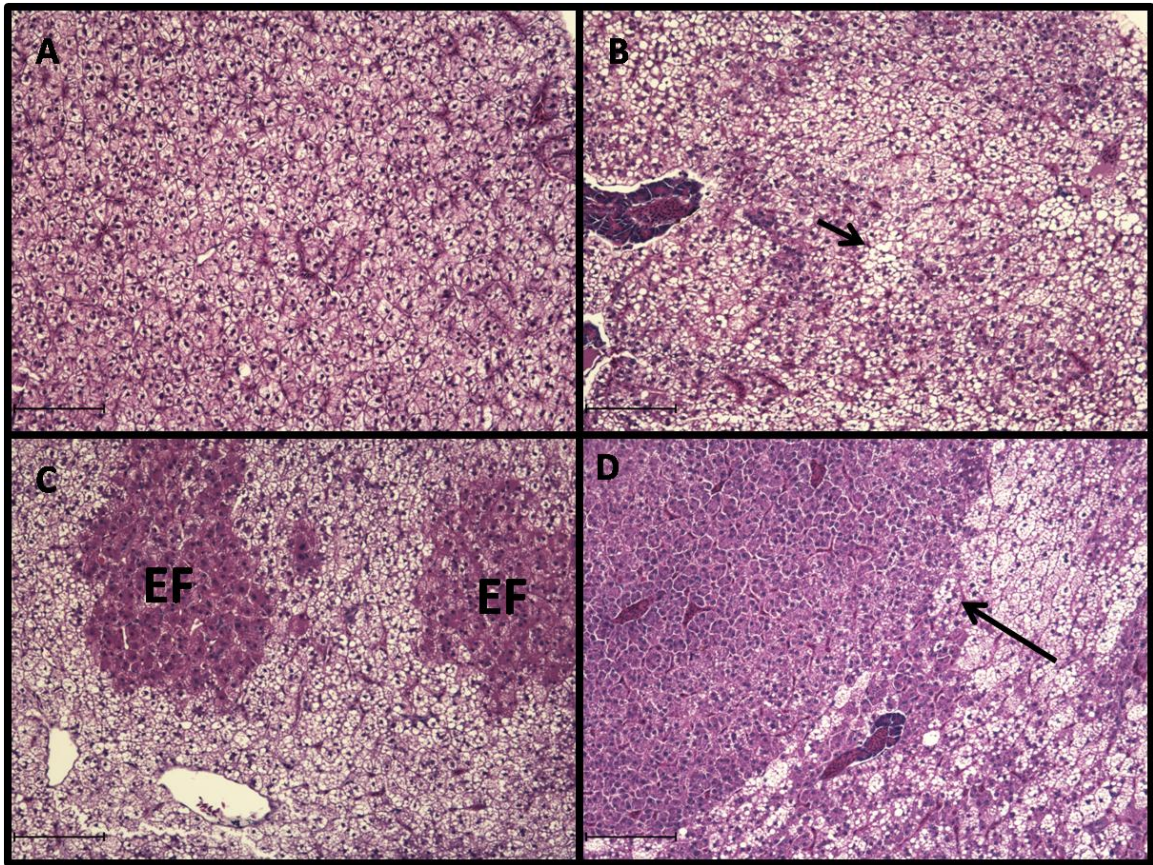


Figure 5.5: Altered liver pathology in KC killifish juveniles 9 months post exposure to BaP

A) Normal liver from a KC killifish juvenile 9 months post exposure to DMSO vehicle control; bar, 100 μm . B) Focal steatosis in liver from a KC killifish juvenile 9 months post exposure to 400 $\mu\text{g/L}$ BaP; arrow, large rounded vacuoles with smooth margin signifying lipid; bar, 100 μm . C) Eosinophilic foci (EF) in liver from KC killifish juvenile 9 months post exposure to 400 $\mu\text{g/L}$ BaP; bar, 100 μm . D) Hepatocellular carcinoma in liver from a KC killifish juvenile 9 month post exposure to 400 $\mu\text{g/L}$ BaP; arrow, irregular and invasive border of the carcinoma; bar, 100 μm . All figures are hematoxylin and eosin stained paraffin sections.

6. Conclusions, future research directions, and implications

6.1 Summary and conclusions

The work presented in this dissertation used two populations of *Fundulus heteroclitus* (killifish) with different exposure histories to examine how alterations in the cytochrome P450-1 (CYP1) family of enzymes affect the biotransformation of polycyclic aromatic hydrocarbons (PAHs) and ultimately their toxicity. Additionally, I examined how early-life exposure to PAHs can result in the onset of chronic toxicity, including carcinogenicity, during later life stages. Toward these goals, I explored three specific aims.

The first aim was to further characterize the mechanisms of PAH biotransformation by investigating the mRNA induction of the CYP1 metabolic enzymes (*CYP1A*, *CYP1B1* and *CYP1C1*) in two populations of the Atlantic killifish (*Fundulus heteroclitus*). One population was collected from a reference site on King's Creek (KC) in Gloucester County, Virginia. The other population was collected from a PAH contaminated superfund site on the Elizabeth River (ER) in Portsmouth, VA. ER killifish have offspring that are resistant to the teratogenic effects of PAHs and recalcitrant to the induction of CYP1A. The recent identifications of the metabolic enzymes CYP1B1 and CYP1C1 provide the potential for a better understanding of PAH induced toxicity and of the ER resistant phenotype. Chapter 2 more fully characterized the ER recalcitrant

phenotype using two model PAHs, benzo[a]pyrene (BaP) and benzo[k]fluoranthene (BkF), and the dioxin-like compound (DLC), 3,3',4,4',5-pentachlorobiphenyl (PCB126). We compared their developmental and molecular responses by screening the embryos for CYP1 protein activity, cardiac deformities, and mRNA expression of *CYP1A*, *CYP1B1*, *CYP1C1*, and the aryl hydrocarbon receptor (*AHR2*). This chapter revealed that in KC embryos the newly characterized *CYP1B1* and *CYP1C1* were both induced in response to BaP, BkF and PCB126. This chapter also revealed that ER embryos had suppressed induction of *CYP1B1* and *CYP1C1*. This data suggests that the adaptation of the ER killifish is further upstream than *CYP1A*, and may be better described as an altered responsiveness of the AHR.

The second aim was to investigate the influence of altered biotransformation on PAH induced embryotoxicity. Chapter 3 examined the effects of the *CYP1A* inhibitor FL on the biotransformation and teratogenicity of BaP. Although FL did enhance the teratogenic effects of BaP, we did not observe any effect of the co-exposure on BaP excretion or metabolic transformation. Chapter 3 also used the resistant embryos of the ER population to examine the effects of the altered responsiveness of the AHR pathway on the biotransformation and teratogenicity of BaP. A higher concentration of parent BaP was recovered from ER embryos compared to those from the reference population. This suggests that the ER population has either a reduced rate of BaP excretion or altered biotransformation. A higher concentration of the metabolite, BaP-9,10-

dihydrodiol was recovered from the ER embryos, suggesting that these fish may shifting their biotransformation either by slowing down elimination or phase II conjugation. It is also possible that the ER embryos are shifting biotransformation away from more reactive metabolites including BaP-7,8-dihydrodiol which can lead to the production of the highly mutagenic BaP-7,8-dihydrodiol-9,10-epoxide. Chapter 4 utilized morpholino technology to examine the effects of knocking down CYP1A activity on the biotransformation of BaP. Although knocking down CYP1A activity did result in an increase in teratogenicity, there was no observed effect of the morpholino on the recovery of parent BaP. The only other metabolite that was recovered above detection limits was BaP-7,8,9,10-tetrahydrodiol (BaP-tetrol). Lower amounts of BaP-tetrol were recovered from killifish embryos injected with both the control and the CYP1A morpholinos. This data suggests that there may be an effect of the injection or of a structural motif within the morpholino itself that alters biotransformation. However, because the amount of BaP-tetrol recovered was so close to the detection limits, no conclusions could be drawn.

The third aim was to investigate the role of altered biotransformation on PAH induced genotoxicity and carcinogenicity. Chapter 5 used the resistant larvae of the ER population to examine the effects of the altered responsiveness of the CYP1 enzymes on the biotransformation and carcinogenicity of BaP. Higher concentrations of BaP were recovered from ER larvae over time compared to KC larvae. This data suggests that the

ER larvae had a slower rate of biotransformation, excretion, or both. KC larvae developed both mitochondrial and nuclear DNA damage after exposure to BaP, with a significantly higher level of damage observed in the mitochondria. The ER larvae did not show any significant PAH-induced increase in either mitochondrial or nuclear DNA damage. Nine months after exposure to BaP, KC juveniles developed eosinophilic and basophilic foci of cellular alteration (FCA), hepatocellular adenoma, and hepatocellular carcinoma. A significantly lower frequency of FCA were observed in BaP exposed ER juveniles, and no adenomas or carcinomas were observed in this population. These data indicate that the ER juveniles are less susceptible to both the genotoxic and the carcinogenic effects of BaP compared to killifish juveniles from a reference site population.

Polycyclic aromatic hydrocarbons (PAHs) are a class of environmental contaminants that pose serious risks to both human and environmental health. The temporal trends in the United States, suggests that PAH concentrations will continue to climb along with the rise in urban development and vehicle usage (Van Metre and Mahler 2005). PAHs can elicit a variety of toxic effects including immunotoxicity, narcosis, teratogenicity and carcinogenicity (Levin et al. 1978, Billiard et al. 1999, Fay et al. 2000, Carlson et al. 2004, Incardona et al. 2004, Uno et al. 2004, Wassenberg and Di Giulio 2004a, Billiard et al. 2006, Incardona et al. 2006) The biotransformation of PAHs is extensively studied because PAHs are common pollutants and many of them elicit their

toxic effects after bioactivation to reactive intermediate metabolites (Miller and Ramos 2001, Schlenk et al. 2008). Although there is a wealth of information available about the biotransformation of PAHs, there is less known about how the chemical or molecular alteration of these metabolic pathways can affect toxicity.

The results of the research presented here provide necessary information pertaining to how alterations in PAH biotransformation can affect both their acute and the chronic toxic effects. I have provided a greater understanding of the mechanism of adaptation in the Elizabeth River killifish population, by characterizing the reduced expression of the newly identified CYP1B1 and CYP1C1 enzymes in ER embryos exposed to AHR agonists. I have shown that embryos born to ER parents have an altered metabolic profile compared to embryos from a reference population, indicating that biotransformation plays a role in their adaptation. By examining the chronic effects of an early-life exposure to BaP, I have shown that in addition to being resistant to the acute toxicity of PAHs, ER larvae are also resistant to PAH-induced genotoxicity and carcinogenicity. Surprisingly, I did not detect any effect of FL co-exposure or morpholino knockdown of CYP1A on the biotransformation of BaP. Further studies in adults and with more sensitive methods of detection are suggested to further elucidate the role of altered biotransformation in mediating the embryotoxicity of PAHs

6.2 Future research directions

The results of the work described in this dissertation have revealed new questions and directions for future research. Many of the studies examining the resistance of the Elizabeth River killifish have focused on the recalcitrant CYP1A phenotype of the embryos, larvae and adults in this population. The hypothesis that the resistance of the ER fish was mediated by inhibition of CYP1A was disproven by the increased severity of PAH induced cardiac deformities observed in embryos in which CYP1A activity was reduced either by chemical inhibition or morpholino technology (Wassenberg and Di Giulio 2004a, Wassenberg et al. 2005, Billiard et al. 2006, Matson et al. 2008a). In Chapter 3 higher concentrations of parent BaP and BaP-9,10-dihydrodiol were recovered from ER embryos. If the resistance of the ER population could be fully explained by CYP1A inhibition, the metabolic profiles should have been similar in KC embryos injected with the CYP1A-MO and those co-exposed to the CYP1A inhibitor fluoranthene (FL). However there was no difference in the amount of parent BaP recovered in either the CYP1A-MO injected embryos or in embryos co-exposed to FL. Additionally BaP-9,10-dihydrodiol was not recovered in either group at concentrations above detection limits.

The work outlined in Chapter 2 suggests that the observed resistance of the ER population is better explained by an alteration in the regulation of CYP1 induction. One possibility is that these fish have developed an altered responsiveness of the AHR. This

hypothesis is supported by work that shows zebrafish embryos injected with an AHR2-MO are protected from PAH induced teratogenicity (Billiard et al. 2006). Future research should include injection of KC embryos with AHR2-MO and an examination of the metabolic profile to determine if it is similar to what is observed in the ER population. Another approach would be to try to use RNA rescue approaches to return normal function of the AHR to ER embryos and attempt to recover sensitivity to embryotoxicity and a normal metabolic profile.

One of the problems consistently encountered in this work was an inability to detect BaP metabolites in killifish embryos and larvae. This difficulty could be explained by the lack of available tissue at these developmental stages. If multiple metabolites were produced, it is possible that no one metabolite was produced in high enough concentrations to be detected by our current methodology. Additionally the small tissue samples prevented us from being able to isolate metabolites that were conjugated by phase II enzymes. To fully characterize altered PAH biotransformation in the ER population, adults can be injected with BaP and their bile can be analyzed for the presence of parent compound and multiple metabolic intermediate compounds. Zhu et al (2008) recovered 7,8-dihydrodiol, 1,6-dione, 3,6-dione and 3-OH, both in free form and as glucuronic acid conjugates, from the bile of KC killifish. It would be interesting to examine the profile differences of both wild-caught and laboratory reared adults from the ER population.

In addition to performing adult experiments, there is a need to better detect and quantify PAH biotransformation within embryos. It is likely that biotransformation within developing embryos is different than what is observed in adult fish. Jonsson et al (2007a) showed that in zebrafish the expression of *CYP1A*, *CYP1B1*, and *CYP1C1* change over the course of development, which will most likely have an effect on biotransformation. 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. They suggested that the difference in bioconcentration kinetics was the primary reason for the increased sensitivity of early life stages to PAH-induced toxicity. Many PAH elicit teratogenic effects and the resistance to embryotoxicity may be driving the adaptation of multiple killifish populations inhabiting contaminated sites.

Studies in medaka (*Oryzias latipes*) have shown that a technique called multiphoton laser scanning microscopy (MPLSM) can detect BaP and specific fluorescent BaP metabolites in the tissues of developing embryos (Hornung et al. 2004, Hornung et al. 2007). The use of this technique may provide insight concerning the synergistic toxicity observed in embryos exposed to PAH mixtures, as well as in the resistance of ER embryos to PAH-induced teratogenicity. In MPLSM, a laser at a long wavelength is pulsed at femtosecond rates to produce the energy required to excite a fluorophore without causing damage to the developing embryo. Once the fluorophore is excited, it

behaves exactly as it would if it was excited by a single photon at a shorter wavelength. The fluorescent properties of PAHs make them model compounds for MPLSM analysis. As part of this dissertation preliminary experiments were performed in which KC embryos were exposed to 200 $\mu\text{g/L}$ BaP 2 days post-fertilization for a period of 24 hours. Forty-eight hours post-exposure, the embryos were rinsed with clean artificial salt water (20 ppt), and the tissue distributions of BaP, BaP- 3-OH, and BaP-3-glucuronide were examined. All of the images were captured using a tunable two-photon laser scanning microscope system with two-photon excitation at 760 nm and 840 nm, and emission collected through a 410-490 nm bandpass filter. For each organism, a series of images were taken at 20 μm intervals from the surface of the organism to a depth of 400 μm (Figure 6.1). The excitation wavelength of 760 nm is the optimal wavelength to observe parent BaP, while 840 nm is ideal for examining the distribution of the metabolites BaP-3-OH and BaP-3-glucuronide.

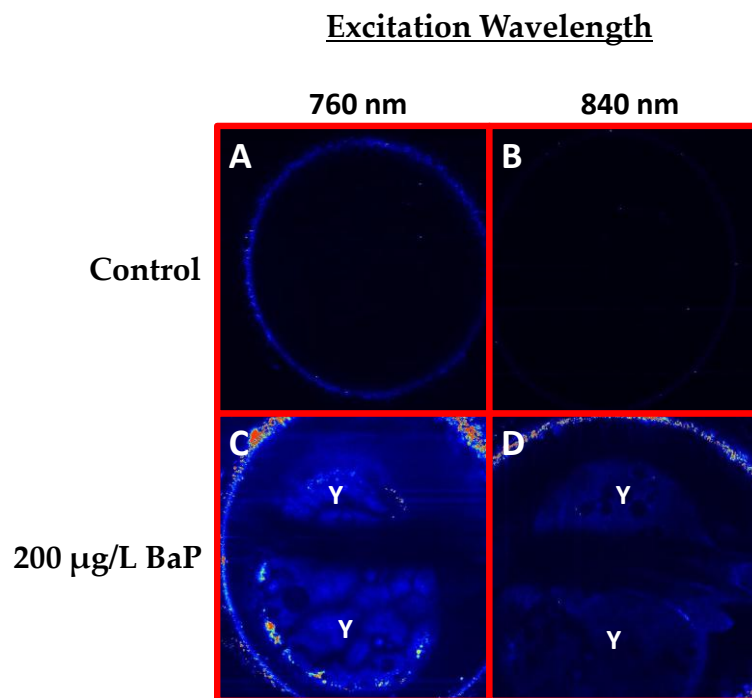


Figure 6.1: Multi-photon microscope images of KC embryos 48 hours after exposure to 200 µg/L BaP

Images (A) and (B) were taken of a control embryo and images (C) and (D) are of an embryo treated with 200 µg/L BaP. The yolk sacs of the embryos are identified by the letter "y". Although the chorion of the control embryo did fluoresce at 760 nm, no BaP was detected in control killifish embryos (Figure 6.1 A, Figure 6.1 B). Forty-eight hours after exposure, parent BaP, BaP-3-OH and BaP-3-glucuronide were detected in the yolk of the embryo (Figure 6.1 C, Figure 6.1 D). These results were similar to what was observed in medaka, indicating that this technique is viable for use in killifish (Hornung et al. 2007). These results show that embryonic tissues are metabolically viable early in

development, and provide evidence that biotransformation plays a role in BaP-induced embryotoxicity.

Although we were able to detect significantly higher concentrations of BaP-9,10-dihydrodiol in the offspring of the ER population, we were not able to detect the production of any BaP metabolites in KC embryos exposed to BaP alone or in co-exposure with FL. BaP and FL co-exposed KC embryos develop a synergic increase in the severity and frequency of cardiac deformities. It is possible that this increase in teratogenesis was caused by a reactive intermediate of BaP biotransformation that has yet to be identified. One possibility for future research would be to determine the identity of that metabolite and expose the embryonic offspring of both ER and KC killifish to the compound and attempt to elicit a teratogenic response.

In KC embryos co-exposed to BaP and FL, the observed teratogenic effects may have been caused by the alteration of FL excretion or bioactivation. Although possible, it is unlikely that FL biotransformation is driving the toxicity of the mixture. The morpholino knockdown of CYP1A in both zebrafish and killifish embryos induced similar cardiac deformities after co-exposure to the PAH-type AHR inducer β -naphthoflavone (BNF) (Billiard et al. 2006, Matson et al. 2008a). This indicates that the embryotoxicity is driven by a disturbance in the biotransformation of the PAH-type AHR agonist. However, the examination the ability of BaP to alter the metabolism of FL would be an interesting area of future research. The biotransformation of FL can produce

reactive metabolites, such as the mutagenic FL-trans-2,3-dihydrodiol, which may have teratogenic effects (Pothuluri et al. 1992). Zebrafish embryos exposed to FL in the presence of hypoxia developed pericardial effusion and severe lordosis suggesting that FL, or a metabolic derivative, can elicit embryotoxic effects under certain environmental conditions (Matson et al. 2008b).

Another potentially interesting line of future research is to determine how alterations in CYP1 induction and activity affect retinoic acid (RA) synthesis. Both CYP1A and CYP1B1 have shown to function in the metabolism of RA (Murray et al. 2001, Denison and Nagy 2003, Choudhary et al. 2004). The concentration of RA and the ability of embryonic cells to respond to it are essential for proper development. Vandersea et al. (1998) exposed killifish embryos to RA for 2 hours during gastrulation. Embryos exposed to 0.5 - 500 μM of RA developed multiple dose-dependent defects in the brain, heart, eye, pectoral fins and cranial formation. Embryos exposed to a concentration of 5 μM RA developed cardiac deformities similar to those observed in killifish embryos exposed to PAHs. One possibility for future work would be to measure the concentration of RA in killifish embryos exposed to mixtures of PAHs to determine a possible role for RA synthesis in mediating the teratogenic effects of PAH exposure.

In Chapter 5, we observed that the larval offspring of ER parents are less susceptible than reference site fish to PAH-induced carcinogenicity; however, wild-caught adult ER fish do have a high prevalence of hepatic lesions and neoplasms. One

possible reason for these observations in the wild is that the adult killifish inhabiting the Elizabeth River have been exposed to the contaminated sediment for multiple years prior to the onset of carcinogenicity. Another possibility is that the complex mixture of PAHs at the Elizabeth River may be a more potent carcinogen than BaP alone. Future studies of this site should include a chronic exposure of KC and ER laboratory reared F1 larvae to the Elizabeth River sediment, and an examination of the resulting hepatotoxicity.

6.3 Implications

Killifish are useful models, in the laboratory and in the field, for examining how individual organisms and populations interact with the environment. This dissertation highlights some of the unique characteristics of this species including their population-specific adaptation to toxicants as well as their well understood developmental and molecular biology. The recent development of the morpholino gene knockdown technique for use in killifish will enable researchers to examine the mechanisms of environmental toxicology in even greater detail. One of the current problems that researchers have to overcome is the lack of genomic resources for this model. As this research highlights, the susceptibility of killifish to various environmental toxicants and the adaptation observed in the ER population, is more mechanistically complex than an alteration of a single metabolic enzyme or pathway. A more complete suite of genomic

tools will enable researchers to more fully explore mechanisms of toxicity in this environmentally relevant model.

The ability of organisms to adapt to environmental contamination is hard to prove and understand. Killifish have limited migration, and therefore provide an opportunity to study the effects of chronic pollution on a population, providing evidence for genetic adaptation in the wild. One concern is that the ability of these populations to remain reproductively active in highly contaminated environments, may give regulators falsely positive assessments about the health of aquatic ecosystems. Interpreting biomarker studies in contaminated waterways necessitates that laboratory studies accompany the surveys of biota in the field. An additional concern is that although adapted killifish populations express compensatory responses to chemical exposure, there are also fitness costs of this rapid adaptation. These costs can include increased sensitivity to immunotoxicants, alterations in salinity, and hypoxia. Therefore, although these populations can survive the effects of the contamination, the growing concerns of global climate change, wide spread hypoxia, and drought may affect their ability to respond to chemical stressors.

The work described in this dissertation further characterizes the adaptation of a population of Atlantic killifish that inhabit the PAH-contaminated Atlantic Wood Industries superfund site on the Elizabeth River in Portsmouth, VA. ER embryos, larvae and adults are refractory to the induction of multiple CYP1 enzymes in the AHR

pathway. These data support the hypothesis that altered responsiveness of the AHR is involved in the resistance of these fish to both PAH induced teratogenicity and carcinogenicity. Embryonic and larval offspring ER killifish also have a slower rate of biotransformation and may have an altered metabolic profile, suggesting a role of biotransformation in mediating the observed resistance of this population to PAH-induced toxicity. Until we develop and utilize alternate forms of energy, the concentrations of PAHs in the environment are going to rise along with the increase in urbanization and vehicle usage. Humans and biota will continue to be exposed to complex mixtures of PAHs that likely contain both compounds that act as AHR agonists and compounds that work as CYP1A inhibitors. The work presented in this dissertation confirms the synergistic teratogenic effects of these mixtures, and contributes to evidence for the need to develop more mechanistic based models in risk assessment. Increasing our understanding about the mechanisms underlying PAH toxicity will enable regulators to better evaluate and address PAH contamination and the potential effects on biota. Although we were not able to confirm the role of biotransformation in mediating the toxicity of PAH mixtures, it is likely that it is a factor and warrants further research.

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Biography

Lauren Patrice Battle was born in Baltimore, Maryland on December 6, 1980. She received a Bachelor of Science in Biology from the University of Maryland Baltimore County in May, 2002. In August of 2002 she came to Duke University to pursue her Ph.D. in Environmental Science. She married Mr. David Anthony Wills Jr. on November 29, 2008.

Publications:

Wassenberg, D.M., Nerlinger, A.L., **Battle, L.P.**, Di Giulio, R.T. 2005. Effects of the PAH-heterocycles, carbazole and dibenzothiophene, on *in vivo* and *in vitro* CYP1A activity and PAH-derived embryotoxicity in *Fundulus heteroclitus*. *Environmental Toxicology and Chemistry* 24: 2526-32.

Fellowships:

1998-2002	Meyerhoff Scholarship, University of Maryland Baltimore County
2000	Leadership Alliance Summer Fellowship, Shoals Marine Laboratory, Cornell University
2001	National Science Foundation, REU Summer Fellowship, Shoals Marine Laboratory, Cornell University
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