Molecular Mechanisms Underlying Adaptation to PAHs in *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 Environment in the Graduate School of Duke University

2010
ABSTRACT

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Abstract

Chronic exposure to toxicant mixtures is a serious threat to environmental and human health. It is especially important to understand the effects of these exposures for contaminants, such as polycyclic aromatic hydrocarbons (PAHs), which are toxic, ubiquitous, and increasingly prevalent. Furthermore, estuarine systems are of particular concern, as they are highly impacted by a wide variety of pollutants; fish there are often exposed to some of the highest levels of contaminants of any vertebrate populations, along with other stressors such as fluctuations in water level, dissolved oxygen, and temperature. A population of *Fundulus heteroclitus* (the Atlantic killifish or mummichog, hereafter referred to as killifish) inhabits a Superfund site heavily contaminated with a mixture of PAHs from former creosote operations; they have developed resistance to the acute toxicity and teratogenic effects caused by the mixture of PAHs in sediment from the site. The primary goal of this dissertation was to better understand the mechanism(s) by which Elizabeth River killifish resist the developmental toxicity of a complex mixture of PAHs and to investigate the tradeoffs associated with this resistance. Because the aryl hydrocarbon receptor (AHR) pathway plays an important role in mediating the effects of PAHs, one major hypothesis of my work was that suppression of the AHR response plays an important role in the resistance of Elizabeth River killifish. For this reason, investigation of the activation of the AHR pathway, as measured by CYP induction, is a unifying thread throughout the work. Another major hypothesis of this work is that adaptation to PAHs has secondary consequences for Elizabeth River killifish, such as altering their response to other xenobiotics. To investigate these hypotheses, a series of experiments were carried out in PAH-adapted killifish from the
Elizabeth River and in reference fish. The morpholino gene knockdown technique was modified for use in killifish; we demonstrated that CYP1A knockdown exacerbates PAH-driven cardiac teratogenesis and AHR2 (but not AHR1) knockdown rescues PAH-driven cardiac teratogenesis. Using acute toxicity tests of larval killifish, we showed that Elizabeth River killifish are less sensitive than reference larvae to chlorpyrifos, permethrin, and carbaryl. These results demonstrated that the adaptation was able to protect from multiple xenobiotics, not just PAHs. Using the in ovo ethoxyresorufin-o-deethylase (EROD) assay and a subjective cardiac deformity screen, we showed that the adaptation was spread throughout the killifish subpopulations of the Elizabeth River estuary. However, the adaptive response varied greatly among the subpopulations, which showed that AHR pathway suppression was not required for some level of protection from PAH toxicity. Finally, using the quantitative real-time PCR, the EROD assay, and cardiac deformity screening, we demonstrated that the adaptation was heritable for two generations of fish reared in clean laboratory conditions. The findings in this dissertation will help to reveal how mixtures of PAHs exert their toxic action in unadapted organisms. Furthermore, these studies will hopefully demonstrate how chronic exposure to PAH mixtures can affect organisms at the population and even evolutionary level. Perhaps most importantly, they will help us to better predict the consequences and tradeoffs for organisms and populations persisting in PAH-contaminated environments.
Dedication

To my wonderful wife Nicole and my incredible parents Bill and Cheryl. Words cannot express the depths of my gratitude. Your love, support, and encouragement have made all the difference.
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1. Introduction

Chronic exposure to toxicant mixtures is a serious threat to environmental and human health. It is especially important to understand the effects of these exposures for contaminants, such as polycyclic aromatic hydrocarbons (PAHs), which are toxic, ubiquitous, and increasingly prevalent (Edwards 1983; Van Metre and Mahler 2005; Van Metre et al. 2000). Furthermore, estuarine systems are of particular concern, as they are highly impacted by a wide variety of pollutants; fish there are often exposed to some of the highest levels of contaminants of any vertebrate populations, along with other stressors such as fluctuations in water level, dissolved oxygen, and temperature (Wirgin and Waldman 2004). Our laboratory studies a population of *Fundulus heteroclitus* (the Atlantic killifish or mummichog, hereafter referred to as killifish) inhabiting a United States Environmental Protection Agency Superfund site heavily contaminated with a mixture of PAHs from former creosote wood treatment and storage operations. These killifish have developed resistance to the acute lethality and cardiac teratogenic effects caused by the mixture of PAHs in sediment from the site (Meyer and Di Giulio 2002; Ownby et al. 2002). This phenomenon raises important questions. How are these fish able to circumvent the severe toxicity of this complex mixture of contaminants? What tradeoffs are associated with developing resistance to PAHs?

Understanding the mechanisms underlying this resistance to PAH toxicity is a central theme of this dissertation. Defining the nature and mechanism(s) of resistance will help to reveal how mixtures of PAHs exert their toxic action in un-adapted organisms. Furthermore, these studies will hopefully demonstrate how chronic exposure to PAH mixtures can affect organisms at the population and even evolutionary level.
Perhaps most importantly, it will help us to better predict the consequences and tradeoffs for organisms and populations persisting in PAH-contaminated environments.

1.1 Polycyclic aromatic hydrocarbons

Polycyclic aromatic hydrocarbons (PAHs) are mutagenic, carcinogenic, and teratogenic contaminants generated by incomplete combustion of organic compounds. PAHs can enter the environment through natural sources such as forest fires, and through anthropogenic activities such as combustion of fossil fuels (Walker et al. 2005). They are ubiquitous and are increasing in the environment, tracking human population growth and the continued burning of fossil fuels (Van Metre and Mahler 2005; Van Metre et al. 2000). Estuarine habitats are vulnerable to PAH contamination via industrial outfalls, oil shipping and refining, wastewater discharges, urban runoff, and atmospheric deposition (Latimer and Zheng 2003).

Classically, PAHs are known as carcinogenic (Baird et al. 2005; Xue and Warshawsky 2005) and immunosuppressive toxicants (Lanckacker et al. 2010; Reynaud and Deschaux 2006) and as non-specific narcotic toxicants (Sikkema et al. 1995). Some PAHs are agonists for the aryl hydrocarbon receptor (AHR, discussed in section 1.2), while others are antagonistic or do not appear to have great affinity for the receptor (Billiard et al. 2004; Billiard et al. 2002; Denison and Nagy 2003; Marlowe and Puga 2005). In addition to the well-established toxicities, recent work has shown that some PAHs cause early life stage lethality and cardiac teratogenesis in fish. For some PAHs, primarily those of higher molecular weight, there is evidence that these effects are mediated through the AHR (Billiard et al. 2006; Clark et al. 2010; Incardona et al.
In these cases, PAH toxicity typically manifests as cranio-facial and cardiac malformations, and pericardial and yolk-sac effusion reminiscent of the “blue-sac syndrome” observed with the related and highly-studied planar halogenated aromatic hydrocarbons (pHAHs; e.g. 2,3,7,8 tetrachlorodibenzo-p-dioxin (TCDD)) (Hahn 2002; Prasch et al. 2003a; Toomey et al. 2001). In addition, some of the lower molecular weight PAHs have been shown to induce similar deformities at high concentrations independent of the AHR (Incardona et al. 2006a; Incardona et al. 2006b; Incardona et al. 2005; Incardona et al. 2004).

1.2 The aryl hydrocarbon receptor pathway

As mentioned previously, a number of PAHs are ligands for the aryl hydrocarbon receptor (AHR). The AHR binds halogenated aromatic hydrocarbons, such as some dioxins and polychlorinated biphenyls (PCBs), and some PAHs (Marlowe and Puga 2005; Schmidt and Bradfield 1996). However, it also binds a variety of plant-derived natural products (such as flavonoids), and its endogenous ligands may be derivatives of tryptophan and tetrapyroles (Denison and Nagy 2003). Gene activation via the AHR pathway has been increasingly well characterized (see reviews by Denison and Nagy 2003; Hahn 2002; Petrulis and Perdew 2002; Schmidt and Bradfield 1996). In the absence of ligand the AHR resides in the cytoplasm in a complex with heat shock protein 90 (Hsp90), X-associated protein 2 (XAP-2), and a co-chaperone p23. Ligand binding triggers a conformational change and translocation into the nucleus. The chaperones dissociate, revealing binding domains for its binding partner the aryl hydrocarbon receptor nuclear translocator (ARNT aka hypoxia inducible factor-1α). This
heterodimer complex binds to specific DNA sequences known as xenobiotic response elements (XREs, aka dioxin response elements or aryl hydrocarbon response elements) in the promoter region to induce transcription of a number of genes, including components of Phase I and II metabolism (i.e. cytochrome P450 (CYP)1A, 1B1, 1C1; glutathione S-transferase (GST); NADP(H):oxidoreductase; UDP-glucuronosyltransferase (UGT) (Nebert et al. 2000). The AHR complex also induces transcription of a factor called the aryl hydrocarbon receptor repressor (AHRR), which down-regulates the pathway, perhaps by competing with the AHR-ARNT complex for XREs (Hahn et al. 2009; Mimura et al. 1999). After the AHR-ARNT complex dissociates from DNA, the AHR is degraded in the nucleus by proteasomes or exported from the nucleus and degraded in the cytosol (Pollenz 2002).

The AHR pathway appears to be fairly well-conserved among mammals and lower vertebrates (Hahn 1998, 2002; Hahn et al. 2006). Two different AHRs, AHR1 and AHR2, have been identified in fish, including killifish (Andreasen et al. 2002; Karchner et al. 1999; Tanguay et al. 1999; Walker et al. 2004). ARNT and the AHRR have also been identified in killifish and other fish (Andreasen et al. 2002; Evans et al. 2005; Karchner et al. 2002; Powell et al. 1999). Importantly, the piscine AHR pathway is also known to be activated by dioxins, PCBs, and PAHs in a similar manner to the mammalian AHR pathway (Billiard et al. 2006; Jonsson et al. 2007; Prasch et al. 2003a; Prasch et al. 2003b; Toomey et al. 2001).

1.3 Fundulus heteroclitus

1.3.1 Basic biology
The killifish is a small teleost fish found in estuarine ecosystems along the Atlantic coast of North America from New Brunswick to Florida (Shute 1980). Killifish live about 3-4 years, are sexually dimorphic, and reach sexual maturity at less than one year old (Abraham 1985). They are one of the most abundant intertidal fishes and are a major component of estuarine food webs (Teo and Able 2003a; Yozzo et al. 1994). Killifish are omnivorous feeders; reported food sources include plant detritus, macroalgae, grass shrimp, crabs, annelids, and other fish (Able et al. 2007; Allen et al. 1994; Kneib 1986b; Kneib and Stiven 1978; McMahon et al. 2005). In turn, killifish are prey for birds, fishes, and invertebrates (Kneib 1982; Nemerson and Able 2003; Post 2008). In general, they spawn near the high tide line on a semilunar schedule concurrent with tidal changes (Taylor et al. 1979).

In addition to their important role in estuarine ecosystems, killifish have a number of attributes that make them a popular research subject (reviewed in Burnett et al. 2007). Killifish are tolerant of significant variation in environmental conditions, including salinity, temperature, oxygen, and pH (Dunson et al. 1993; Gonzalez et al. 1989; Nordlie 2006; Smith and Able 2003; Stierhoff et al. 2003; Wood and Marshall 1994). They are easy to capture and maintain, are amenable to manual spawning, and are highly fecund, with a single female able to produce up to several hundred eggs at once. Eggs (about 1-2 mm in diameter) have a transparent chorion that facilitates observation of staged embryonic development including organogenesis (Armstrong and Child 1965). Despite wide distribution of killifish populations along the Atlantic coast of North America, individual killifish have relatively small home ranges (Lotrich 1975; Skinner et al. 2005) and are ideal for studying the impacts of local contamination and other stressors (Burnett et al. 2007; Eisler 1986; Mulvey et al. 2002; Mulvey et al. 2003; Teo and Able 2003a, b).
Furthermore, their tolerance of a wide variety of environmental conditions has made them extremely useful for the study of a wide range of adaptations to environmental changes.

**1.3.2 Recent development as a genomic and molecular model**

Because of the aforementioned adaptability of killifish to a wide variety of environmental conditions, they also have great potential for investigation of genome-environment interactions. In addition to the advantages of studying wild outbred populations that face wide daily variation in temperature, salinity, and dissolved oxygen, killifish can be compared to their diverse relatives in the family Fundulidae or to other popular fish models such as zebrafish (*Danio rerio*). Currently these advantages of killifish have not been fully exploited, but there has been great progress despite the lack of a sequenced genome (see review by Burnett et al. 2007).

Several studies used differential display or subtractive hybridization to examine gene expression in response to contaminants. For example, Meyer et al. (2005) employed this approach to identify genes differentially expressed in PAH-tolerant and susceptible populations. Suppressive subtractive hybridization was used to develop “fingerprints” of exposure to the PAHs anthracene and pyrene (Peterson and Bain 2004; Roling et al. 2004) and to examine the impact of arsenic exposure on gene expression in offspring (Gonzalez et al. 2006). More recent studies have used cDNA arrays to investigate the effects of chromium, both in laboratory exposures and in fish from contaminated sites (Roling et al. 2006; Roling et al. 2007; Roling and Baldwin 2006). In addition, Oleksiak and co-workers have compared gene expression among populations from a series of polluted and reference sites; these studies will be discussed in more
detail in section 1.4.5 (Fisher and Oleksiak 2007; Oleksiak 2008; Williams and Oleksiak 2008).

Killifish microarrays have also been used to investigate evolution of regulation of gene expression (reviewed by Crawford and Oleksiak 2007; Whitehead and Crawford 2006b). Studies in killifish allow researchers to compare variation in expression among individuals within distinct populations, between populations, and across related Fundulidae. A series of studies by Crawford and colleagues have demonstrated a high degree of variation in gene expression among and within populations and between taxa that is likely to be evolutionarily important (Oleksiak et al. 2002; Oleksiak et al. 2005; Whitehead and Crawford 2006a).

As mentioned previously, the killifish genome is not yet sequenced but work is progressing in that direction (see Burnett et al. 2007). There is a collection of expressed sequence tags (ESTs) and complete cDNAs; these make up about 55,000 nucleotide sequences comprising about 18,000 unique sequences (Paschall et al. 2004). Burnett et al. highlight the development of a number of nuclear markers, such as microsatellites and amplified fragment length polymorphisms, which can be used to help develop a framework for genome sequencing (Adams et al. 2005; McMillan et al. 2006).

Finally, a number of molecular techniques are beginning to be used in killifish, despite the lack of a fully-sequenced genome. The feasibility of transgenesis was demonstrated by (Winn et al. 1995) during development of a model of in vivo mutagenesis in killifish. Additionally, morpholino gene knockdown has recently been adapted for efficient use in killifish. Morpholinos are antisense oligonucleotides that can be used to transiently block translation of targeted mRNAs. They are very useful for studying gene function during development in fish. In killifish, morpholino knockdown of
CYP1A was used to validate the technique and to investigate the synergistic effects generated by AHR agonists and CYP1A inhibition (Matson et al. 2008a). Recently, morpholino knockdown was used to demonstrate which of the two known killifish AHRs mediates PAH toxicity during development (Clark et al. 2010). Following sequencing of the genome, there appear to be few barriers to the use in killifish of these and other molecular methods used in more traditional vertebrate models.

1.4 Elizabeth River killifish

1.4.1 First report of PAH-resistance in Elizabeth River killifish

As described previously, initial studies in and around the Atlantic Wood Superfund Site on the Elizabeth River focused on the severe acute toxicity, including cataracts, skin lesions, immune disfunction, and lethality, affecting a wide variety of fishes. The work clearly showed that organisms in the estuary were highly impacted by creosote contamination. Other early studies also identified a high incidence of lesions in liver, kidney, and pancreas in killifish (Fournie and Vogelbein 1994; Van Veld et al. 1991; Van Veld et al. 1992; Vogelbein and Fournie 1994; Vogelbein et al. 1990). After the observation of neoplasia in killifish, further attention was focused on that particular species. Given their relatively non-migratory nature, the Atlantic Wood killifish population appeared likely to be a stable, persistent population that was exhibiting tumors primarily due to long-term residence under high exposure to creosote-contaminated sediments. Despite the severity of tumorigensis in killifish resident in the site, it appeared that Atlantic Wood killifish were somehow capable of circumventing
many of the acute toxic effects seen in spot and other fish and were managing to thrive despite severe pollutant stress.

Evidence of resistance by Elizabeth River killifish to the effects of PAHs was first published by Van Veld and Westbrook (1995). In this study, cytochrome P4501A (CYP1A) response was compared in adult fish collected from the Atlantic Wood site and a reference site on the nearby York River. CYP1A is a major enzyme involved in Phase I, oxidative biotransformation of xenobiotics. It is highly induced via the AHR by exposure to a wide-range of chemicals, including PAHs and other aryl hydrocarbons. As might be expected, CYP1A levels in freshly-caught fish were elevated in Atlantic Wood fish in comparison to reference fish (5-fold in liver, 56-fold in gut). However, following intraperitoneal (ip) injection of the PAH 3-methylcholanthrene (3-MC), hepatic expression of CYP1A protein increased 418-fold in reference fish but was not significantly changed in Atlantic Wood fish. The authors also compared CYP1 activity using the ethoxyresorufin-o-deethylase (EROD) assay, a fluorescence-based measurement of CYP1 catalysis, and EROD activity followed the same trend as CYP1A protein. Additionally, when each group was exposed to contaminated Elizabeth River sediments, hepatic CYP1A protein expression and EROD activity were greatly elevated in fish from the reference site compared to the levels measured in fish from the Atlantic Wood site. In total, this study demonstrated that fish from the Atlantic Wood site exhibit a remarkable recalcitrance to induction of CYP1A by PAHs.

1.4.2 Characterization of PAH resistance in Elizabeth River killifish

After identification of striking resistance by Atlantic Wood killifish to one of the major biological responses to PAH exposure, greater attention was focused on characterizing the breadth of PAH resistance of Elizabeth River killifish. Due to the
probability of close contact with contaminated sediments and the potential for increased sensitivity in early life stages, effects on embryos were, and continue to be, of particular interest. Furthermore, it is likely that heritable PAH adaptation is driven by acute toxicity and early life stage effects which could prevent reproduction, rather than chronic effects like carcinogenesis. Ownby et al. (2002) investigated early life stage toxicity caused by PAH-contaminated sediments in killifish embryos from populations inhabiting four sites on the Elizabeth River, including the Atlantic Wood site, and the York River reference population. Sediment PAH concentration measured among the Elizabeth River sites ranged from 3.9±3.2 to 264±115 µg/g (dry weight, select PAHs). Field-collected adults were manually spawned in the lab and the tolerance of their offspring to Atlantic Wood site sediment was compared. Embryos obtained from the reference population suffered from a variety of cardiac abnormalities (tube hearts, reduced circulation, and pericardial swelling), but embryos from Atlantic Wood parents showed a dramatic and near-complete resistance to these effects. The observed cardiovascular defects were similar to the suite of deformities known as blue-sac disease caused by some halogenated aromatic hydrocarbons (HAHs), such as 3,3′,4,4′,5-pentachlorobiphenyl (PCB-126) and 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) (reviewed by Peterson et al. 1993). The authors also showed that embryos from other Elizabeth River populations showed an intermediate degree of tolerance to the same effects. The level of resistance was roughly associated with contaminant levels at the sites of collection, perhaps reflecting population exposure history. In addition, the authors tested the heritability of tolerance by exposing F2 embryos obtained from lab-reared F1 adults. The F2 embryos exhibited a similar degree of tolerance to F1 embryos, indicating that resistance was heritable.
The resistance of Atlantic Wood killifish to PAHs (Meyer and Di Giulio 2003; Meyer et al. 2002) and PCB-126 (Meyer and Di Giulio 2002) was further investigated in the Di Giulio laboratory. In addition to investigating resistance to toxicity from contaminated sediments and aryl hydrocarbons, these studies further investigated the pattern and heritability of the recalcitrant CYP1A response previously observed by Van Veld and Westbrook in adult Elizabeth River killifish (1995). Despite sediment contamination dominated by PAHs and not HAHs, F1 Atlantic Wood embryos were highly resistant to cardiac teratogenesis and induction of CYP1 activity generated by PCB-126 exposure (Meyer and Di Giulio 2002). In fact, recent data show that both F1 and F2 Elizabeth River killifish were even more resistant to PCB-126 than adapted killifish from HAH-contaminated sites in Newark Bay, NJ and New Bedford Harbor, MA (Nacci et al. 2010). Atlantic Wood F1 and F2 embryos were also resistant to Elizabeth River pore water generated developmental abnormalities and mortality observed in reference embryos (Meyer and Di Giulio 2003; Meyer et al. 2002). In addition, Atlantic Wood F1 embryos were recalcitrant to induction of CYP1A by the AHR agonist-type PAHs 3-MC and β-naphthoflavone (BNF). However in these studies, the resistance was not consistently heritable to subsequent generations. PCB-126-induced EROD activity in F3 embryos (F2 were not tested) returned to levels similar to those of reference embryos; at the highest doses tested, resistance by F3 embryos to the teratogenic effects of PCB-126 was intermediate between the highly resistant F1 embryos and the susceptible reference embryos. Likewise, recalcitrance to CYP induction was largely lost in F3 embryos and F2 larvae dosed with 3-MC, BNF, or sediment pore water (Meyer and Di Giulio 2003; Meyer et al. 2002). In addition, hepatic EROD activity in adult Atlantic Wood F2s and even F1 adults exposed to Elizabeth River sediments was found
to be similar to that of adult reference fish. In contrast to the results of Ownby et al. (2002) and Nacci et al. (2010), studies by Meyer and colleagues seemed to show that the resistance was heritable but not fully genetic. Meyer and coworkers proposed that this might be achieved through epigenetic regulation of CYP1A. However, Timme-Laragy et al. (2005) found no difference between the methylation status of CpG sites in the CYP1A promoter of Elizabeth River and reference fish. It is difficult to reconcile the results of Meyer et al. with those of Ownby et al. and Nacci et al., although it is notable that some data obtained by Meyer and colleagues supported a conclusion of genetic heritability, but other data do not. In general, the strongest evidence for full genetic heritability was obtained for resistance to teratogenesis in embryos, while studies of heritable resistance in juveniles and adults yielded mixed results. Refractory CYP response and resistance to toxicity tended to fade with age, perhaps indicating that components of the adaptation are developmental stage specific. It is also possible that because resistant fish were less fit under clean conditions, the laboratory population could have undergone “reverse” selection, yielding less resistant offspring in later generations (Meyer and Di Giulio 2003). Interestingly, hybrid embryos generated by crossing Atlantic Wood fish of either sex with reference fish demonstrated a BNF-induced EROD response intermediate between those of reference and Atlantic Wood embryos (Meyer et al. 2002). In a subsequent study, survival of hybrid larvae exposed to diluted Elizabeth River pore water was intermediate between that of Atlantic Wood and reference larvae (Meyer and Di Giulio 2003). Furthermore, in both cases the response of the two hybrid lines was nearly indistinguishable, regardless of the sex of the parent from the Atlantic Wood population. These results seem to be more consistent
with a hypothesis of genetically heritable resistance, transmitted by both male and female Atlantic Wood fish.

### 1.4.3 Mechanisms of PAH-resistance in Elizabeth River killifish

A significant number of studies have addressed the mechanisms underlying the adaptation of Elizabeth River killifish to the acute toxicity and teratogenesis caused by PAH mixtures. Early in the study of the Atlantic Wood population, investigators discovered elevated expression of a number of xenobiotic metabolizing enzymes. Phase II enzymes, such as glutathione-S-transferases (GSTs), act to conjugate xenobiotics, increasing their solubility and enhancing their excretion from the body.

During investigation of the hepatic lesions in Atlantic Wood killifish, Van Veld et al. (1991) noted that hepatic and intestinal GST activity was elevated 3-4 fold in Atlantic Wood fish with respect to reference fish. Armknecht et al. (1998) hypothesized that elevation in GST might contribute to the PAH resistance. They confirmed the earlier finding of elevated GST activity in Atlantic Wood fish and also showed a concomitant elevation of protein. In both studies, the GST elevation was found to be intermediate in fish from a site with intermediate levels of PAHs, perhaps indicating that elevated GST was a marker of exposure rather than an adaptive change. However, hepatic GST activity was not induced in reference fish fed a BNF-doped diet (Van Veld et al. 1991).

In another study, Gaworecki et al. (2004), observed elevated activity of the conjugation enzyme UDP-glucuronosyltransferase (UGT) in Atlantic Wood killifish when compared to reference fish. They also showed a small elevation in sulfotransferase (SULT) activity, although its overall level of activity measurable in killifish was low. Finally, Cooper et al. (1999) demonstrated that the xenobiotic transporter p-glycoprotein (Pgp, also known as ABC transporter B1) was expressed 2-3 fold higher in Atlantic Wood fish than in
reference killifish. Pgp is sometimes referred to as a Phase III metabolizing enzyme; they are membrane transport ATPases involved in efflux of compounds. Although identified in a variety of normal tissues, Pgp is frequently elevated in multidrug-resistant cell lines and chemotherapy-resistant tumors (Litman et al. 2001). Thus far, the heritability of these elevated Phase II and III responses has not been investigated, so it is unclear if they are components of heritable adaptation or simply acclimatory responses to PAH exposure.

Even more striking than this upregulation of Phase II metabolism, however, is the dramatic recalcitrance to induction of the Phase I metabolizing enzyme CYP1A (described previously). This conspicuous down-regulation, along with the knowledge of the role CYP1 enzymes can play in activation of PAHs to more toxic and reactive metabolites, led our laboratory to hypothesize that suppression of CYP1A activity was a major component of resistance in Atlantic Wood killifish. To test this hypothesis and to investigate the ramifications of exposure to PAH mixtures, reference population killifish embryos were exposed to AHR agonists while CYP1A activity was suppressed using chemical inhibitors (Wassenberg and Di Giulio 2004a, b; Wassenberg et al. 2005). Rather than reducing toxicity, however, combined exposure of embryos to an AHR agonist and a CYP inhibitor resulted in a dramatic synergistic increase in cardiac teratogenesis. For example, the combination of 11 µg/L of the AHR agonist BNF with 100 µg/L of the CYP1A inhibitor α-naphthoflavone (ANF) resulted in a near-maximal score on the subjective deformity index used, despite the fact that neither compound elicited any observable teratogenicity on its own (Wassenberg et al. 2005). Comparable results were observed using several other AHR agonists (BaP, BNF, and PCB-126) and CYP1A inhibitors (ANF, fluoranthene, 2-aminoanthracene, piperonyl butoxide,
carbazole, and dibenzothiophene) (Wassenberg and Di Giulio 2004a; Wassenberg et al. 2005). Similar dramatic enhancement of toxicity was observed when embryos were exposed to a mixture of diluted Elizabeth River pore water and ANF. To further investigate this phenomenon and to confirm the role of CYP1A inhibition, targeted morpholino knockdown of CYP1A was performed. Billiard et al. (2006) demonstrated in zebrafish (Danio rerio) that, as with chemical inhibition, morpholino knockdown of CYP1A drastically exacerbated BNF toxicity. Importantly, our laboratory recently developed the morpholino gene knockdown technique for use in killifish and confirmed that knockdown of CYP1A exacerbates BNF toxicity in killifish (Matson et al. 2008a). Collectively, these results have a number of implications. Clearly, suppression of CYP1A alone is not only insufficient to provide the resistance observed in Elizabeth River killifish but in fact increases the hazard of AHR agonist-type PAHs. Furthermore, at least with respect to embryotoxicity, the results strongly call in to question the model of additive toxicity commonly assumed for PAHs.

The mechanism by which CYP1A inhibition synergizes PAH toxicity is not certain. It is possible that blockage of metabolism by CYP1A prevents degradation of the PAH and prolongs its persistence. This could serve to make PAHs resemble HAHs, perhaps explaining why cardiac teratogenesis generated by PAH mixtures closely resembles that caused by dioxin-like compounds. Another possibility is that inhibition of CYP1A shifts metabolism of PAHs to a different route resulting in more toxic metabolites. To address these hypotheses, Elizabeth River and reference embryos were exposed to a mixture of BaP and the CYP1A inhibitor PAH fluoranthene (Fl) from 24 hours post fertilization (hpf) to 120 hpf, at which point the presence of parent BaP and specific metabolites were measured (Wills et al. 2009). Co-exposure to Fl had very little
effect on the BaP metabolism measured in either population. Elizabeth River embryos retained slightly more parent BaP than did reference embryos. Additionally, more of the only metabolite detected, BaP-9,10-dihydrodiol, was measured in Elizabeth River embryos. Since 9,10-dihydrodiol and its subsequent metabolite, 9,10-diol,7,8-epoxide, are less mutagenic than the alternatives, Wills et al. (2009) hypothesized that this possible shift in metabolism could contribute to resistance in Elizabeth River killifish (Peltonen and Dipple 1995; Stegeman and James 1985). If increased production of the 9,10-dihydrodiol is a consistent difference between the populations, this could indicate an increase in the ratio of epoxide hydrolase activity relative to CYP1A (Bauer et al. 1995; James and Little 1983; Kleinow et al. 1998; Oesch 1988).

Given the observed effects of blocking CYP1A on PAH toxicity, it appears likely that the refractory CYP1A response in Elizabeth River killifish could be part of a broader down-regulation of the entire AHR pathway. The pivotal role of the AHR in the toxicity of both DLCs and some PAHs (Marlowe and Puga 2005) makes it a likely target for development of resistance. Other populations of fish, including killifish, have been identified that are resistant to HAHs (reviewed in Wirgin and Waldman 2004) and several demonstrate resistance to both PAHs and HAHs. As stated previously, Elizabeth River killifish are resistant to PCB-126 and to PAHs (Meyer and Di Giulio 2002; Nacci et al. 2010; Ownby et al. 2002). Killifish from New Bedford Harbor, MA are resistant to the high levels of PCBs at the site and also to PAHs (Bello et al. 2001; Nacci et al. 1999; Nacci et al. 2002c). While tomcod (Microgradus tomcod) from the Hudson River, NY are resistant to high levels of PCB contamination, their CYP response is refractory to induction by PCBs but not PAHs (Roy et al. 2002). However, they are less susceptible to DNA damage by BaP (Sorrentino et al. 2004). One hypothesis for development of
resistance to different but highly related classes of contaminants in multiple populations and species is through modification of a single conserved gateway, such as the AHR.

Further evidence that PAH resistance in Elizabeth River killifish occurs through modification at the top of the AHR pathway was provided by analysis of transcriptional expression of hepatic CYP1A, AHR1, AHR2, ARNT2, and AHRR in adult fish treated with BNF (Meyer et al. 2003b). CYP1A, AHRR, and AHR2 expression were induced in reference fish, but not in Elizabeth River fish; AHR1 and ARNT2 were not differentially expressed. This suggests suppression at the level of the AHR, since both CYP1A and AHRR are AHR-regulated. It is also notable that there were no differences between populations in basal levels of expression of any genes studied, which implies that a difference in initial mRNA expression of AHR pathway genes does not confer resistance. Furthermore, it is noteworthy that AHR2 was inducible in reference fish but not Elizabeth River fish. Although there is debate as to whether AHR is inducible by chemical treatment, one would expect that if it were, Elizabeth River fish direct from the field would have elevated basal levels due to PAH exposure in situ. This lack of induction of AHR2 in Elizabeth River fish, coupled with statistically indistinguishable basal levels of expression may indicate that resistance is conferred through an insensitive or non-inducible AHR2 in Elizabeth River fish. It is important to note, however, that expression of AHR2 was increased detectably in male fish from the Elizabeth River, indicating that these changes may have a sex specific component. Thus far, these comparisons have only been made at the level of mRNA, not protein. Recent assessment in our laboratory of PAH-induced CYP mRNA expression in embryos provides further evidence that the alteration is prior to transcription of individual AHR-responsive genes (L.P. Wills et al. 2010). CYP1A, CYP1B1, and CYP1C1 were all induced by BaP, BkF, and PCB-126.
exposure in reference embryos but not Elizabeth River embryos. It seems likely that alteration at the level of the AHR is causing changes in expression of the CYPs and AHRR rather than separate alterations for each gene.

Morpholino knockdown studies provide evidence supporting AHR down-regulation as a potential mechanism of resistance to PAHs and PAH mixtures. In zebrafish, morpholino knockdown of AHR2 was protective from synergistic cardiac toxicity caused by a mixture of BNF and ANF (Billiard et al. 2006) and by high doses of pyrene or benz[a]anthracene (Incardona et al. 2006b; Incardona et al. 2005). Furthermore, AHR2 knockdown in zebrafish protects from teratogenesis following exposure to multiple PAH mixtures, including BaP and FL, benzo[k]fluoranthen (BkF) and FL, Elizabeth River sediment extract, and coal tar (Lindsey Van Tiem, personal communication). Recently, we demonstrated that knockdown of AHR2, but not AHR1, protects killifish from cardiac teratogenesis due to BNF, BkF, and PCB-126 (Clark et al. 2010).

PAHs can cause toxicity by metabolism or photo-modification yielding products that cause oxidative stress (Arfsten et al. 1996). For this reason, Meyer et al. (2003a) hypothesized that upregulation of antioxidant defenses could be an important component of resistance to stressors in the Elizabeth River environment. Meyer et al. verified that exposure to Elizabeth River sediments led to increases in several antioxidant defenses, including total glutathione (GSH), glutathione reductase activity, and manganese superoxide-dismutase (MnSOD) protein, in both Elizabeth River and reference fish. Studies with zebrafish established that, as with Elizabeth River sediments, a mixture of BNF and ANF causing cardiac malformations induced expression of a battery of antioxidant response genes (Timme-Laragy et al. 2009). To
further examine the role of oxidative stress in the synergistic toxicity of BNF and ANF, Timme-Laragy et al. used morpholino knock down of NF-E2-related factor 2 (Nrf2; a transcription factor that controls expression of a variety of antioxidant defenses) (Kaspar et al. 2009). Blockade of Nrf2 resulted in exacerbation of BNF and ANF mixture toxicity.

Given the data showing the potential for PAH mixtures to generate oxidative stress, it was not surprising that the Elizabeth River killifish differ from reference fish in their response to oxidative stressors. Elizabeth River F1 and F2 larvae were more resistant than reference larvae to the model pro-oxidant t-butyl hydroperoxide (Meyer et al. 2003a). Compared to reference fish, whole-body homogenates of Elizabeth River F1 or F2 larvae also showed greater total oxygen scavenging capacity (TOSC), slightly elevated total GSH, and greater MnSOD protein expression. Total GSH levels were particularly elevated in juvenile liver. In a subsequent study, wild-caught Elizabeth River adults exhibited higher hepatic total GSH and selenium-independent GSH peroxidase activity (Bacanskas et al. 2004). However, selenium-dependent GSH peroxidase was higher in reference fish. Interestingly, mitochondrial lipid peroxidation was elevated in Elizabeth River fish relative to reference fish, despite the observed increases in antioxidant defenses. Overall, these studies show that Elizabeth River sediments and PAH mixtures generate oxidative stress and that killifish exhibit a variety of short-term physiological responses to this stress. Furthermore, some antioxidant responses in Elizabeth River killifish appear to be heritable to the F2 generation, so they may contribute to the overall resistant phenotype.

1.4.4 Fitness trade-offs and cross-resistance in PAH-adapted killifish

It is generally accepted that adaptation to a stressor is accompanied by concurrent fitness costs to populations (Clements and Rohr 2009; Coustau et al. 2000;
Kinnison and Hairston 2007). Strong selective pressure is thought to drive reduction in genetic variation and overall fitness. Reduced fecundity, growth rate, and survival are considered classical fitness costs, but a broader interpretation could also include greater sensitivity to other stressors, both chemical and physical. Despite the dramatic PAH-resistance exhibited by Elizabeth River killifish, they are clearly not wholly unaffected by the contamination. As described previously, Elizabeth River killifish suffer from a variety of hepatic and extra-hepatic neoplasms (Fournie and Vogelbein 1994; Vogelbein et al. 1990). They also seem to be more susceptible to disease and have documented alterations in immune function (Faisal et al. 1991; Frederick et al. 2007; Kelly-Reay and Weeks-Perkins 1994; Meyer and Di Giulio 2003; Meyer et al. 2005; Weeks et al. 1988). This may also be a direct effect of PAH exposure, as there is evidence that PAHs can have a variety of effects on the immune system of fish (reviewed by Reynaud and Deschaux 2006). Some of these effects are clearly an effect of long-term persistence in a heavily polluted environment, but others may be costs of adaptation.

To explore the consequences and fitness costs of the adaptation, Meyer and Di Giulio (2003) compared the response of Elizabeth River and reference killifish to several stressors and to clean conditions. In clean conditions, survivorship of Elizabeth River F1 larvae was significantly lower than reference larvae after 9 months. Interestingly, survivorship of F2 larvae was no different than reference fish. A similar trend was observed for growth. Additionally, reference larvae tolerated phototoxicity mediated by combined exposure to fluoranthene and UV light for longer than Elizabeth River larvae. However, Elizabeth River F1 larvae still survived longer when exposed to Elizabeth River sediments, regardless of UV exposure. In addition, Elizabeth River F1 and F2 larvae tolerated low oxygen (0.5 mg/L) conditions for significantly less time than
reference larvae. One explanation for this trade-off could be cross-talk between the AHR and hypoxia signaling pathways, since the dimerization partner for the AH receptor, the ARNT (aka hypoxia-inducible factor 1β), is shared by both pathways (Carlson and Perdew 2002). The potential effect of this crosstalk on toxicity of PAH mixtures was further investigated in our laboratory. Zebrafish embryos were exposed to the AHR agonists BaP and BNF, the CYP inhibitors FL and ANF, and to synergistically toxic mixtures of BaP and FL or BNF and ANF, with or without hypoxia (Matson et al. 2008b). Hypoxia did not effect the toxicity of either BaP and BNF, but coexposure to hypoxia and the CYP inhibitors FL and ANF led to significant increases in pericardial edema. Furthermore, hypoxia and FL caused severe lordosis (curvature of the spine).

Meyer et al. (2005) used differential display to compare gene hepatic gene expression in Elizabeth River killifish and a reference population. The genes that were most strikingly decreased in the Elizabeth River fish were related to immune function, including Factor XI, a clotting factor also involved in complement activation, and the complement components C3 and C9. Additionally, expression of UDP-glucose pyrophosphorylase was lower and expression of glucose 6-phosphatase was higher in Elizabeth River fish, suggesting potential impairment of aerobic energy metabolism and a shift away from energy production in mitochondria. This result is consistent with the reduced performance of Elizabeth River killifish in hypoxia described previously.

A series of studies by Jung and coworkers further explored the effect of PAHs and PAH-adaptation on the mitochondria of Elizabeth River and reference killifish. The recalcitrant CYP1A phenotype of Elizabeth River fish is consistent in mitochondria. Jung and Di Giulio (2010) identified mitochondrial CYP1A and showed that it was induced by BaP in reference fish, but not Elizabeth River killifish. They used long-amplicon-PCR to
demonstrate that, as might be expected, wild-caught Elizabeth River adults have far
greater levels of both mitochondrial DNA (mtDNA) and nuclear DNA (nDNA) damage in
liver, brain, and muscle than reference fish (Jung et al. 2009a; Jung et al. 2009b).
Interestingly, the levels of mtDNA and nDNA damage were similar in a given tissue,
despite evidence that mtDNA is more susceptible to damage by PAHs (Allen and
Coombs 1980; Backer and Weinstein 1980). Although wild-caught Elizabeth River
killifish exhibited a higher basal level of DNA damage, they may actually be less
susceptible to induction of DNA damage by PAHs. Jung et al. (2009a) showed that i.p.
injection of BaP resulted in a 2-fold to 7-fold increase in DNA damage in reference fish,
but less than a 2-fold increase in Elizabeth River fish. Reduced activation of BaP by
mitochondrial CYP1A probably contributes to the observed resistance to mtDNA
damage. These observations may fit with the observations of Wills et al. (2009)
described previously indicating that Elizabeth River fish could be shifting metabolism of
BaP away from the formation of DNA-damaging metabolites. Furthermore, this
correlates strongly with recent work in our laboratory showing that Elizabeth River larvae
are less susceptible to BaP-induced chronic liver toxicity and carcinogenesis (Wills et al.
accepted). The results of Wills et al. suggest that adult Elizabeth River killifish suffer the
tumors previously described after lifelong PAH exposure, in spite of apparent resistance
to induction of carcinogenesis in early life stages.

1.4.5 Population genetic and genomic effects of PAH adaptation

Thus far, much of the work reviewed here has focused on the nature and
underlying mechanism of PAH-tolerance in Elizabeth River killifish. However, the
existence of the adapted Elizabeth River population along with contaminant-adapted
killifish populations in New Bedford Harbor, MA and Newark Bay, NJ, provides unique
opportunities for investigating the effects of pollutants on selection and genome-level response to environmental stress.

There are several explanations for the persistence of a killifish population in the highly contaminated Atlantic Wood Superfund site. One is that they have developed heritable adaptations that enable their survival; much of the work described previously supports that conclusion. Alternately, the Atlantic Wood sub-population could suffer heavy mortality, but continually replenish via immigration from nearby sub-populations. Mulvey and coworkers used allozyme frequency (Mulvey et al. 2002) and mtDNA haplotype diversity (Mulvey et al. 2003) to test these possibilities. Genetic diversity was compared among sub-populations throughout the Elizabeth River estuary and reference populations in the nearby York River. The Elizabeth River sub-populations were located in sites that varied in degree of sediment PAH contamination from 1-200 µg/g total PAHs. In both studies they found that fish collected from the Atlantic Wood site were genetically distinct from the other sub-populations investigated. Fish from a second, moderately contaminated Elizabeth River site were less distinct from other sub-populations. Overall, genetic distance between sub-populations was not correlated with geographic distance, but was correlated with degree of PAH contamination at the site of collection. This suggests that contamination did have a significant effect on genetic structure, at least at the geographic scale examined. In contrast, genetic structure was linked to geographic distance, but not to contamination level, in comparison of HAH-adapted and reference killifish populations from New Bedford Harbor, MA (McMillan et al. 2006; Roark et al. 2005). Recently, our laboratory compared the level of resistance to a variety of aryl hydrocarbons among embryos of Elizabeth River killifish sub-populations from throughout the estuary, most of which were collected in or near the
sites used by Mulvey and coworkers (Chapter 5). Unlike the Atlantic Wood population, some of the sub-populations exhibited a high degree of AHR pathway activation, as measured by EROD activity, but were still resistant to aryl hydrocarbon-induced teratogenesis. The degree of aryl hydrocarbon resistance was not highly correlated with the level of PAHs at the sub-population collection site. Furthermore, each sub-population exhibited a unique pattern of response to the various hydrocarbons tested. These unique patterns of sub-population response and lack of correlation to contaminant level are notable, given the strong correlations between PAH level and genetic distance (Mulvey et al. 2002; Mulvey et al. 2003) and resistance (Ownby et al. 2002). Interestingly, Mulvey et al. estimated a rate of migration high enough to maintain significant gene flow among sub-populations; the modeled effective migration rate for juveniles was 9.6 migrants per generation and for adults was 17.5 migrants per generation. The authors hypothesized that the Atlantic Wood population remains genetically distinct despite the migration rate because immigrants are unable to survive in the harsh conditions. However, it is possible that emigrants from the Atlantic Wood site could be driving the appearance of adaptation at sites with low levels of PAH contamination. Moreover, the transmission and fixation of only some components of the adaptation to other sub-populations might explain the unique patterns and incomplete resistance exhibited by the individual sub-populations. High migration rates may also serve to help maintain overall genetic diversity. Strong selection for a particular adaptive parameter can lower overall genetic diversity. Furthermore, pollutant stress can reduce genetic variability in populations by reducing population size through loss of sensitive individuals and reduced fecundity. However, Mulvey and coworkers observed no loss of genetic diversity in either study.
In a series of studies comparing aryl hydrocarbon-adapted killifish populations from the Atlantic Wood Superfund site and Superfund sites in New Bedford Harbor, MA and Newark Bay, NJ, Oleksiak and co-workers searched for gene pathways or genomic loci linked to contaminant exposure and adaptation. Under the hypothesis that pollutant exposure would necessitate changes in metabolic gene expression to provide energy for protection against toxicity, they utilized a metabolic cDNA array to compare gene expression in brain (Fisher and Oleksiak 2007) and liver (Oleksiak 2008) between each pollution-adapted population and two nearby matched reference populations. Compared to its reference sites, 13 of 260 examined genes (5%) were differentially expressed in brains of Elizabeth River killifish. This was reduced to only one gene after a Bonferroni correction. In addition, only two genes were shared among all three Superfund populations (NADH-ubiquinone oxidoreductase AGG subunit precursor, a subunit in oxidative phosphorylation complex I, and thioredoxin, an oxidoreductase that facilitates the reduction of other proteins). In livers of Elizabeth River killifish, 20 of 250 examined genes (8%, reduced to 2 of 250 after Bonferroni correction) were differentially expressed compared to reference populations. Again, a small number of genes were identified that were differentially expressed in all three aryl hydrocarbon adapted populations (acyl-CoA-binding protein, the MNLL subunit of NADH-ubiquinone oxidoreductase, and thioredoxin). Interestingly, none of these genes were consistently expressed in the polluted populations. For example, thioredoxin was more highly expressed in the Elizabeth River population and less highly expressed in the New Bedford Harbor and Newark Bay populations. The authors state that the relative lack of overlap among the gene expression patterns of the three aryl hydrocarbon adapted populations reflects different routes of adaptation and different pollutant exposure. However, previous
studies have shown a great deal of overlap in the adaptive responses of the three populations (see review by Wirgin and Waldman 2004), so it is also possible that the limited number of genes in the array was unable to fully capture the adaptive changes. Overall, many of the differentially expressed genes in both studies appear to be consistent with responses to contaminant exposure, such as genes involved in oxidative phosphorylation and oxidative stress response. Finally, as with the work by Mulvey and coworkers, these studies also did not demonstrate any reduction in variance in gene expression due to contaminant exposure.

To identify altered loci in the genome that could be signatures of selection, Williams and Oleksiak (2008) compared allele frequencies of amplified fragment length polymorphisms (AFLPs) among the same pollutant-adapted and paired reference populations used in the gene expression studies. In this study the goal was to identify genomic loci that had large differences in allele frequency between the aryl hydrocarbon-adapted and reference populations, but that were not also highly different between the pair of reference populations; these outlier loci could be involved in adaptation and pollutant response. A total of 9 of 299 loci scored (3%) were identified as outliers in comparison of the Elizabeth River killifish to two reference populations. Of these, six were found in individual comparisons of the Elizabeth River population to each reference population that were not also found in comparison of reference populations to each other. The Elizabeth River population had two of its outlier loci in common with the New Bedford Harbor fish and two with the Newark Bay fish. No outliers were shared among all three adapted populations; rather, most of the identified loci were unique to each adapted population. The authors state that this could be caused by linked loci dragging different polymorphisms to fixation in each population, even if the locus driving selection
was actually the same in each population. It is also possible that the adaptation is
specific and different between the populations. However, as stated previously, the
populations are faced with many of the same contaminants and exhibit many similar
adaptive responses so it is surprising that more shared outlier loci were not identified.
The authors also mention that other selective pressures, such as predation or food
availability, could drive differences among populations. It is interesting to consider that
these differences could also be secondary effects of the contamination. Finally, as with
the Oleksiak laboratory’s gene expression studies and the work of Mulvey et al., genetic
diversity was not reduced in the Elizabeth River populations.

1.5 Dissertation objective and outline

Chronic exposure to PAH mixtures is a serious and ongoing threat to
environmental and human health. Killifish are an excellent model for study of the
impacts of chronic environmental pollution. The existence of contaminant-adapted
killifish provides unique opportunities for investigating the mechanisms of toxicity and
resistance to chronic pollution stress. Furthermore, adapted populations provide us with
opportunities to learn how pollutant stress affects organisms at a population and even
evolutionary level.

The primary goal of this dissertation is to better understand the mechanism(s) by
which Elizabeth River killifish resist the toxicity of a complex mixture of PAHs and to
investigate the tradeoffs associated with this resistance. As discussed in section 1.4.3,
the AHR pathway plays an important role in mediating the effects of PAHs. One major
hypothesis of my work was that suppression of the AHR response plays an important
role in the resistance of Elizabeth River killifish. For this reason, investigation of the activation of the AHR pathway, as measured by CYP induction, is a unifying thread throughout the work. Another major hypothesis of this work is that adaptation to PAHs has secondary consequences for Elizabeth River killifish, such as altering their response to other xenobiotics.

This dissertation is organized into five research chapters that contribute to understanding of the mechanism(s) and consequences of adaptation to chronic exposure to PAH mixtures:

- In Chapter 2, I adapted the morpholino gene knockdown technique for use in killifish. Morpholinos are valuable tools for manipulating gene expression in investigation of mechanisms of developmental toxicity in fish.

- Using this morpholino knockdown approach in Chapter 3, I investigated the role of the two killifish AHRs in mediating cardiac teratogenesis caused by PAHs and PCB-126. I showed that AHR2 mediates these effects in killifish, suggesting that down-regulation of AHR2 could be a mechanism by which Elizabeth River killifish resist aryl hydrocarbon toxicity.

- In Chapter 4, I compared the response of PAH-adapted killifish and reference killifish to neurotoxic pesticides. The goal of this work was to determine if adaptation had tradeoffs for response to a different class of xenobiotics. This work showed that Elizabeth River killifish are resistant to multiple chemicals in addition to aryl hydrocarbons. Furthermore, the results suggested that factors beyond AHR pathway suppression are likely to play a role in contaminant resistance in Elizabeth River killifish.
• In Chapter 5 we examined the resistance of killifish subpopulations from throughout the Elizabeth River to activation of the AHR pathway and cardiac teratogenesis caused by aryl hydrocarbons, singly and in mixtures. This study demonstrated a complex pattern of resistance to toxicity and AHR pathway activation in subpopulations from throughout the estuary.

• In Chapter 6 we showed that resistance to aryl hydrocarbon-induced cardiac teratogenesis is heritable for multiple generations. We also showed that recalcitrance to CYP induction is largely heritable, although investigation of the F2 generation revealed a subset of individuals that were more responsive than F1 individuals.

Finally, the findings of this dissertation and their implications are summarized in Chapter 7. In addition, preliminary data from ongoing investigations are provided in brief in the appendix.
2. Development of the morpholino gene knockdown technique in Fundulus heteroclitus: a tool for studying molecular mechanisms in an established environmental model

This chapter was published under the same title in Aquatic Toxicology, Volume 87, pages 289-295, in the year 2008. The authors are Cole W Matson, Bryan W. Clark, Matthew J. Jenny, Carrie R. Fleming, Mark E. Hahn, and Richard T. Di Giulio. The article was written by Cole Matson, but all experimental design, experimental work, and data examination were contributed to equally by Cole Matson and Bryan Clark.

2.1 Introduction

The Atlantic killifish (Fundulus heteroclitus) is one of the most abundant estuarine fish along the Atlantic coast of the United States; as such it is ecologically very important (Burnett et al. 2007; Kneib 1986b; Meredith and Lotrich 1979). Although killifish are widespread, they have small home ranges, which make them ideal for studying the effects of local stressors (Lotrich 1975). They are tolerant of significant changes in many environmental conditions, including salinity (Griffith 1974; Nordlie 2006; Wood and Marshall 1994), pH (Gonzalez et al. 1989), temperature (Dunson et al. 1993; Nordlie 2006; Smith and Able 1994), and oxygen (Nordlie 2006; Smith and Able 2003; Stierhoff et al. 2003; Wannamaker and Rice 2000). Specific killifish populations have adapted to a variety of anthropogenic stressors, including polycyclic aromatic hydrocarbons (PAH) (Meyer and Di Giulio 2002; Meyer and Di Giulio 2003; Ownby et al. 2002), 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) (Prince and Cooper 1995a, b), polychlorinated biphenyls (PCB)(Bello et al. 2001; Nacci et al. 1999), and mercury (Weis 1984). For
these reasons, killifish have become one of the premier teleost environmental models, and certainly one of the most well-studied estuarine environmental models (Burnett et al. 2007). While we know a great deal about killifish physiology, a lack of genomics data and the resulting paucity of genetic tools available hinder our ability to investigate mechanisms of adaptation and chemical response. To adapt existing genetic tools for use in this valuable environmental model, we developed and evaluated antisense morpholino oligo (MO) gene knockdown methods for use in killifish. Morpholino oligos are chemically modified oligonucleotides (generally 25 bases) that bind to specifically targeted mRNA (Nasevicius and Ekker 2000). Morpholinos reduce gene expression via steric blocking of translation or pre-mRNA splicing.

Of the killifish populations known to have adapted to environmental contaminants, several have adapted specifically to chemicals that act via AhR-dependent mechanisms, and alterations to the AhR pathway seem to be a critical component of the adaptation (Bacanskas et al. 2004; Bello et al. 2001; Hahn et al. 2004; Meyer et al. 2003b; Nacci et al. 1999; Ownby et al. 2002; Powell et al. 2000; Prince and Cooper 1995a, b). Specifically, all of these populations exhibit a marked reduction in CYP1A inducibility in response to a variety of AhR agonists (Arzuaga and Elskus 2002; Bello et al. 2001; Elskus et al. 1999; Meyer and Di Giulio 2003; Meyer et al. 2002; Nacci et al. 1999; Prince and Cooper 1995a). To more fully understand alterations to the AhR pathway, CYP1A was chosen as the target for morpholino gene knockdown in killifish from a reference site.

CYP1A is an excellent target gene for assessing the utility of MO technology in killifish for several reasons. First, CYP1A is known to be a critical determinant of embryotoxicity caused by PAH mixtures (Billiard et al. 2006; Wassenberg and Di Giulio
Second, we can easily test for protein activity via the *in ovo* EROD assay. This is particularly important given the inability to use mRNA to quantify the effectiveness of MOs that act by inhibiting translation. Third, knockdown of CYP1A via chemical inhibition in *Fundulus* (Wassenberg and Di Giulio 2004a) or with morpholinos in zebrafish (Billiard et al. 2006) has been shown to exacerbate the embryotoxicity of AhR agonists and PAH mixtures. Therefore, we could also use deformity screens to test whether the MO gene knockdown was successful. Finally, immunochemical techniques have been shown to be useful in assessing induced CYP1A protein levels in *Fundulus* embryos (Toomey et al. 2001).

Antisense morpholino oligo technology has primarily been used in zebrafish (*Danio rerio*) and clawed frogs (*Xenopus laevis* and *X. tropicalis*). While MO based experiments are most often conducted in zebrafish, there are several significant differences between zebrafish and killifish eggs. Killifish eggs are larger, have a more durable chorion, have higher internal pressure, have mucous-like material on the exterior surface of the chorion that can contain large amounts of debris, and develop more slowly (Armstrong and Child 1965; Brummett and Dumont 1981; Marteinsdottir and Able 1988; Morin and Able 1983; Taylor 1999). Egg size does not pose a significant challenge for microinjection of morpholinos; however, the tougher chorion and high internal pressure are considerable challenges. The mucous-like membrane surrounding the chorion is a significant problem with regard to clogging injection needles.

Killifish have a long development time (~14 days) compared to zebrafish (2-3 days). The slower developmental rate in killifish can be both an advantage and disadvantage. The several hours that it takes embryos to reach the 8-cell stage allows for the injection of large numbers of eggs, but the extended development period greatly
increases the amount of time a morpholino must remain effective. Since zebrafish develop rapidly, the duration of morpholino effectiveness has not been a primary concern. However, reports of successful use of this technology in rainbow trout, *Oncorhynchus mykiss* (Boonanuntanasarn et al. 2002) and sea lamprey, *Petromyzon marinus* (McCauley and Bronner-Fraser 2006), which have significantly longer development times (>2 weeks), suggest that MO usage in killifish would be feasible. Antisense morpholino oligo technology remains the only thoroughly tested method of *in vivo* gene expression knockdown in fish embryos. Thus, the development of MO technologies in *Fundulus* would provide a powerful tool for studying gene function in this established environmental model. Subsequent research will make use of this technology to further our understanding of the aryl hydrocarbon receptor (AhR) pathway and its role in killifish adaptation to PAH-contaminated environments.

### 2.2 Materials and methods

#### 2.2.1 Fish care

Adult killifish were collected at a reference site on King’s Creek, a tributary of the James River which feeds into the lower Chesapeake Bay in southeastern Virginia (37°17′52.4″N, 76°25′31.4″W). Fish were maintained at 23-25°C in 25 ppt artificial seawater (ASW; Instant Ocean, Foster & Smith, Rhinelander, WI) and fed a mixture of TetraMin® Tropical Flakes (Tetra, Blacksburg, VA) and freshly hatched brine shrimp (*Artemia*, Brine Shrimp Direct, Ogden, UT). Eggs were manually collected from females and fertilized *in vitro* by milking sperm from males directly into a beaker containing the eggs in ASW. Eggs were set aside for 30 minutes for fertilization, and then washed in
0.3% hydrogen peroxide. Eggs are subsequently “rolled” on durable wet paper towels with wet fingers to remove mucous-like material and debris from the exterior of the chorion, as per standard medaka egg separation techniques (Marty et al., 1990). At this point the embryos are ready for injection. Adult care and reproductive techniques are non-invasive and have been reviewed and approved by the Duke University Institutional Animal Care & Use Committee (A250-04-09).

2.2.2 Morpholino and microinjection

Morpholino antisense oligos were designed and manufactured by Gene Tools (Philomath, OR). The CYP1A morpholino was designed to target *F. heteroclitus* cytochrome P4501A (Morrison et al., 1998)(GenBank, AF026800); the CYP1A MO sequence was 5′-ATGCCATGATGACAACTTTTCTCTG-3′. This morpholino overlaps the start codon (underlined) and acts as a translational blocker. Gene Tools’ standard control morpholino (Control MO, 5′-CCTCTTACCTCAGTTACAATTATA-3′) was used as a morpholino injection control. All morpholinos used were tagged with fluorescein to monitor embryonic incorporation. Morpholinos were diluted to 250 μM injection stocks in RNase free water. Working injection stocks were maintained at 4°C. Morpholino injection stocks were briefly vortexed and centrifuged for 5 minutes prior to use.

The microinjection system consisted of a Nikon SMZ-1500 zoom stereomicroscope (Nikon Instruments Inc., Lewisville, TX), MDI PM 1000 Cell Microinjector (MicroData Instrument Inc., S. Plainfield, NJ), course manipulator (Narishige International USA, Inc., New York, NY), and a three-axis joystick oil hydraulic micromanipulator (Narishige). A PC-10 Puller (Narishige) was used to pull filtered ethanol-rinsed 1.0 mm O.D., 0.5 mm I.D., 10 cm borosilicate glass tubing (Sutter Instrument, Novato, CA) into microinjection needles using a single pull with a heater
output setting of 53. Embryos were placed in a glass Petri dish filled with ASW. For injections, embryos were immobilized by wedging them between a 44.7 μL calibrated “holding” pipette (VWR, West Chester, PA) and the bottom of a glass Petri dish. Eggs were oriented so that the blastodisc was aligned for injection directly into the cell. Morpholino injection volumes ranged from 2-5 nL. Injection pressure was varied to compensate for variable needle opening size, thus insureng an appropriate injection volume. Confirmation of proper injection and full incorporation was performed immediately following injections and at 24 hpf, respectively, using a Leica MZ FLIII fluorescence zoom stereomicroscope (Leica Microsystems Inc., Bannockburn, IL) and GFP/FITC filter. Only normally developing embryos that displayed strong uniform fluorescence were included in subsequent dosing experiments.

2.2.3 Chemicals and dosing

β-naphthoflavone (BNF), benzo[a]pyrene (BaP), ethoxyresorufin, and dimethyl sulfoxide (DMSO) were purchased from Sigma-Aldrich (St. Louis, Mo), and 3,3’,4,4’,5-pentachlorobiphenyl (PCB126) was purchased from AccuStandard (New Haven, CT). BNF, BaP, PCB126, and ethoxyresorufin were all dissolved in DMSO. Embryo dosing was conducted in 25 ppt ASW, with individual embryos being exposed in 10 mL of solution in 20-mL glass scintillation vials (VWR). Ethoxyresorufin was added to all exposure vials for a final concentration of 21 μg/L for subsequent EROD assays. BNF exposures were performed at a final concentration of 10 μg/L. DMSO concentrations were held constant at < 0.002% across all experimental groups. Individual embryos were placed in 10 mL of dosing solution from 24 hpf through deformity screening at 144 hpf. Morpholino duration experiments were conducted using a sliding window approach. In addition to the standard dosing described above, independent groups of embryos
were dosed at 96, 144, 196, or 240 hpf, with CYP1A protein activity assessed 48 hours after dosing. A later larval window was added in view of the fact that morpholino duration exceeded the normal 14 day incubation period. Hatchlings were dosed at 18 dpf and screened at 20 dpf. Although primary dosing was performed with the model AhR agonist BNF, we also performed dosing experiments with both BaP (100 μg/L) and PCB126 (1 μg/L).

2.2.4 EROD assay

CYP1A activity was measured via an in ovo EROD (7-ethoxyresorufin-O-deethylase) assay modified from Nacci et al. (1998). A detailed description of the modified method is provided in Wassenberg and Di Giulio (2004a). This assay quantifies the fluorescence of resorufin, the product of CYP1A metabolism of ethoxyresorufin, which accumulates in the bi-lobed urinary bladder in Fundulus embryos. Upon hatching, resorufin no longer accumulates in the urinary bladder. Therefore, an in vivo EROD assay was used to measure CYP1A activity in Fundulus larvae. The in vivo and in ovo EROD assays differ only in that resorufin fluorescence is quantified in the gall bladder of larvae, rather than the urinary bladder of embryos. EROD assays were performed 48 hours after dosing for all time points except the 24 hpf dosing, for which larvae were screened 72 hours later. The in vivo EROD assay required anesthetization of larvae with MS-222 (Sigma-Aldrich) and immobilization in 3% methylcellulose for proper orientation during quantification (Billiard et al. 2006).

2.2.5 Deformity assessment

Embryos were dosed with BNF at 24 hpf as described previously. Heart deformities were scored blind at 144 hpf. Deformities were scored as 0, 1, or 2.
Common heart abnormalities with BNF exposure were heart elongation and pericardial edema. This scale represented normal (0), mild deformities (1), and severe deformities (2). Representative images for each deformity class have been included in Figure 4 inset. Our distinction between mild and severe deformities is important in that embryos with severe heart abnormalities do not hatch, whereas mild deformities prevent hatching in roughly one half of affected embryos (data not shown). However, we do not have data regarding the long-term survival of *Fundulus* with mild heart abnormalities. Embryos scored as a 1 on the deformity scale have a range of heart abnormalities from moderate orientation changes to slight elongation of the atrium.

**2.2.6 Confirmation of CYP1A morpholino efficacy by in vitro protein synthesis**

The TNT® T7 Quick Coupled Reticulocyte Lysate System (Promega, Madison, WI) was used to synthesize [35S]methionine-labeled proteins as per manufacturer protocols. Briefly, 20 μL of T7 TNT Quick Master Mix was combined with 1 μL of [35S]methionine (> 1,000 Ci/mmol at 10 mCi/mL), 2 μL *Fh*CYP1A-pBluescript II KS(-) (0.5 μg/μL), and adjusted to a final volume of 25 μL with H2O. To test the efficacy of the CYP1A MO, 0.5 μL of a 25 μM stock of the standard control morpholino (Control MO) or CYP1A MO was added to the reaction, for a final concentration of 500 nM. Mixtures were incubated at 30°C for 30 minutes to allow for sufficient *in vitro* transcription of CYP1A. To facilitate translation of the membrane-associated CYP1A protein, 1 μL of canine pancreatic microsomal membranes (Promega) was added to each 11 reaction and incubated at 30°C for an additional 60 minutes. Fifteen microliters of the labeled protein were resolved by SDS-PAGE. Fluorography was used to amplify the signal and
visualize proteins on film. Densitometric analysis was performed with the ImageJ software package (National Institutes of Health, Bethesda, MD).

### 2.2.7 Western blot analysis

Whole embryos were homogenized in 2X sample treatment buffer (15 μL of buffer per embryo) and proteins were further denatured by boiling samples for 3 minutes. Homogenates were centrifuged at 14,000 rpm for 5 minutes. Equivalent volumes of lysate were subjected to SDS-PAGE and blotted to nitrocellulose. Blots were probed with monoclonal antibody 1-12-3 (3 μg/mL) against *Stenotomus chrysops* (scup) CYP1A (Kloepper-Sams et al. 1987; Park et al. 1986). Blots were subsequently probed with goat anti-mouse IgG horseradish peroxidase (Bio-Rad, Hercules, CA) secondary antibody (1:1000 dilution). The blots were visualized on film by enhanced chemiluminescence (ECL Plus ™, GE HealthCare, Piscataway, NJ). Densitometric analysis was performed with the ImageJ software package. The relative densitometric units were determined by normalizing the data from PCB-only and PCB plus CYP1A MO treatments to the values from DMSO treatments after all densitometric values were adjusted for local background.

### 2.2.8 Data analysis

All data were analyzed using SPSS ver. 15 (Chicago, IL). EROD data were not normally distributed according to the Kolmogorov-Smirnov test. As such, EROD data were rank transformed and a non-parametric ANOVA was performed to test for differences among treatments. A Bonferroni-corrected post hoc comparison was conducted to determine which pairwise comparisons were statistically significant. Deformity data were non-parametric in nature and as such were analyzed similarly to our
2.3 Results and discussion

2.3.1 Assessment of injection techniques and morpholino effectiveness

To examine the effectiveness of MOs in Fundulus embryos, we tried using standard zebrafish injection techniques, which involve morpholino injection into the yolk up to the four cell stage (Nasevicius and Ekker 2000). Fluorescence screening of these embryos at 24 hpf revealed that the morpholino failed to fully incorporate into the embryo; the vast majority of fluorescence was observed distributed throughout the yolk. In fact, fluorescence remained in the yolk throughout the 14-16 day incubation period. Early hatchlings (18 dpf) still had observable fluorescence in what was left of their yolk, clearly showing that yolk-injected morpholino was not efficiently incorporated into the developing embryo. The results of EROD assays used to test for functional knockdown of CYP1A activity are shown in Figure 1. Embryos injected into the yolk with the CYP1A MO showed a slight, nonsignificant reduction in BNF-induced CYP1A activity relative to non-injected embryos.

In light of these results showing that the CYP1A MO was ineffective when injected into the yolk, we altered our protocols to inject MO directly into the blastomere, similar to the technique used to inject MO into Xenopus embryos (Heasman et al. 2000). Injections were performed on embryos up to the 4-cell stage, with 4-cell embryos being injected into two separate cells. When morpholino was injected directly into the cell, it
fully incorporated into the developing embryo. Fluorescence screening at 24 hpf was used to confirm normal embryonic development and full morpholino incorporation. Individuals with evenly distributed but minimal fluorescence were evaluated separately as “low incorporation” embryos representing a reduced-dose morpholino group.

CYP1A MO function was tested in cell-injected embryos via an *in ovo* EROD assay. EROD assay results for high-incorporation embryos demonstrate that the CYP1A MO significantly reduced the induction of CYP1A activity in 4 dpf BNF-exposed embryos (Fig. 1). CYP1A activity in BNF-exposed morphants was reduced by 70%, relative to non-injected or control morpholino-injected embryos exposed to BNF. This level of knockdown is critical, given that previous work has shown that greater than 50% knockdown of CYP1A activity is associated with the synergistic teratogenicity of some PAH combinations (Wassenberg and Di Giulio 2004a). Induction of EROD activity was also significantly reduced in low incorporation embryos relative to controls; however, the knockdown was not as efficient as seen in the high-incorporation embryos (Fig. 1).

### 2.3.2 Confirmation of CYP1A morpholino efficacy by *in vitro* and *in vivo* analysis

Reduced EROD induction in CYP1A MO-treated embryos suggested that the MO was effective at reducing CYP1A translation in the embryos. To directly assess the effectiveness of the MO, we measured its effect on CYP1A protein synthesis *in vitro* and *in vivo*.

A TNT® assay system was used to confirm the MO efficacy at inhibiting the *in vitro* translation of CYP1A. Membrane associated proteins do not perform optimally in the *in vitro* TNT reactions (see manufacturer protocols); therefore, microsomal membranes were added to the reactions after the initial transcription phase to facilitate
translation. Although the protein yield was low compared to non-membrane associated proteins, detectable amounts of CYP1A protein were observable by SDS-PAGE of \(^{[35]}\)Met-labeled proteins (Figure 2). Based on densitometric analysis, the CYP1A MO was able to inhibit translation by approximately 98% compared to the No MO and Ctrl MO control treatments.

Western blot analyses were performed to confirm the knockdown of endogenous CYP1A protein in *Fundulus* embryos at various developmental time points. CYP1A protein was not detectable in initial experiments with BNF treated embryos (4 and 6 dpf) in which lysate from 6 embryos was loaded in each lane. Additional gels with lysate from 12 embryos per lane allowed for the minimal detection of CYP1A in both DMSO controls and BNF treatments but were not sensitive enough to detect any changes in protein levels (data not shown). To optimize detection capabilities, *Fundulus* embryos were exposed to a stronger AHR agonist (PCB126). While a strong induction of CYP1A was observed in 8 dpf embryos exposed to PCB126, CYP1A protein was barely detectable in PCB126-exposed embryos injected with the CYP1A MO (Figure 3A). To further assess the degree of inhibition over time, western blot analyses for CYP1A were performed on PCB126-treated embryos at 6, 8, and 12 dpf. Significant decreases in induced CYP1A expression (79-86%) were observed in embryos injected with the CYP1A MO at these time points (Figure 3B).

### 2.3.3 Phenotypic evaluation of morpholino function

The functionality of the CYP1A MO was also tested in terms of its ability to enhance BNF-induced embryotoxicity. BNF has been shown to cause cardiovascular deformities in developing fish embryos at concentrations as low as 10 μg/L (Billiard et al. 2006). It has also been demonstrated that reducing CYP1A protein activity, via chemical
inhibition or morpholino knockdown, during BNF exposures results in increased severity of cardiovascular developmental abnormalities in fish embryos (Billiard et al. 2006; Wassenberg and Di Giulio 2004a). We confirmed that 10 μg/L BNF is sufficient to induce cardiovascular abnormalities of mild to intermediate severity in Fundulus embryos (Fig. 4). More importantly, the morpholino injected embryos revealed a dramatic increase in the number and severity of cardiovascular abnormalities in BNF-dosed, but not control, groups. The control morpholino had no observable effect on the number or severity of deformities in Fundulus embryos exposed to BNF.

2.3.4 Morpholino functional duration

Functional duration was tested by dosing with BNF and conducting EROD assays on embryos at increasing intervals following injection of the CYP1A MO. In ovo assays were performed at two day intervals from 4-12 dpf, and in vivo assays were performed on newly hatched Fundulus at 20 dpf.

Injection of CYP1A-MO at the 1-4-cell stage caused a persistent reduction in EROD induction by BNF, with 70% decrease at 4-dpf and a slow recovery of inducibility (Fig. 5). In newly hatched Fundulus larvae (20 dpf), BNF-induction of EROD activity was still reduced as compared to control (non-injected) larvae exposed to BNF. A curve estimation was performed to determine which model best fit the data (Fig. 5). A logarithmic model provided the best estimate of mean EROD activity as a function of time. The logarithmic model (Fig. 5) yielded a 10 dpf estimate for the point at which CYP1A activity in MO-injected embryos, as measured via EROD assay, was back to 50% of its normal response, with complete recovery predicted around day 58.

The longevity of morpholino-induced knockdown is a critical concern of scientists using morpholino techniques to study development or processes that only occur after a
certain developmental stage. Since development occurs much more slowly in Fundulus than in zebrafish, there was some concern that the duration of morpholino effects in Fundulus might be insufficient for practical use of this technique. However, these data clearly demonstrate that significant morpholino-induced knockdown lasts beyond hatching (~14 dpf), and thus persists throughout the development of all major organ systems. In combination, the EROD (Fig. 5) and western blot (Fig. 3) data provide a convincing argument for the long duration of morpholino-induced knockdown in Fundulus. Thus, it appears that morpholinos will be just as useful in Fundulus as they are in zebrafish.

2.4 Conclusions

We have conclusively shown, via molecular and phenotypic methods, that MO technologies can be adapted to the teleost estuarine environmental model, Fundulus heteroclitus. The development and evaluation of MO technologies in Fundulus has revealed several critical changes that must be made when working with this species, relative to zebrafish methods. The hardness of the chorion and high internal pressure make the use of an agarose ramp for injection ineffective. A holding pipette seems to be necessary to adequately immobilize Fundulus eggs for proper microinjection, because intracellular injections are necessary for proper incorporation of morpholino and thus proper alignment of the blastomere is critical. This has the consequence of slowing down the injection process. However, time is not a critical concern with Fundulus injections, given the slower rate of division. The hardness of the chorion is also important in this regard, as non-perpendicular injections often result in broken needles.
We have also shown that the duration of MO-mediated gene knockdown in *Fundulus* is comparable to that achieved in zebrafish, in terms of developmental stages during which MO knockdown persists. Gene knockdown continues well into the larval stage, as seen in zebrafish. One additional point to note with regard to morpholino duration is that the gene we selected for evaluation of MO technologies in *Fundulus* (CYP1A) is highly inducible (Handley-Goldstone et al. 2005; Timme-Laragy et al. 2007). As such, it provides a very conservative test of morpholino duration.

The development of MO technologies in *Fundulus* will allow researchers to more fully investigate a number of interesting aspects of killifish, including their adaptation(s) to contaminated environments. The methodology developed here also paves the way for complementary methods including RNA rescue, and siRNA, all of which will help promote *Fundulus* as the premier teleost model in environmental biology (see review by Burnett et al. 2007).
Figure 1: CYP1A activity as measured via *in ovo* EROD assay for both uninjected and morpholino-injected *Fundulus* embryos.

EROD data are presented as percent activity relative to non-injected BNF control. BNF exposures were conducted at a concentration of 10 μg/L, and DMSO was used as a vehicle control. EROD activity was measured in 4 dpf embryos. Letters above bars represent statistically significant groupings ($p \leq 0.05$; Bonferroni corrected non-parametric ANOVA). Cell and yolk denotations represent location of morpholino injections. Low inc. represents cell injected embryos that had incomplete or low incorporation of the morpholino.
Figure 2: Inhibition of *in vitro* translation of CYP1A by CYP1A MO.

The ability of a CYP1A MO targeting the translation start site for CYP1A to inhibit translation was tested via an *in vitro* translation assay using the TNT® Coupled Reticulocyte System (Promega). The standard control MO (Ctrl MO) from Gene Tools was used to detect any non-specific interference in the transcriptional/translational process. A final MO concentration of 500 nM was used in each of the reactions. Densitometric analysis was used to calculate percent inhibition of the CYP1A MO compared to No MO (97.7%) or Ctrl MO (98.8%).
The ability of a CYP1A start site MO to inhibit translation *in vivo* was assessed by western blot analysis using lysates from pooled embryos. A) Representative CYP1A western blot for 8 day post fertilized embryos exposed to PCB126 (1 μg/L). Lysate from 4 embryos was loaded in each lane. Equal protein loading was confirmed by Ponceau staining of nitrocellulose membrane and Coomassie blue staining of SDS-PAGE gels after transfer. B) Densitometric analysis of CYP1A western blots for three different time points during *Fundulus* embryo development. Percent reduction of CYP1A induction at each time point: 6 dpf, 79%; 8 dpf, 82%; 12 dpf, 86%. Lysate from 6 embryos was loaded in each lane. For each time point, data from PCB-only and PCB+CYP1A-MO-treated embryos were normalized to the value for the DMSO control.
Figure 4: Mean deformity scores for both uninjected and morpholino injected *Fundulus* embryos.

Increasing deformity score represents increasing severity of deformities. BNF exposures were conducted at a concentration of 10 μg/L, and DMSO was used as a vehicle control. Letters above bars represent statistically significant groupings (*p* ≤ 0.05; Bonferroni corrected non-parametric ANOVA). Inset contains representative images for each deformity class. Hearts have been traced in white for visualization purposes.
Figure 5: Mean CYP1A activity in CYP1A MO injected embryos in response to BNF exposure (10µg/L), relative to the non-injected BNF exposed embryos.

The data demonstrate the functional duration of the morpholino in *Fundulus* embryos. DMSO was used a vehicle control. Error bars represent standard error of the mean (SEM). Sample sizes are included at the base of each bar. The logarithmic curve that best explains the data is also provided.
3. AHR2 mediates cardiac teratogenesis of polycyclic aromatic hydrocarbons and PCB-126 in Atlantic killifish (Fundulus heteroclitus)

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3.1 Introduction

The Atlantic killifish or mummichog (Fundulus heteroclitus), found in estuaries along the Atlantic coast of North America from Newfoundland to Florida (Shute 1980), has a variety of attributes that make it an important field and laboratory toxicology model (Burnett et al. 2007). Fundulus are tolerant of significant variation in environmental conditions, including salinity, temperature, oxygen, and pH (Dunson et al. 1993; Nordlie 2006; Smith and Able 2003; Stierhoff et al. 2003; Wood and Marshall 1994). Because of their wide distribution and abundance, Fundulus are important components of estuarine ecosystems (Kneib 1986a; Meredith and Lotrich 1979). However, despite wide distribution, individual Fundulus have relatively small home ranges (Lotrich 1975) and are ideal for studying the impacts of local contamination and other stressors (Burnett et al. 2007). Fundulus have been used in studies of a wide variety of contaminants including toxicity of polycyclic aromatic hydrocarbons (PAHs) (e.g. Wassenberg and Di Giulio 2004a), polychlorinated biphenyls (PCBs) (e.g. Jonsson et al. 2007; Nacci et al. 1999), metals (e.g. Roling et al. 2006), and pesticides (e.g. Fortin et al. 2008); Fundulus have also been used to study processes such as endocrine disruption (e.g. Kelly and Di
Giulio 2000), environmental carcinogenesis (e.g. Vogelbein et al. 1990; Wills et al. accepted), and evolutionary adaptation (e.g. Schulte et al. 2000). Additionally, individual Fundulus populations have been identified that are resistant to a number of anthropogenic stressors, including mercury (Weis and Weis 1984), 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) (Prince and Cooper 1995b), PCBs (Meyer and Di Giulio 2002; Nacci et al. 1999), and PAHs (Meyer et al. 2002; Ownby et al. 2002).

Of the Fundulus populations adapted to environmental contaminants, most are resistant to the acute toxicity and teratogenic effects caused by PCBs and PAHs, which are known to interact with the aryl hydrocarbon receptor (AHR) pathway. PCBs and dioxin have long been known to cause a suite of deformities collectively known as “blue sac disease” (reviewed in Peterson et al. 1993) and some PAHs and PAH mixtures also induce similar cardiac deformities (Billiard et al. 1999; Wassenberg and Di Giulio 2004a). Furthermore, alteration of the AHR pathway appears to be an important component of adaptation to these contaminants. Notably, these populations exhibit a marked recalcitrance to induction of cytochrome P4501A (CYP1A) in response to exposure to a variety of AHR agonists (Arzuaga and Elskus 2002; Bello et al. 2001; Elskus et al. 1999; Meyer et al. 2002; Nacci et al. 1999; Prince and Cooper 1995a; Van Veld and Westbrook 1995; L. P. Wills et al. 2010).

The AHR mediates responses to halogenated hydrocarbons and PAHs, such as CYP1A induction, by recognizing and binding these ligands and driving gene expression (Schmidt and Bradfield 1996). Following activation by ligand binding, the AHR dimerizes with the aryl hydrocarbon receptor nuclear translocator (ARNT); this complex associates with specific DNA sequences known as xenobiotic response elements (XREs) or AHR response elements (AHREs) to induce transcription of a number of genes, including
components of Phase I and II metabolism (i.e. CYP1A, 1B1, 1C1; some glutathione S-
transferases (GSTs); NADP(H):oxidoreductase; UDP-glucuronosyltransferase (UGT)
(Nebert et al. 2000). The AHR complex also induces transcription of a factor called the
aryl hydrocarbon receptor repressor (AHRR), which down-regulates the pathway by
competing with the AHR-ARNT complex for XREs (Karchner et al. 2002).

In order to further increase the utility of Fundulus for the study of aryl
hydrocarbon toxicity and adaptation, better understanding of the components of the
molecular pathways governing PAH response in Fundulus is crucial. The AHR pathway
appears to be well-conserved among mammals and lower vertebrates (Hahn 2002;
Hahn et al. 2006) and a variety of genes in the AHR pathway have been characterized in
Fundulus. Due to gene and genome duplication events, multiple AHRs have arisen and
been identified in fish, including two in Fundulus, AHR1 and AHR2 (Andreasen et al.
2002; Karchner et al. 1999; Tanguay et al. 1999). ARNT and the AHRR have also been
identified in Fundulus and other fish (Andreasen et al. 2002; Evans et al. 2005; Karchner
et al. 2002; Powell et al. 1999). In zebrafish, AHR2 mediates teratogenesis induced by
PAH mixtures (Billiard et al. 2006), 3,3’,4,4’,5-pentachlorobiphenyl (PCB-126) (Jonsson
et al. 2007), and TCDD (Prasch et al. 2003b; Teraoka et al. 2003). Currently, it is
unclear whether AHR1, AHR2, or both perform this role in Fundulus. In vitro, both AHR1
and AHR2 have been demonstrated to bind TCDD and the model PAH β–
naphthoflavone (BNF) and to activate a luciferase reporter upon TCDD binding
(Karchner et al. 2002; Karchner et al. 1999).

In the current study, we used a morpholino gene knockdown approach to
investigate the roles of AHR1 and AHR2 in mediating cardiac teratogenesis induced by
PAHs and PCB-126 in Fundulus heteroclitus. We confirmed knockdown of AHR1 and
AHR2 protein by Western blot analysis. Our findings lead us to conclude that AHR2 and not AHR1 is the primary mediator of this response in *Fundulus*. We also verified the lack of effect of AHR1 knockdown using a second morpholino directed at a splice junction, which allowed confirmation of knockdown via RT-PCR.

### 3.2 Materials and methods

#### 3.2.1 Fish

Adult killifish were collected with minnow traps on King’s Creek, a relatively-uncontaminated tributary of the Severn River which feeds into the lower Chesapeake Bay in southeastern Virginia (37° 17’52.4”N, 76° 25’31.4”W). In the lab, fish were maintained at 23-25 °C, in 20‰ artificial sea water (ASW; Instant Ocean, Foster & Smith, Rhinelander, WI, USA), with a 14:10 light:dark cycle, and were fed pelleted fish feed (Aquamax © Fingerling Starter 300, PMI Nutritional International, LLC, Brentwood, MO, USA). Eggs were collected by manual spawning of females and fertilized *in vitro* by expressing sperm from males directly into a beaker containing eggs in ASW. Eggs were set aside for approximately one hour for fertilization, then washed briefly with 0.3% hydrogen peroxide in ASW. Eggs were then rolled on durable wet paper towels to remove mucous and debris from the chorion (Matson et al. 2008a). Adult care and reproductive techniques were non-invasive and approved by the Duke University Institutional Animal Care & Use Committee (A234-07-08).

#### 3.2.2 Morpholino and microinjection

Morpholino antisense oligos were designed and manufactured by Gene Tools (Philomath, OR, USA). Translation blocking morpholinos were designed against *F.*
heteroclitus AHR1 (GenBank, AF024591) and AHR2 (GenBank, U29679) (Karchner et al. 1999); the AHR1 MO sequence was 5'-TTCTCCTCTTGCGTCCAGCATACAT-3' and the AHR2 MO sequence was 5'-GGTTCCACAGACATCTTGCCGCTCGG-3'. Additionally, a splice-junction morpholino was used to further demonstrate the efficient knockdown of AHR1. The morpholino targeted the exon 8-intron 8 boundary (S. Karchner, personal communication) and began at nucleotide 1004 (counting started with ATG) and continued into the intron (AHR1-spl MO, 5'-GACCTCCATTTAATATGCACTTACT-3'). Splice-junction morpholinos cause aberrant splicing of pre-mRNA; unlike translation blocking morpholinos, knockdown via splice-junction morpholinos can be quantified using PCR. This morpholino targeted the ligand binding domain of AHR1, which should result in a non-functional receptor. Finally, Gene Tools’ standard control morpholino (control-MO, 5'-CCTCTTACCTCAGTTACAATTTATA-3') was used as a control for effects of morpholino injection. All morpholinos were tagged with fluorescein to visualize incorporation. Individual morpholinos were prepared as 250 µM injection stocks in RNase-free water and maintained at 4 °C. When injected in combination, the concentration of each individual morpholino was halved so that the total concentration of morpholino was maintained at 250 µM. Injection stocks were briefly vortexed and centrifuged for two minutes prior to use. Injection volume was approximately 4 nL, yielding morpholino doses of 1 pmol/embryo (250 µM) or 0.5 pmol/embryo (125 µM).

Microinjection was performed as described previously (Matson et al. 2008a) using a system consisting of a Nikon SMZ-1500 zoom stereomicroscope (Nikon Instruments, Inc, Lewisville, TX, USA), MDI PM 1000 Cell Microinjector (MicroData Instrument, Inc., S. Plainfield, NJ, USA), course manipulator (Narishige International USA, Inc., New York, NY, USA), and a three-axis joysick oil hydraulic micromanipulator.
Borosilicate glass tubing (1.0 mm o.d., 0.5 mm i.d.) was pulled into microinjection needles on a PC-10 Puller (Narishige) with heater output set at 53. All injections were into a cell during the 1-4 cell stages. Embryos were maintained overnight in an incubator at 27 °C; confirmation of morpholino incorporation and proper early development was performed at 22-24 hours post fertilization (hpf) using a Leica MZ FLIII fluorescence zoom stereomicroscope (Leica Microsystems Inc., Bannockburn, IL, USA) with GFP/FITC filter. Only normally developing embryos with strong, uniform morpholino incorporation were used.

### 3.2.3 Chemicals and dosing

β-naphthoflavone (BNF) and dimethyl sulfoxide (DMSO) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Benzo[k]fluoranthene (BkF) and benzo[a]pyrene (BaP) were purchased from Sigma-Aldrich (St. Louis, MO, USA) or from Absolute Standards, Inc. (Hamden, CT, USA) and 3,3',4,4',5-pentachlorobiphenyl (PCB-126) was purchased from AccuStandard (New Haven, CT, USA). Stocks were prepared by dissolving BNF, BkF, BaP or PCB-126 in DMSO. All chemicals used in buffers for Western blotting or for gel electrophoresis were purchased from Sigma-Aldrich.

Following manual spawning and microinjection as described previously, morpholino-injected and non-injected embryos were dosed individually in 20-mL glass scintillation vials (VWR, West Chester, PA) beginning at 24 hpf. Exposures were conducted in 10 mL of dosing solution with final concentrations of 10 µg/L BNF, 300 µg/L BkF, 1 µg/L PCB-126, and 100 µg/L BaP. All dosing solutions were made in 20 ppt ASW. Control embryos were exposed to DMSO at a concentration (v/v) equal to that in the dosed group; DMSO concentrations were held at <0.03% across all treatments. Embryos were maintained in dosing solution in the incubator at 27 °C from 24 hpf until
the appropriate time for the given analysis. All exposures consisted of at least three experimental replicates with n≥10 embryos per treatment group unless otherwise noted.

3.2.3.1 Dosing for deformities

To determine the role of each AHR in the cardiac deformities caused by several AHR agonists, morpholino-injected (control MO, AHR1 MO, AHR2 MO, or combination of AHR1&2 MO) and non-injected embryos were exposed as described previously with deformity assessment occurring at 144 hpf. In a second experiment, non-injected embryos and embryos injected with an AHR1 splice morpholino (AHR1-spl MO) or AHR2 MO were dosed only with DMSO, 10 µg/L BNF, or 300 µg/L BkF and assessed in the same manner.

3.2.3.2 Dosing for Western blot analysis

Morpholino-injected (AHR1 MO, AHR2 MO, and combination of AHR1&2 MO) and non-injected embryos were exposed to 10 µg/L BNF at 24 hpf and flash frozen in groups of ten at 144 hpf. Because exposure at 24 hpf may have resulted in induction of AHR protein expression and subsequent degradation prior to protein analysis at 144 hpf (see review by Pollenz 2002), a second experiment was conducted with dosing beginning later; morpholino-injected (AHR1 MO, AHR2 MO, and control MO) and non-injected embryos were exposed to 100 µg/L BaP at 96 hpf and flash frozen in groups of ten at 144 hpf. Embryos were stored at -80 °C until analysis.

3.2.3.3 Dosing for reverse transcription PCR

Finally, to further demonstrate efficient knockdown of AHR1 and confirm the results observed with the translation-blocking morpholino, AHR1-spl MO-injected, AHR2 MO-injected, and non-injected embryos (n≥12 embryos per treatment group) were dosed
individually with 300 μg/L BkF at 24 hpf. At 48 hpf, embryos were paired, placed in 50 μL RNAlater (Qiagen, Valencia, CA, USA), and flash frozen for reverse transcription PCR (RT-PCR) analysis. Embryos were stored at -80 °C until analysis.

3.2.4 Deformity assessment

Following morpholino injection and dosing, cardiac teratogenesis was scored blind at 144 hpf under light microscopy. Deformity severity was scored as a 0 (normal), 1 (moderate deformity), or 2 (severe deformity) as described previously (Matson et al. 2008a). Observed heart abnormalities consisted primarily of heart elongation, improper atrial-ventricular alignment, and pericardial edema. Embryos scored as a 1 on the deformity scale have a range of heart abnormalities from moderate changes in atrial-ventricular alignment to misalignment of the chambers accompanied by elongation of the atrium and sinus venosus (the inflow vessel of the heart). All embryos scored as a 1 maintained some blood flow as observed by light microscopy. Embryos scored as a 2 on the deformity scale exhibited severe misalignment and elongation of the atrium and ventricle or complete alteration of the heart to a single, elongated tube. Many of the individuals scored as a 2 did not have any apparent blood flow, but some exhibited severely reduced blood flow. Representative images (144 hpf) of each deformity class are shown in Figure 1.

3.2.5 Confirmation of morpholino efficacy by Western blot analysis

After thawing on ice, samples were homogenized in homogenization buffer (300 mM sucrose, 50 mM KCL, 50 mM NaCl, 8 mM EGTA, 30 mM HEPES, pH 7.5) with 1 mM phenylmethanesulfonyl fluoride, 1 μg/mL leupeptin, and 1 μg/mL aprotinin with a hand-held tissue homogenizer. The homogenate was centrifuged at 500 g for 15 min at
4 °C to remove the chorion and non-homogenized tissues. Supernatant was moved to a new tube and spun at 18,000 g for 15 min at 4 °C to remove the nuclei and mitochondria. The supernatant was aliquoted and stored at ~80 °C till further analyses. SDS-Page and Western blot analysis were performed as described by Jung and Di Giulio (2010) for CYP1A, with the following differences for AHR analysis. AhR1 (1:50 dilution) and AhR2 (1:500 dilution) polyclonal antibodies raised against the killifish (Merson et al. 2006) were used as primary antibody, and goat anti-rabbit IgG horseradish peroxidase antibody (1:10,000 dilution, Jackson laboratory, Bar Harbor, ME, USA) was used as secondary antibody. Blots were visualized on X-ray film by enhanced chemiluminescence (SuperSignal® West Pico Chemiluminescence Substrate, Thermo Scientific, Rockford, IL, USA).

3.2.6 Confirmation of AHR1 splice-MO efficacy by reverse transcription PCR analysis

Paired embryos were thawed on ice, homogenized with RNA-Bee for 30 seconds, and mRNA was extracted by modified phenol-chloroform extraction according to the RNA-Bee protocol (Tel-Test, Inc., Friendswood, Texas, USA). RNA quantity and quality was analyzed using a NanoDrop ND-1000 (NanoDrop Technologies, Wilmington, DE). Using the Omniscript cDNA synthesis kit for Reverse Transcription (Qiagen), cDNA was prepared according to manufacturer’s instructions using 500 ng of RNA, random hexamers, and RNAse inhibitor, and carried out in a Biometra T1 thermocycler (Göttingen, Germany) for 1 h at 37°C. RT-PCR was performed in a 25 µL reaction containing 62.5 ng template, 12.5 µL AmpliTaq Gold PCR master mix (Applied Biosystems, Foster City, CA, USA), 9 µL distilled H₂O, and 0.5 µL each of 10 µM primer. Thermocycler conditions were 5 min at 95°C, 35 cycles of 15 s at 95°C, 15 s at 58°C,
and 4 min at 72°C, followed by 4 min at 72°C. Two fragments were amplified from each sample. To amplify the region targeted by the splice morpholino, the forward primer was located in exon 7 (5’-AACATCCAGGGCAGGCTCAAGTTT-3’) and the reverse in exon 9 (5’-CATTCCAGCGATTCTTTTAGTGAG-3’). A reference fragment from a region untargeted by the morpholino was amplified for comparison with the forward primer located in exon 2 (5’-ACCAAGTCCAATCCGTCCAAACGA-3’) and the reverse in exon 3 (5’-GCTGCTCCATTCCACTGTTCA-3’). All reactions were performed in triplicate. Gel electrophoresis of 10 µL of PCR product was performed in 2% agarose gels stained with ethidium bromide. Gel images were captured with a Bio-Rad Molecular Imager Gel Doc (Bio-Rad, Hercules, CA, USA).

### 3.2.7 Statistical analysis

All analyses were performed using JMP 8.0 (SAS Institute Inc, Cary, NC, USA). All data were analyzed by non-parametric analysis of variance (ANOVA) controlling for morpholino and dose, followed by least square means (LSMeans) procedures. As stated previously, experiments were replicated a minimum of three times; no differences between experimental replicates were observed for any test. Tukey-adjusted pairwise comparisons were conducted to determine differences between groups. Statistical significance was accepted at $p \leq 0.05$ for all tests.

### 3.3 Results

#### 3.3.1 Deformity assessment

Targeted morpholino knockdown of AHR1, AHR2, or the combination was used to determine the role of each of the *Fundulus* AHRs in mediating the cardiac deformities
induced by PAHs and PCB-126. Morpholino-injected and non-injected embryos were exposed to BNF, BkF, and PCB-126 as described previously and the effect of gene knockdown on response was assessed.

We have previously demonstrated that 10 µg/L of the model PAH BNF induces cardiac deformities of mild to intermediate severity in naïve Fundulus (Matson et al. 2008a) and this was confirmed in this study. BNF-exposed embryos generally exhibited a heart phenotype of misalignment of the atrium and ventricle; this was accompanied by some overall lengthening of the heart, reduction of the apparent size of the chambers, and pericardial edema (see representative images Fig 1). The mean deformity scores of BNF-exposed embryos are shown in Figure 2A. As expected, non-injected embryos exposed to BNF displayed a mean deformity score of 0.59±0.04. Embryos injected with AHR1 MO and dosed with BNF had a mean deformity score (0.71±0.06) that was not statistically different from that of non-injected, BNF-dosed embryos (p=0.0962). In contrast, AHR2 knockdown resulted in a mean deformity score (0.15±0.04) in BNF-dosed embryos that was approximately 21% of that for non-injected, BNF-dosed embryos. Additionally, the deformity score for AHR2 MO-injected, BNF-dosed individuals was not statistically different from that of the DMSO-dosed non-injected control group (p=0.1089). Embryos with both AHR1 and AHR2 knocked down had a mean deformity score of 0.37±0.10, which was intermediate between the non-injected BNF treatment and the AHR2 knockdown BNF treatment, but was not statistically different from the control morpholino BNF-dosed group (p=0.2155). DMSO-dosed morpholino treatments were not statistically different from the non-injected DMSO treatment (0.06±0.03); these groups are not shown in the figure for simplicity.
Additionally, the severity of cardiac deformities in BNF-dosed individuals injected with control MO was no different from non-injected, BNF-dosed embryos ($p=0.4620$).

To determine the effect of AHR knockdown on response to a PAH found in the environment, embryos were exposed to BkF at 300 µg/L. At this concentration, BkF induces a much more severe deformity phenotype in naïve embryos than does 10 µg/L BNF. Typically, there was significant misalignment of the atrium and ventricle, accompanied by lengthening of the heart and pericardial edema; individuals ranged in severity from a 1 to 2 on the deformity scale (see Figure 1). The results of BkF exposure are shown in Figure 2B. Non-injected embryos exposed to BkF had a mean deformity score of 1.23±0.08. Control MO-injected embryos exposed to BkF had a slightly lower mean deformity score (1.08±0.14), but were not statistically different from non-injected individuals ($p=0.9964$). AHR1 MO-injected embryos exhibited a mean deformity score (0.86±0.09) that was approximately 70% of that of the non-injected BkF dosed control group. However, the mean deformity score of AHR1 MO-injected embryos was not statistically different from that of the non-injected or control MO-injected individuals dosed with BkF ($p=0.0782$ or $p=0.9481$, respectively). In contrast, AHR2 knockdown resulted in a BkF-induced mean deformity score of 0.33±0.07, which was significantly lower than that of both the non-injected and control MO-injected BkF-dosed groups ($p<0.0001$ and $p=0.0002$, respectively). The BkF-induced deformity score for AHR2 MO-injected embryos was approximately 27% of the non-injected group and 31% of the control MO-injected group. BkF-dosed embryos with both AHR1 and AHR2 knocked down had a mean deformity score of 0.68±0.09, which was statistically lower than the deformity score for non-injected embryos dosed with BkF ($p=0.0002$), but not those with injected with control MO ($p=0.3076$). Additionally, this reduction was not as great as that
for the individuals with only AHR2 knocked down. Again, DMSO-dosed embryos injected with any morpholino were not statistically different (\( p > 0.05 \)) from the non-injected DMSO treatment (0.07±0.05); these groups are not shown in Figure 2B for simplicity. Additionally, injection of control MO did not affect the severity of cardiac deformities compared to non-injected embryos in BkF-dosed treatments (\( p = 0.9964 \)).

To investigate the role of the *Fundulus* AHRs in mediating teratogenesis caused by a halogenated aromatic hydrocarbon, embryos were exposed to 1 µg/L PCB-126. At this concentration, greater than 70% of dosed, non-injected embryos are scored as a 2 on the deformity scale. Typically, the hearts are greatly elongated tubes with no recognizable chambers. This is accompanied by little to no observable blood flow and a large volume of pericardial edema (see Figure 1). The results of PCB-126 exposure are shown in Figure 2C. Both non-injected and AHR1 knockdown embryos dosed with PCB-126 exhibited severe deformities (1.80±0.12 and 1.76±0.11, respectively), which did not differ statistically (\( p = 0.9999 \)). AHR2 knockdown resulted in a statistically reduced (\( p < 0.0001 \)) mean deformity score of 0.96±0.11 after PCB-126 exposure, which was about 53% of that for the non-injected individuals. Knockdown of both AHR1 and AHR2 resulted in a mean deformity score of 1.00±0.16 in PCB-126 dosed embryos, which was a statistically significant reduction compared to the non-injected, PCB-126-dosed group (\( p = 0.0027 \)). However, knockdown of AHR1 and AHR2 was not different from AHR2 knockdown alone in protecting from PCB-126 exposure (\( p = 0.9999 \)).

Because AHR1 knockdown did not appear to result in deformity rescue, a second morpholino targeting AHR1 was utilized to confirm that AHR1 knockdown was achieved and was not protective from the cardiac teratogenesis. The use of a splice-junction morpholino allows confirmation of knockdown by PCR. The results of this experiment,
shown in Figure 3, were similar to the BNF and BkF exposures with translation-blocking morpholino described previously. Non-injected and AHR1-spl knockdown embryos dosed with BNF had mean deformity scores that were not statistically different ($p=0.9999; 0.64\pm0.08$ and $0.62\pm0.09$, respectively). AHR2 knockdown again resulted in a statistically significant protection from BNF compared to non-injected embryos ($p=0.0100$; mean deformity score $0.18\pm0.10$). When dosed with BkF, non-injected and AHR1-spl knockdown embryos exhibited deformity scores that were not statistically different ($p=0.9999; 1.14\pm0.07$ and $1.16\pm0.06$, respectively), whereas AHR2 knockdown was again protective from BkF ($p<0.0001$; mean deformity score $0.31\pm0.12$).

### 3.3.2 Confirmation of morpholino efficacy by Western blot analysis

To confirm morpholino knockdown, AHR1 and AHR2 protein was measured in two exposure experiments. In the first, morpholino-injected and non-injected embryos were dosed individually with 10 µg/L BNF or DMSO at 24 hpf and flash frozen in pools of ten at 144 hpf. In the second, embryos were dosed individually with 100 µg/L BaP at 96 hpf and flash frozen in pools of ten at 144 hpf.

As shown in the representative gel image (Figure 4) for BNF exposure, the greatest expression of AHR1 and AHR2 protein was measured in DMSO control treatments and appeared to decrease in BNF treatments. Compared to the non-injected DMSO-dosed group, AHR1 MO injection resulted in a reduction of AHR1 protein; this reduction was somewhat less apparent for comparison of the BNF-dosed groups due to the lower overall expression of AHR protein. In BNF-dosed treatment groups, there was a reduction of AHR2 protein in the AHR2 MO-injected group and the group injected with both AHR1 and AHR2 morpholinos. Additionally, induction of CYP1A protein expression
by BNF was observed in the non-injected and AHR1 MO-injected groups, but was prevented in the groups injected with AHR2 MO or both AHR1 MO and AHR2 MO. Because of the reduced AHR protein levels in individuals dosed with BNF at 24 hpf and analyzed at 144 hpf, a second experiment was conducted with BaP wherein embryos were dosed later (96 hpf) and flash frozen only 48 hours later (144 hpf). As shown in the representative gel image (Figure 5), this resulted in similar levels of AHR protein in both DMSO control and BaP-dosed treatment groups. Compared to the other treatments, AHR1 protein was reduced in the two AHR1 MO groups. Additionally, AHR2 protein was greatly reduced in the two AHR2 MO groups compared to the other treatments.

3.3.3 Confirmation of AHR1 splice-MO efficacy by reverse transcription PCR analysis analysis

Knockdown of AHR1 with a splice morpholino was demonstrated with RT-PCR of AHR1 cDNA (Figure 6). The image is representative of a minimum of five pairs of embryos per group. Knockdown was demonstrated by comparing amplification of a cDNA fragment in the region targeted by the morpholino (exon 7 to exon 9) to amplification of a fragment in exons 2 and 3 that is unaffected by the splice morpholino. In both DMSO and BkF dosed groups, non-injected embryos exhibited strong amplification of a band in both regions of the gene. In contrast, AHR1-spl MO injection resulted in a loss of the band in the morpholino targeted region, but no loss of the band in exon 2.
3.4 Discussion

*Fundulus* are an important and highly-studied model in environmental toxicology, in part due to identification of multiple populations adapted to contamination from the AHR agonists PAHs, PCBs, and dioxin. Because populations adapted to these contaminants demonstrate a recalcitrant AHR pathway, it is logical to suspect that alteration of the AHR pathway plays a crucial role in circumventing toxicity. However, it was previously unclear what role AHR1 and AHR2 played in mediating the toxic effects of these contaminants in *Fundulus*. Karchner et al. (1999) identified two AHRs in *Fundulus*; both AHR1 and AHR2 were able to bind TCDD with high affinity fashion and were able to bind DNA in the presence of ligand. Subsequently, they showed that both AHR1 and AHR2 also bound BNF (Karchner et al. 2002). They further demonstrated that both AHR1 and AHR2 could mediate TCDD-induced expression of luciferase *in vitro*. Collectively, these results indicated that both AHR1 and AHR2 had the potential to mediate effects of AHR agonists in *Fundulus*. It has been suggested that the presence of two AHRs in fish could contribute to their high degree of sensitivity to AHR agonists (Karchner et al. 1999). However, results from the current study indicate that AHR2 is the primary mediator of cardiac teratogenesis due to a variety of AHR agonists.

3.4.1 The role of AHR1 and AHR2 in cardiac teratogenesis

In these experiments, only AHR2 knockdown was protective from the effects of BNF and PCB-126. Furthermore, the combination of AHR1 and AHR2 knockdown was no different than AHR2 knockdown alone in protection from PCB-126. AHR2 knockdown was also protective from teratogenesis caused by BkF, but the results of the experiments using translation-blocking morpholinos yielded some ambiguity regarding
the effect of AHR1 knockdown. The mean deformity score in AHR1 MO injected individuals was statistically lower than that of the non-injected individuals dosed with BkF. However, it was not lower than the control MO-injected individuals dosed with BkF. This may indicate a role for both AHR1 and AHR2 in mediating BkF toxicity, but injection with a combination of both morpholinos did not improve protection when compared to knockdown of AHR2 alone. An intermediate level of protection in embryos with concurrent AHR1 and AHR2 knockdown also occurred in BNF-dosed embryos. It is likely that the lower dose of morpholino used for the combination knockdowns is responsible for the reduction in rescue compared to embryos with AHR2 knockdown alone. It is also possible that the lower dose of morpholino masked a combined protective effect of AHR1 and AHR2 knockdown. However, this seems unlikely considering the results of the second set of experiments utilizing a splice junction morpholino. Individuals injected with AHR1-spl MO and dosed with BNF or BkF exhibited mean deformity scores no different from non-injected controls, while the AHR2 morpholino was again protective from BNF and BkF. In view of the weight of evidence from these experiments, it seems likely that the difference in mean deformity scores between AHR1 MO-injected individuals and non-injected individuals dosed with BkF in the first experiment is artifactual.

Previous studies provide support that AHR2 is the primary mediator of the effects of AHR agonists in Fundulus. While Karchner et al. (2002) showed that both receptors bound agonists, they also demonstrated that AHR2 was somewhat better able to drive luciferase expression in vitro. In adult killifish, AHR2 transcripts were similarly abundant in multiple tissues, whereas AHR1 transcripts were found primarily in brain, heart, ovary, and testis; the authors hypothesized that this suggests a more specialized role for AHR1
in certain tissues (Karchner et al. 1999; Powell et al. 2000). It is possible that there is a tissue-specific role for AHR1 in mediating response to AHR agonists that could not be addressed due to the approach of the current study. It is also possible that AHR1 and AHR2 mediate toxicity in a compound-specific fashion. In the current study we utilized a variety of AHR agonists (BNF, BaP, BkF, and PCB-126) and found no evidence to support this conclusion. However, there is great diversity of structure and activity among potential AHR agonists and it is possible that compounds not investigated here act through AHR1 or both AHR2 and AHR1.

Our results are significantly informed by comparison of Fundulus AHRs to those of zebrafish (Danio rerio), which have been used extensively to investigate the mechanisms of induction of cardiac toxicity by PAHs, PCB-126, and TCDD. Zebrafish AHR2 is an ortholog of Fundulus AHR2; unlike Fundulus, they also have two identified AHR1s. In zebrafish, morpholino knockdown of AHR2 was protective from cardiac toxicity caused by TCDD (Prasch et al. 2003b; Teraoka et al. 2003), PCB-126 (Jonsson et al. 2007), the PAHs pyrene and benz[a]anthracene (Incardona et al. 2006b; Incardona et al. 2005), and PAH mixtures (Billiard et al. 2006). In zebrafish exposed to high doses of the tricyclic PAHs dibenzothiphene and phenanthrene, cardiac toxicity was not altered by either AHR1a or AHR2 knockdown (Incardona et al. 2005). Subsequently, that research group showed that embryos with AHR1a knockdown also exhibited pyrene resistance, but that it did not affect vascular CYP1A induction (Incardona et al. 2006b). Currently, the role of AHR1b in cardiac toxicity in zebrafish has not been demonstrated, although in vitro it binds TCDD with high affinity and activates a luciferase reporter (Karchner et al. 2005). Collectively, findings in zebrafish further support the conclusion that Fundulus AHR2 is the primary mediator of similar effects. Phylogenetically,
Fundulus AHR2 is most closely related to zebrafish AHR2 and Fundulus AHR1 is most closely related to zebrafish AHR1b; zebrafish AHR1a is isolated from the other identified zebrafish and Fundulus AHRs (Hahn et al. 2006; Karchner et al. 2005).

### 3.4.2 Consequence of AHR1 and AHR2 knockdown for normal development

Beyond its adaptive functions, the AHR serves poorly understood physiological roles. AHR homologs have been identified in invertebrates, but they lack the ability to bind TCDD and other aryl hydrocarbons, indicating that they serve a purpose other than xenobiotic response (Hahn 2002; Hahn et al. 2006). Studies in AHR null mice and mice with constitutively active AHR have show that the mouse AHR plays a role in normal development and function of the liver, heart, immune system, vasculature, and reproductive system (reviewed in Nguyen and Bradfield 2008). In the current study, no developmental effects of knockdown of either AHR1 or AHR2 were observed. Each embryo was observed carefully, but assessment was focused on cardiac teratogenesis. It is possible that the effects of AHR knockdown were not apparent in this gross assessment. However, it is important to note that morpholinos result in gene knockdown and not gene knockout. It is therefore possible that sufficient AHR remained to carry out its physiological role(s) in morpholino-injected embryos.

### 3.4.3 Assessment of morpholino efficacy

In this study use of several AHR agonists resulted in a range of severity of cardiac malformation. Although AHR2 knockdown was protective in all cases, it only reduced the effects of PCB-126 by about half and did not rescue the BkF-induced effects to control levels. This is probably due to the severity of the effects and the fact that morpholino knockdown is not complete. Similar incomplete rescue has been observed
in studies utilizing morpholinos to investigate aryl hydrocarbon teratogenesis in zebrafish (Billiard et al. 2006; Jonsson et al. 2007). However, it was important in the current study to assess the efficacy of the morpholino knockdown process, particularly due to the relative lack of use of morpholino knockdown in *Fundulus*. With Western blots we demonstrated that both translation blocking morpholinos reduced expression of their respective targeted proteins. However, it was difficult to measure AHR protein levels early in development, at least in part due to interference by the relative excess of vitellogenin. As a result, we analyzed protein from pooled 144 hpf embryos, which allowed good measurement of AHR. However, when embryos were dosed at 24 hpf, we observed lower AHR protein levels in BNF-dosed fish compared to controls. The rapid degradation of AHR protein following binding of a ligand has been described previously (reviewed in Pollenz 2002) and is not unexpected given the amount of time between dosing and protein assessment. While dosing at 24 hpf still allowed assessment of morpholino efficacy, it made comparison of the morpholino effects on protein levels under dosed conditions difficult. When individuals were dosed at 96 hpf and protein measured at 144 hpf in a second experiment, morpholino efficacy was more apparent under both control and dosed conditions.

Use of the AHR1-spl MO provided a second method (RT-PCR) for confirmation of AHR knockdown and also allowed us to demonstrate knockdown much earlier than was possible with Western blot analysis. Although the splice morpholino-injected individuals clearly lost the targeted region of AHR1, we were unable to amplify an altered sequence created by the action of the splice morpholino. This may be due to a large increase in target size generated by inclusion of the intron, which is one of the possible outcomes reported by GeneTools. This is the first report of the use of a splice
morpholino in *Fundulus*, further demonstrating the application of molecular tools in this environmental model.

### 3.4.4 The role of AHR1 and AHR2 in aryl hydrocarbon-resistant Fundulus populations

The pivotal role of the AHR in the toxicity of both dioxin-like compounds (DLCs) and PAHs makes it a likely target for development of resistance to these contaminants in field populations. Recalcitrance to CYP1A induction, indicative of depression of the AHR pathway, is shared by aryl hydrocarbon-resistant *Fundulus* populations from the Elizabeth River, VA (Meyer et al. 2002; Van Veld and Westbrook 1995), New Bedford Harbor, MA (Bello et al. 2001; Nacci et al. 1999), and Newark Bay, NJ (Prince and Cooper 1995a). However, there has been limited work investigating the specific role of the AHRs in these resistant populations.

In one study, *Fundulus* from Newark Bay exhibited lower hepatic expression of AHRs than reference fish, possibly contributing to resistance (Arzuaga and Elskus 2002). However, the use of photoaffinity labeling to measure AHR protein did not distinguish between AHR1 and AHR2. Previous work in our lab analyzed transcriptional expression of AHR pathway genes in liver of BNF-treated adult *Fundulus* from the PAH-adapted Elizabeth River population and a reference population (Meyer et al. 2003b). AHR2 expression was induced in reference fish, but not in the Elizabeth River population. Overall, expression of AHR1 was low and the populations did not differ in their level of AHR1 expression. Importantly, there were no differences in basal mRNA expression of AHR pathway genes, so this was not the source of resistance. It is notable that AHR2 was moderately inducible in reference *Fundulus*. One would then expect that *Fundulus* directly from the Elizabeth River might have elevated basal levels
due to exposure to high levels of PAHs in situ. In keeping with results from the current study demonstrating the importance of AHR2 in mediating PAH toxicity, the lack of induction of AHR2 in Elizabeth River Fundulus and statistically indistinguishable basal levels of expression may indicate that resistance is conferred through an alteration of AHR2 activation. Recent work also supports the conclusion that an alteration at the top of the AHR pathway confers resistance on the Elizabeth River population fish. Wills et al. (2010) demonstrated that multiple AHR pathway genes (CYP1A, CYP1B1, and CYP1C1) are induced in reference Fundulus embryos, but not in Elizabeth River embryos, suggesting suppression via the shared transcription factor AHR.

In contrast to Meyer et al., Powell et al. (2000) observed no differences in basal or contaminant-induced mRNA expression of AHR1 or AHR2 between embryos from DLC-resistant New Bedford Harbor Fundulus and a reference population. Interestingly, AHR1 expression was distributed in all tissues investigated in wild-caught adults from the New Bedford Harbor population, but only in brain, heart, and ovary of reference Fundulus. However, the unusual pattern of AHR1 expression was not maintained in lab-reared offspring of New Bedford Harbor Fundulus, indicating that the altered AHR1 expression pattern was probably not responsible for the heritable resistance to DLCs. Subsequently, the same lab investigated polymorphisms of the Fundulus AHR1 locus and reported differences in frequency of the major AHR1 allele types between the New Bedford Harbor population and a reference population (Hahn et al. 2004). They expressed representative proteins from the two most divergent allele groups and compared their functional properties in vitro. Both AHR1 alleles exhibited similar TCDD binding and ability to activate a luciferase reporter upon TCDD-binding. Although there are a number of potential explanations for these findings, including that the activity of the
AHR1 alleles differs in ways that were not assessed by the functional assays, it is possible that AHR1 polymorphism is not driving the resistance. Hahn et al. (2004) also point out that AHR1 could be linked to another polymorphic gene and they identify AHR2 as one candidate. AHR2 and AHR1 are arranged in tandem in the genome of Takifugu rubripes (pufferfish) and zebrafish (Hahn et al. 2006), but the arrangement of these genes is not yet determined in Fundulus. Given our findings on the role of Fundulus AHR2 in aryl hydrocarbon response, it is interesting to speculate that polymorphisms in AHR2 could be driving the observed variation in AHR1 allele frequency via linkage disequilibrium. Currently, an assessment of variation of AHR2 allele frequency in the New Bedford Harbor population has not been published.

3.4.5 Conclusions

In summary, we have shown through targeted morpholino gene knockdown that AHR2 mediates PCB-126 and PAH responses in Fundulus, including cardiac teratogenesis and CYP1A protein expression. Although our results do not eliminate the possibility that AHR1 or a combination of AHR1 and AHR2 may play a role in response to other aryl hydrocarbons or through other endpoints, we did not find any evidence to support this conclusion. Results from this study aid in further characterization of the mechanism of aryl hydrocarbon toxicity in Fundulus and other fish and will enhance investigation of the molecular mechanisms underlying adaptation of Fundulus populations to severely contaminated environments.
Figure 6: Representative images of embryos at 144 hpf exhibiting each score on the deformity scale.

A score of 0 corresponds to a normal heart, 1 to a moderately deformed heart, and 2 to a severely deformed heart. Clearly visible in the normal heart are the ventricle (V), atrium (A), and sinus venosus (Sv). Pericardial edema (Pe, margin marked by dashed line) surrounds the elongated hearts in embryos scored as 1 and 2.
Figure 7: Effect of AHR1 and AHR2 knockdown on cardiac teratogenesis due to BNF, BkF, and PCB-126.

Effect of 250 µM AHR1 MO, 250 µM AHR2 MO, a combination of 125 µM AHR1 MO and 125 µM AHR2 MO, or 250 µM control MO on mean deformity score (± SEM) of Fundulus embryos exposed to DMSO or 10 µg/L BNF (A), 300 µg/L BkF (B), or 1 µg/L PCB-126 (C) compared to non-injected (NI) controls. Embryos were exposed at 24 hpf and scored at 144 hpf. None of the MO-injected, DMSO control groups differed from the non-injected, DMSO controls in any experiment; for figure clarity only the non-injected, DMSO controls are shown. Bars not marked by the same letter are statistically different at p≤0.05 (ANOVA, Tukey adjusted LSMeans).
Figure 8: Effect of AHR1 splice morpholino and AHR2 morpholino on cardiac teratogenesis due to BNF and BkF.

Effect of 250 µM AHR1-spl MO and 250 µM AHR2 MO on mean deformity score (± SEM) of Fundulus embryos exposed to DMSO, 10 µg/L BNF, or 300 µg/L BkF compared to non-injected (NI) controls. Embryos were exposed at 24 hpf and scored at 144 hpf. Bars marked by * are statistically different from the respective non-injected dosed group $p \leq 0.05$ (ANOVA, Tukey adjusted LSMeans).
Figure 9: Effect of AHR1 and AHR2 morpholino on BNF-induced expression of AHR1, AHR2, and CYP1A protein at 144 hpf.

Effect of 250 µM AHR1 MO, 250 µM AHR2 MO, and a combination of 125 µM AHR1 MO and 125 µM AHR2 MO on expression of AHR1, AHR2, and CYP1A protein in Fundulus embryos exposed individually to DMSO or 10 µg/L BNF at 24 hpf. Embryos were pooled in groups of ten for Western blot analysis at 144 hpf.
Figure 10: Effect of AHR1, AHR2, and control morpholino on BaP-induced expression of AHR1 and AHR2 protein.

Effect of 250 µM AHR1 MO, 250 µM AHR2 MO, or 250 µM control MO on expression of AHR1 and AHR2 protein in *Fundulus* embryos exposed individually to DMSO or 100 µg/L BaP at 96 hpf. Embryos were pooled in groups of ten for Western blot analysis at 144 hpf.
Figure 11: Demonstration of knockdown of BkF-induced AHR1 mRNA using AHR1 splice morpholino.

Loss of mRNA fragment targeted by AHR1-spl MO in embryos exposed to DMSO or 300 µg/L BkF at 24 hpf. Embryos were sacrificed at 48 hpf and paired for mRNA extraction and analysis by RT-PCR. AHR1 knockdown is demonstrated by comparing amplification of a cDNA fragment in the region targeted by the splice morpholino (exon 7 to exon 9, 374 nucleotide expected band size) to amplification of a fragment in exons 2 and 3 (191 nucleotide expected band size) that is unaffected by the morpholino. Representative image shows both RT-PCR amplifications for 2 of ≥6 pairs of embryos per treatment group. Ld = 1 kb DNA ladder.
4. *Fundulus heteroclitus* adapted to PAHs are cross-resistant to multiple insecticides

This chapter is a collaborative effort of Bryan W. Clark and Richard T. Di Giulio.

### 4.1 Introduction

The population of *Fundulus heteroclitus* (the Atlantic killifish or mummichog; hereafter referred to as killifish) inhabiting the Atlantic Wood Industries Superfund site on the Elizabeth River, Virginia, USA are resident in an area heavily contaminated with a complex mixture of polycyclic aromatic hydrocarbons (PAHs) from former creosote operations. Total PAH concentrations of 100-500 µg/g dry sediment are commonly reported for the site (Mulvey et al. 2002; Mulvey et al. 2003; Chapter 5; Vogelbein and Unger 2003; Walker et al. 2004), placing it among the more heavily PAH-polluted sites in the world (Walker et al. 2004). However, Elizabeth River killifish have developed resistance to the acute toxicity and severe cardiac teratogenesis caused by Elizabeth River sediments, PAHs, and PCB-126 (3,3’,4,4’,5-pentachlorobiphenyl) (Meyer and Di Giulio 2002; Meyer et al. 2002; Ownby et al. 2002).

Elizabeth River killifish exhibit alterations in several xenobiotic metabolism and excretion pathways that may contribute to their survival in a contaminated habitat, including elevated levels of glutathione S-transferase (Armknecht et al. 1998; Van Veld et al. 1991), hepatic P-glycoprotein (Cooper et al. 1999), UDP-glucuronosyl transferase (UGT), and sulfotransferase (Gaworecki et al. 2004). Elizabeth River killifish also have upregulated antioxidant defenses (Bacanskas et al. 2004; Meyer et al. 2003a). Perhaps
the most dramatic difference between Elizabeth River killifish and naïve killifish is their recalcitrance to induction of cytochrome P450 (CYP) metabolic enzymes by aryl hydrocarbon receptor (AHR) agonists (Meyer and Di Giulio 2002; Meyer et al. 2002; Van Veld and Westbrook 1995).

Much attention has focused on this recalcitrance to CYP induction by AHR agonists in Elizabeth River killifish. Lack of CYP induction is generally considered a marker of down-regulation of the AHR pathway. In the Elizabeth River Killifish and other fish populations exposed to dioxin-like compound (DLC) pollution, recalcitrance to CYP induction is correlated with marked resistance to the toxic effects of the contaminants (Bello et al. 2001; Nacci et al. 2002b; Powell et al. 2000; Prince and Cooper 1995b; Roy et al. 2002). Furthermore, morpholino knockdown of AHR in zebrafish (Billiard et al. 2006; Prasch et al. 2003b) and killifish (Clark et al. 2010) has shown that blockade of AHR can provide protection from cardiac teratogenesis caused by aryl hydrocarbons such as 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD), PCB-126, and a variety of PAHs. However, it is noteworthy that in some cases this protection is incomplete. This may indicate a role for additional adaptive changes, such as the elevations in phase II and phase III xenobiotic metabolism and antioxidant defenses observed in Elizabeth River killifish. Although clearly important, the relative roles of the AHR pathway and other adaptive genes in the toxicity of PAH mixtures and the resistance of Elizabeth River killifish remains uncertain.

Moreover, although Elizabeth River killifish have developed resistance to some of the acute effects of PAHs, they are not wholly unaffected by the contaminants. They exhibit hepatic neoplasms (Vogelbein et al. 1990), have altered immune function and elevated disease susceptibility (Faisal et al. 1991; Frederick et al. 2007; Meyer and Di
Giulio 2003; Meyer et al. 2005; Weeks et al. 1988), are more sensitive to hypoxia and fluoranthene-mediated phototoxicity (Meyer and Di Giulio 2003), and have reduced growth and survivorship compared to reference fish (Meyer and Di Giulio 2003).

Other than the aforementioned studies, limited data are available concerning the cost of PAH resistance with respect to sensitivity to other environmental pollutants and stressors. This is potentially very important, as estuarine fish are faced with a wide variety of chemical stressors. Furthermore, the biochemical and physiological changes that engender or are coincident with resistance to PAHs are likely to alter toxic responses to other contaminants; these alterations may manifest as both costs and benefits. For example, the refractory CYP1 phenotype may lead to prolonged presence of a toxic parent compound, increasing the effect. This might be expected for compounds such as pyrethroid insecticides, which are highly toxic to fish, but are much more so as parent compounds than metabolites resulting from CYP1 activity (Coats et al. 1989). Conversely, compounds activated by CYP1s, such as the organophosphate insecticide chlorpyrifos, might be expected to be more toxic to reference killifish than Elizabeth River killifish.

The goal of the current study was to gain better understanding of the nature of the contaminant adaptation exhibited by Elizabeth River killifish and to investigate the consequences of PAH-adaptation for response to contaminants other than aryl hydrocarbons. Our hypothesis was that the adaptive changes necessitated by long-term exposure to a complex mixture of PAHs would alter the response of Elizabeth River killifish when faced with other contaminants. In particular, compounds activated by CYP1s, such as chlorpyrifos, would be less toxic to Elizabeth River killifish and that compounds that are generally inactivated by CYP1s, such as permethrin and carbaryl,
would be more toxic to Elizabeth River killifish. To investigate this hypothesis, we challenged offspring of the PAH-adapted Elizabeth River killifish and a reference population with acutely neurotoxic insecticides – the organophosphate chlorpyrifos, the pyrethroids permethrin and fenvalerate, and the carbamate carbaryl. To further illustrate the role of CYP1s in this toxicity, larvae were also co-exposed to β-naphthoflavone, a known CYP1 inducer, with the individual insecticides. In addition, because there is evidence that carbaryl can act as an AHR agonist (Bohonowych and Denison 2007; Denison et al. 1998), we compared the in ovo susceptibility of the two populations to cardiac teratogenesis and CYP induction mediated by carbaryl. Use of these insecticides as probes of resistance will help to demonstrate if the adaptation in Elizabeth River killifish is narrow and AHR-focused or broad, such as increased excretion of many xenobiotics due to elevated phase II and phase III metabolism.

4.2 Materials and methods

4.2.1 Fish

Adult killifish from the PAH-adapted population were collected with wire mesh minnow traps at the Atlantic Wood Industries Superfund Site (36°48'27.2" N, 76°17'38.1" W). Adult killifish from a reference population were collected on King’s Creek, a relatively uncontaminated tributary of the Severn River, Virginia, USA (37°18'16.2"N, 76° 24'58.9"W). After transport to the laboratory, fish were maintained in 20‰ artificial sea water (ASW; Instant Ocean, Foster & Smith, Rhinelander, WI, USA), at 23-25 °C, on a 14:10 light:dark cycle. They were fed pelleted fish feed ad libidum (Aquamax® Fingerling Starter 300, PMI Nutritional International, LLC, Brentwood, MO, USA). All
experiments were conducted with F1 offspring of wild-caught Elizabeth River and King’s Creek adults. Eggs were obtained by manual spawning of females and fertilized \textit{in vitro} by expressing sperm from males into a beaker containing eggs in ASW. Following spawning, embryos were set aside for a minimum of one hour to allow fertilization, then washed briefly with 0.3% hydrogen peroxide solution. For experiments utilizing larvae, embryos were maintained in petri dishes (VWR International, West Chester, PA, USA) lined with absorbent filter paper (No. 3MM chromatography paper, Whatman International Ltd., Maidstone, England). Enough ASW was added to the dishes to keep the embryos moist but not completely submerged. Embryos were maintained in an incubator for 12-14 days at 27 °C. For hatching, more ASW was added to the petri dishes, the absorbent paper was removed, and the dishes were gently rocked in a shaker. After hatching, larvae were maintained in ASW in the incubator at 27 °C and fed \textit{Artemia} nauplii. All larval experiments were initiated at five days post hatch (dph). All adult care, reproductive, and rearing techniques were non-invasive and approved by the Duke University Institutional Animal Care & Use Committee (A234-07-08).

4.2.2 Chemicals and dosing

\(\beta\)-naphthoflavone (BNF), ethoxyresorufin, dimethyl sulfoxide (DMSO), chlorpyrifos, permethrin, carbaryl, and fenvalerate were purchased from Sigma-Aldrich (St. Louis, MO, USA). Stocks were prepared by dissolving chlorpyrifos, permethrin, fenvalerate, carbaryl, or BNF in DMSO. For both larval and \textit{in ovo} experiments, dosing solutions were prepared in 20‰ ASW. Larval exposures were conducted in 5 mL of dosing solution (embryo exposures in 10 mL), with final concentrations of 5 \(\mu\)g/L and 10 \(\mu\)g/L of chlorpyrifos, 400 \(\mu\)g/L and 600 \(\mu\)g/L permethrin, 1 mg/L and 10 mg/L carbaryl, and 10 \(\mu\)g/L and 25 \(\mu\)g/L fenvalerate. In treatments with BNF co-exposure, the final
concentration of BNF was 1 μg/L. These challenge doses were based on values from the literature (Coats et al. 1989; Goodman et al. 1992; Hemmer et al. 1992; Shea and Berry 1983) and range-finding experiments with lower numbers of individuals (data not shown). The carbaryl challenge doses used were notably higher than the other insecticides, consistent with literature reports (Ferrari et al. 2007; Lin et al. 2007; Shea and Berry 1983; Solomon and Weis 1979). Control groups were exposed to DMSO at a concentration (v/v) equal to that in the appropriate dosed groups for a given experiment; in all cases DMSO concentrations were held at <0.03% across all treatments.

4.2.3 Larval susceptibility – chlorpyrifos, permethrin, fenvalerate, and carbaryl with and without co-exposure to BNF

To determine the acute response to neurotoxic pesticides, larvae (5 dph) from each population were placed in 20-mL glass scintillation vials (VWR, West Chester, PA, USA) in groups of five with approximately 5 mL of ASW. Larvae were allowed to acclimate for approximately 30 minutes; all individuals that died due to handling or appeared to be in distress were replaced. For dosing, the original ASW was removed from the scintillation vial using a transfer pipet (VWR, West Chester, PA, USA) and replaced with 5 mL of the appropriate dosing solution. Following dosing, the larvae were again observed briefly for stress or mortality due to handling and were replaced if necessary. Larvae were maintained in an incubator at 27 °C for 24 hours, after which percent mortality in each vial was recorded. Initial experiments investigated the response to chlorpyrifos, permethrin, and carbaryl. Additional experiments were added with fenvalerate because of unexpected results observed with permethrin. Each experiment consisted of n ≥ 15 vials per treatment group and experiments were performed in triplicate.
4.2.4 *In ovo* response to carbaryl

To investigate the ability of carbaryl to induce CYP1 activity and generate cardiac deformities, embryos from each population were dosed individually in 10 mL of solution in 20-mL glass scintillation vials. Embryos were exposed to DMSO or carbaryl beginning at 24 hours post fertilization (hpf) through 144 hpf. In addition, all dosing solutions contained 21 μg/L ethoxyresorufin, a substrate for the ethoxyresorufin-o-deethylase (EROD) assay. EROD activity was assessed at 96 hpf and embryos were screened for cardiac deformity at 144 hpf. All experiments consisted of \( n \geq 10 \) individuals per treatment group and experiments were performed in triplicate.

4.2.4.1 EROD assay

CYP1 activity was measured via the *in ovo* EROD assay modified from Nacci et al. (1998). In brief, embryos were exposed to ethoxyresorufin with dosing solutions, as described in section 4.2.4. Cleavage of the ethoxy group by CYP1s yielded resorufin, a fluorescent product, which collected in the urinary bladder of the embryo. At 96 hpf, the fluorescence due to resorufin in the urinary bladder was visualized using fluorescent microscopy (50X magnification, rhodamine red filter set; Axioskop, Zeiss, Thornwood, NY, USA). EROD activity was measured as intensity of fluorescence within the bladder normalized to intensity in a region outside the bladder and quantified using IPLab software (BD Biosciences, Rockville, MD, USA).

4.2.4.2 Deformity assessment

Embryos dosed with carbaryl at 24 hpf as described previously were scored for cardiac deformities at 144 hpf. Deformity assessment was performed blind using a scale
shown in detail previously (Clark et al. 2010; Matson et al. 2008a). The scale consists of three scores categorized as normal (0), mild deformities (1), and severe deformities (2). Embryos receiving a score of 0 had hearts with a normal appearance including properly aligned and sized chambers, no visible pericardial edema, and unrestricted blood flow. Embryos receiving a score of 1 had slightly elongated hearts, with generally distinct but misaligned chambers, and visible pericardial edema. Embryos with a score of 2 had greatly elongated hearts often with no identifiable chambers and an extremely reduced or complete absence of blood flow.

4.2.5 Statistical analyses

All analyses were performed using JMP 8.0 (SAS Institute Inc, Cary, NC, USA). For all analyses, the individual vial was the unit of replication. Data were analyzed by non-parametric analysis of variance (ANOVA), followed by least square means (LSMeans) procedures. As stated previously, experiments were replicated a minimum of three times; no differences between experimental replicates were observed for any test. For post hoc comparisons, Tukey-adjusted pairwise comparisons were conducted to determine differences between all groups. For in ovo carbaryl exposure, Dunnett’s adjustment was used to make post hoc comparisons to the appropriate control group. Statistical significance was accepted at $p \leq 0.05$ for all tests.

4.3 Results

4.3.1 Larval susceptibility – chlorpyrifos, permethrin, fenvalerate, and carbaryl without co-exposure to BNF
As described previously, susceptibility of Elizabeth River and King’s Creek F1 larvae (5 dph) was compared in response to a 24-hour challenge with a set of neurotoxic insecticides. Additionally, some larvae were co-exposed to BNF to determine if CYP induction altered the acute toxicity of the insecticides.

4.3.1.1 Larval susceptibility to chlorpyrifos

As hypothesized, chlorpyrifos was significantly more toxic to King’s Creek larvae than to Elizabeth River larvae, at least at the higher dose used (Figure 1). In both populations, mortality due to 5 μg/L chlorpyrifos was not statistically different ($p>0.05$) from that in DMSO-dosed control larvae. In King’s Creek larvae, 10 μg/L chlorpyrifos caused an average percent mortality of 46±5.6%, which was statistically greater than control ($p<0.0001$). In contrast, the average percent mortality of Elizabeth River larvae exposed to 10 μg/L chlorpyrifos (9.3±1.4%) was significantly lower than for King’s Creek ($p<0.0001$). Furthermore, when larvae were co-exposed to BNF to induce CYP activity, the average percent mortality caused by 10 μg/L chlorpyrifos was nearly doubled to 98±1.4% in King’s Creek larvae. However co-exposure to BNF and 10 μg/L chlorpyrifos had no obvious effect on Elizabeth River larvae, which were still no different than the control groups. Co-exposure to BNF did not have a statistically significant effect on average percent mortality for any of the other treatment groups ($p>0.05$). Interestingly, co-exposure to BNF did not cause increased mortality at 5 μg/L chlorpyrifos in King’s Creek larvae, even though it demonstrated the ability to increase mortality at the higher dose of chlorpyrifos.
4.3.1.2 Larval susceptibility to permethrin

Overall, killifish larvae were not as susceptible to permethrin as expected based on literature values for fish toxicity. In contrast to chlorpyrifos, the relative levels of susceptibility of Elizabeth River and King’s Creek larvae did not confirm the original hypothesis. Rather, as with chlorpyrifos, Elizabeth River larvae were much less susceptible to permethrin challenge than were King’s Creek larvae (Figure 2). The average percent mortalities of King’s Creek larvae exposed to 400 μg/L permethrin and 600 μg/L permethrin were 91±2.5% and 96±1.5%, respectively. In contrast, Elizabeth River larvae exhibited significantly lower average percent mortality at both doses (p<0.0001, both comparisons). The average percent mortality of Elizabeth River larvae exposed to 400 μg/L permethrin and 600 μg/L permethrin were 6.0±2.2% and 4.0±1.8%, respectively. Co-exposure with BNF demonstrated that CYP activation in King’s Creek larvae could play a role in detoxifying permethrin; addition of BNF caused a statistically significant decrease in toxicity at both doses of permethrin (p<0.0001, both comparisons). At 400 μg/L permethrin, co-exposure to BNF reduced the average percent mortality in King’s Creek larvae to near control levels (6.7±2.4%). Co-exposure to BNF with 600 μg/L permethrin reduced the average percent mortality in King’s Creek larvae to 69±7.2%. As with chlorpyrifos, co-exposure to BNF had no effect on mortality of Elizabeth River larvae exposed to either dose of permethrin.

4.3.1.3 Larval susceptibility to fenvalerate

Because the experiments with permethrin yielded unexpected results, additional challenge experiments were conducted with a second pyrethroid, fenvalerate (Figure 3). Mortality was observed in both populations at lower doses of fenvalerate than with permethrin. There was little difference in response between the two populations (main
effect of population \( p=0.7719 \); the average percent mortality of King’s Creek larvae exposed to 10 μg/L fenvalerate was 18±5.4% and for Elizabeth River larvae it was 15±6.0%. When exposed to 25 μg/L fenvalerate, the average percent mortality was 75±5.8% for King’s Creek larvae and 90±3.5% for Elizabeth River larvae. Co-exposure to BNF had little effect on the toxicity of fenvalerate in either population. The average percent mortality of King’s Creek larvae co-exposed to BNF was 23±4.4% with 10 μg/L fenvalerate and 89±3.4% with 25 μg/L fenvalerate. For Elizabeth River larvae, the average percent mortality in BNF co-exposed treatments was 17±4.1% with 10 μg/L fenvalerate and 71±6.2% with 25 μg/L fenvalerate.

4.3.1.4 Larval susceptibility to carbaryl

As with chlorpyrifos and permethrin, carbaryl was more toxic to King’s Creek larvae than to Elizabeth River larvae (Figure 4). When exposed to 1 mg/L carbaryl neither population exhibited an average percent mortality that was above control levels. However when exposed to 10 mg/L carbaryl, the average percent mortality of King’s Creek larvae was statistically greater than that of Elizabeth River larvae at the same dose \( p<0.0001; 69±2.7\% \text{ vs.} 30±2.5\% \). Similar to fenvalerate but unlike the other previous challenges, co-exposure with BNF had only a small effect on the toxicity of carbaryl. Mortality due to 10 mg/L carbaryl was slightly increased with BNF co-exposure in both King’s Creek (78±5.2%) and Elizabeth River (41±3.6%) larvae; however, the increase over carbaryl alone was statistically significant in Elizabeth River larvae \( p=0.0337 \), but not King’s Creek \( p=0.2317 \) larvae.
4.3.2 In ovo response to carbaryl

In ovo exposure to carbaryl resulted in induction of EROD activity and cardiac teratogenesis in a similar manner to more prototypical AHR agonists. As in the larval experiments, King’s Creek embryos were more susceptible to carbaryl than were Elizabeth River embryos (Figure 5). When exposed to 1 mg/L carbaryl, King’s Creek embryos exhibited an average deformity score of 0.50±0.11, which was significantly elevated above DMSO-dosed individuals ($p=0.0211$). In addition, the CYP1 response as measured by EROD activity was greatly elevated above that of DMSO-dosed embryos (597±96% of KC DMSO; $p=0.0327$). For Elizabeth River embryos, no effect of 1 mg/L carbaryl was observed for either average deformity score or EROD activity ($p>0.05$). At 10 mg/L, carbaryl induced severe cardiac deformities and a robust EROD response in King’s Creek embryos; the average deformity score of 2.0±0.0 and mean EROD activity of 1757±243% of KC DMSO were both significantly greater than in controls ($p<0.0001$ for both). In contrast, the average deformity score of 0.35±0.12 exhibited by Elizabeth River embryos was not statistically different from DMSO-dosed embryos ($p>0.05$). However, EROD activity was elevated approximately 10-fold in Elizabeth River embryos exposed to 10 mg/L carbaryl (280±16% of KC DMSO) compared to DMSO-dosed individuals ($p<0.0001$).

4.4 Discussion

4.4.1 General susceptibility of larval killifish to insecticides

In general, the insecticides used in these experiments caused acute mortality at similar doses to those reported in the literature for early life stages of other fish (Coats et
al. 1989; Ferrari et al. 2007; Goodman et al. 1992; Hemmer et al. 1992) with the possible exception of permethrin. The acute (24 hour) LC$_{50}$ of permethrin has been reported in the range of 0.843-75.0 µg/L across a wide variety of fish species (reviewed in Baser et al. 2003; Coats et al. 1989), whereas little toxicity was observed at comparable doses in the current study. However, Baser et al. (2003) do report a 48-hour LC$_{90}$ in adult guppies (*Poecelia reticulate*) that is comparable to that seen in this study. It is noteworthy that the majority of the data are for adult fish, rather than larvae as used herein. Also of note is that we observed great inter-experimental variability in range-finding exposures when newly-hatched (0 or 1 dph) were utilized (data not shown). In those preliminary experiments some individuals still had visible yolk-sacs. When all of the offspring were maintained for 5 dph prior to exposure and allowed to finish utilization of the yolk-sac, the observed toxicity was much more consistent. This may reflect differential uptake or sequestration of the insecticides in individuals still bearing a yolk-sac.

4.4.2. Cross-resistance of Elizabeth River Fundulus offspring to insecticides

Due to the dramatic suppression of CYP inducibility and activity observed in Elizabeth River killifish (Meyer and Di Giulio 2002; Meyer et al. 2002; Nacci et al. 2010; Van Veld and Westbrook 1995), we hypothesized that some contaminants would be more or less toxic to Elizabeth River killifish depending on their relative activation or inactivation by CYP. Instead, challenge of Elizabeth River and King’s Creek killifish demonstrated that PAH-adapted Elizabeth River offspring were consistently less susceptible than reference fish to all of the insecticides tested except fenvalerate. At the
challenge doses used, King’s Creek killifish suffered approximately 3-20 fold higher average percent mortality.

When challenged with the organophosphate insecticide chlorpyrifos, the two populations responded as predicted. Conversion of chlorpyrifos to chlorpyrifos oxon, the active metabolite, is catalyzed by multiple CYP enzymes and the greater average percent mortality of King’s Creek larvae was likely due to their greater CYP metabolic activity. This conclusion is supported by the observed ability of BNF co-exposure to potentiate chlorpyrifos toxicity in King’s Creek larvae but not in the non-inducible Elizabeth River larvae.

In contrast, permethrin and carbaryl are more active in their parent form and can be detoxified by oxidative metabolism, so it was expected that Elizabeth River larvae would exhibit greater mortality upon exposure to those insecticides. However, as stated previously, the Elizabeth River larvae exhibited a markedly lower average percent mortality than did King’s Creek larvae in response to permethrin and carbaryl. With permethrin, co-exposure to BNF caused a decrease in average percent mortality of King’s Creek larvae, demonstrating that CYP induction could play a role in its detoxification. Permethrin has been demonstrated to be metabolized by oxidative metabolism in rainbow trout (Oncorhynchus mykiss), mice (Mus musculus), and rats (Rattus norvegicus) (Bradbury and Coats 1989). Additionally, enhanced oxidative metabolism by CYP has been found to contribute to resistance to pyrethroids, including permethrin and fenvalerate, in cotton bollworm (Helicoverpa armigera) (Chen et al. 2005; Yang et al. 2004), Colorado potato beetle (Leptinotarsa decemlineata) (Soderlund et al. 1987), and mosquito (Culex pipiens) (Hardstone et al. 2009). While previous data have shown that many vertebrate CYP isoforms, including CYP1A, can also metabolize
carbaryl (Schmidt et al. 2006; Tang et al. 2002), co-exposure to BNF did not decrease the toxicity of carbaryl. CYPs have been implicated in resistance of the western corn rootworm (*Diabrotica virgifera virgifera*) (Scharf et al. 1999) to carbaryl and in resistance of the tobacco budworm (*Heliothis virescens*) to pyrethroids, carbamates, and organophosphates (Zhao et al. 1996). It is worth noting that many of the cases of insecticide resistance involving increased oxidative metabolism have been linked to the CYP6 gene family (Hemingway et al. 2004). The CYP6 family is found exclusively in insects but is phylogenetically related to the CYP3 family found in mammals and in fish, including killifish (McArthur et al. 2003; Nelson 1998).

Because fenvalerate has similar properties to permethrin, it is interesting that fenvalerate is more toxic and does not exhibit differential toxicity in the two populations. Hemmer et al. (1992) compared the acute toxicity of a number of pesticides to larval stages of two estuarine fish, topsmelt (*Atherinops affinis*) and inland silverside (*Menidia beryllina*), to literature data for sheepshead minnow (*Cyprinodon variegatus*), fathead minnow (*Pimephales promelas*), bluegill (*Lepomis macrochirus*), and rainbow trout. In all cases fenvalerate and permethrin exhibited similar toxicity within a given species. In addition, fenvalerate can be metabolized in a similar manner to permethrin; biotransformation occurs via hydrolysis at ester bonds and oxidative metabolism in multiple organisms (Ohkawa et al. 1979; Soderlund et al. 1987). One difference between the two is that permethrin is considered a type I pyrethroid and fenvalerate is considered a type II pyrethroid. Type I and II pyrethroids have been described as having differences in effect, although there is debate as to the mechanism underlying these differences (Soderlund et al. 2002). Fenvalerate is a racemic mixture of four different optical isomers; the toxicity in fish has been attributed primarily to the S isomers
It may be that the protective factors in Elizabeth River killifish are less able to circumvent type II pyrethroids or they could be less capable of biotransformation of the S isomers of fenvalerate in particular. Structurally permethrin and fenvalerate are similar. However, as is typical of type II pyrethroids, fenvalerate has a cyano group near the ester linkage. For that reason, one hypothesis is that the ester linkage of permethrin might be the target of differential metabolism in Elizabeth River killifish. To better test whether the cyano group prevents Elizabeth River larvae from tolerating fenvalerate, future studies could compare the toxicity of permethrin and cypermethrin. Cypermethrin is structurally identical to permethrin, except for the addition of the cyano group at the ester linkage.

The greater tolerance of Elizabeth River larvae to insecticides both activated and detoxified by CYPs indicates that their xenobiotic resistance is not simply based in suppression of CYP activity via AHR pathway down-regulation. As stated previously, a number of other protective mechanisms are elevated in Elizabeth River killifish compared to reference populations and the current work suggests that these factors play an important role in xenobiotic resistance in Elizabeth River offspring.

In addition to the dramatic down-regulation of the Phase I metabolizing enzyme CYP, a number of Phase II enzymes are elevated in Elizabeth River killifish. Phase II enzymes, such as glutathione-S-transferases (GSTs), act to conjugate xenobiotics, increasing their solubility and enhancing their excretion from the body. Van Veld et al. (1991) noted that hepatic and intestinal GST activity was elevated 3-4 fold in intestine, liver, and liver lesions of adult Elizabeth River killifish compared to reference killifish. Along with increased GST activity, elevated levels of GST protein have been observed (Armknecht et al. 1998). In addition, Elizabeth River killifish exhibited elevation of UDP-
glucuronosyltransferase (UGT) activity and a slight elevation in sulfotransferase (SULT) activity (Gaworecki et al. 2004).

It is possible that upregulation of Phase II enzymes could play a significant role in the observed pesticide resistance of Elizabeth River killifish larvae. GSTs have been shown to participate in O-dealkylation and O-dearylation of organophosphates (Chiang and Sun 1993; Oppenoorth et al. 1979). GSTs have not been shown to have as direct a role in metabolism of pyrethroids, but they may play a role in the conjugation of the products of ester cleavage. In fact, GST upregulation by pyrethroids has been demonstrated in rats (Otitoju and Onwurah 2007), amphibians (Greulich and Pflugmacher 2004), shore crabs (Carcinus maenas) (Gowland et al. 2002), and rainbow trout (Davies et al. 1994). Furthermore, GST has been implicated in invertebrate resistance to a number of insecticide classes, including organophosphates, carbamates, and pyrethroids (Hemingway et al. 1991; Kostaropoulos et al. 2001; Wu et al. 1998; Yu and McCord 2007). However, GSTs also play an important role in responding to oxidative stress, which some believe is more important in insecticide resistance than its role in conjugative metabolism (reviewed in Hemingway et al. 2004).

Previous studies demonstrated that Elizabeth River F1 and F2 larvae were more resistant than reference larvae to the pro-oxidant t-butyl hydroperoxide; additionally their whole-body homogenates showed greater total oxygen scavenging capacity, slightly elevated glutathione (GSH), and greater expression of manganese superoxide-dismutase (MnSOD) (Meyer et al. 2003a). Increased total hepatic GSH was also observed in wild-caught Elizabeth River adults compared to reference fish (Bacanskas et al. 2004).
These elevated antioxidant defenses could play a role in insecticide resistance in Elizabeth River killifish. Rats treated with the pyrethroids fenvalerate or cypermethrin exhibited elevated levels of lipid peroxidation (Kale et al. 1999; Maiti et al. 1995; Raina et al. 2009) as did goldfish (Carassius auratus gibelio) exposed to the pyrethroid deltamethrin (Dinu et al. 2010). Furthermore, insect resistance to pyrethroids has been attributed to elevated antioxidant defenses (Muller et al. 2008; Ortelli et al. 2003; Vontas et al. 2001). Carbamates have also been linked to oxidative stress in a hamster ovary cell line (Maran et al. 2009), liver of Nile tilapia (Oreochromis niloticus) (Matos et al. 2007), and in liver and kidney of juvenile rainbow trout (Ferrari et al. 2007). Exposure to the carbamate carbofuran induced lipid peroxidation in the brain of exposed rats, along with reduction of GSH levels (Kamboj et al. 2006). Interestingly, these effects were attenuated by co-treatment with the antioxidant N-acetylcysteine, which acts as a precursor for GSH. This may demonstrate a role for elevated GSH in the observed protection of Elizabeth River killifish from carbaryl.

Another protective factor that may contribute to insecticide resistance in Elizabeth River killifish is P-glycoprotein (Pgp), also known as ATP-binding cassette transporter B1 (ABCB1). The Pgps are sometimes referred to as Phase III metabolizing enzymes; they are membrane transport ATPases that are involved in the efflux of compounds. Although they have been identified in a variety of normal tissues, they are also frequently elevated in multidrug-resistant cell lines and chemotherapy-resistant tumors (Litman et al. 2001). Cooper et al. (1999) demonstrated that Pgp was expressed 2-3 fold higher in liver and liver tumors of adult Atlantic Wood fish than in reference killifish.
It is likely that elevated Pgp induced in PAH-resistant killifish could play a protective against a number of pesticides. Pgp is believed to transport a wide variety of pesticides, including the pyrethroids cypermethrin and fenvalerate, the organophosphate methyl parathion, and the carbamate thiodicarb (reviewed in Buss and Callaghan 2008). Lanning et al. (1996b) showed that chlorpyrifos oxon, the neurotoxic metabolite of chlorpyrifos, interacted with Pgp in vitro and increased Pgp expression in rats. Interestingly, chlorpyrifos was shown to interact with Pgp, but acted to inhibit efflux of a substrate through human Pgp transfected in a mouse cell line (Bain and LeBlanc 1996). In contrast, Pgp did contribute to efflux of another organophosphate, diazinon, in a mammalian intestinal cell line (Cavret et al. 2005; Lecoeur et al. 2006). Furthermore, Pgp is suspected to play a role in pesticide resistance of a number of insect populations, including mosquitoes resistant to the pyrethroid cypermethrin (Buss et al. 2002) and tobacco budworms resistant to the carbamate thiodicarb (Lanning et al. 1996a). Cotton bollworms resistant to fenvalerate, cypermethrin, and the organophosphate methyl paraoxon had significantly higher expression of Pgp detected by immunoblot (Srinivas et al. 2004). However, the same insects also exhibited higher levels of esterases and a decreased sensitivity to acetylcholinesterase inhibition that likely contributed to resistance. Overall, the literature suggests that Pgp could contribute to pesticide resistance in Elizabeth River killifish. However, heritability of increased Pgp expression has not been investigated in Elizabeth River killifish, so it is unclear if Pgp could play a role in the resistance of F1 larvae observed in the current study.

4.4.3. In ovo susceptibility to carbaryl

The developmental effects due to carbaryl observed in the current study are only somewhat consistent with other studies. Previous work demonstrated an inconsistent
effect of carbaryl on cardiac development of killifish (Weis and Weis 1974). Exposure of embryos to 10 mg/L carbaryl resulted in cardiac abnormalities in one experiment in that study, but not in several others. Effects on circulatory development were also observed in exposure of Japanese medaka (Oryzias latipes) to carbaryl (Solomon and Weis 1979). Recent attempts to investigate the effect of carbaryl on development in zebrafish (Danio rerio) did not replicate the gross cardiac teratogenesis, but did cause some pericardial edema, tail malformations, and bradycardia (Lin et al. 2007).

There has been some debate as to whether or not carbaryl is truly an AHR ligand. Ledirac et al. (1997) reported that carbaryl could induce CYP1A1 gene expression in human hepatoma and keratinocyte cell lines, but did not appear to bind to the AHR. However, Denison and co-workers showed that carbaryl could drive in vitro AHR-dependent luciferase expression, induce AHR to bind to DNA, and compete with 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) for AHR binding (Bohonowycz and Denison 2007; Denison et al. 1998). The authors estimated that carbaryl was about 300,000-fold less potent than TCDD, a prototypical AHR ligand. Additionally, computational modeling demonstrated that carbaryl could assume the planar confirmation that is typical of AHR ligands (Casado et al. 2006). The overall weak interaction of carbaryl with the AHR may explain the inconsistent results seen in previous studies and the high doses required to induce EROD activity and cardiac teratogenesis in the current study.

Although effects were only observed at mg/L doses in the current study, carbaryl was clearly able to induce cardiac malformations similar to those previously observed in killifish and other fish exposed to a variety of AHR agonists (Antkiewicz et al. 2005; Clark et al. 2010; Matson et al. 2008a; Prasch et al. 2003b; Toomey et al. 2001; Wassenberg and Di Giulio 2004a). In addition, the aryl hydrocarbon-resistant F1 Elizabeth River
embryos were resistant to cardiac teratogenesis caused by carbaryl. Interestingly, 10 mg/L carbaryl induced a roughly 10-fold increase in EROD activity in Elizabeth River embryos. Except for slight induction of EROD activity by Elizabeth River pore water (Meyer et al. 2002), induction of EROD activity in F1 Elizabeth River embryos has rarely been observed. It is possible that this indicates that carbaryl is able to induce EROD activity through AHR-independent means, or that the mechanism of EROD suppression in the PAH-adapted Elizabeth River killifish is not suited to blocking the effects of high concentrations of carbaryl. However, if these effects are mediated through the AHR, the previously described suppression of the AHR pathway in Elizabeth River killifish appears likely to contribute to the protection from carbaryl observed in Elizabeth River larvae.

4.4 Conclusions

It is generally accepted that adaptation to a stressor is often accompanied by concurrent fitness costs to populations (Kinnison and Hairston 2007; Wirgin and Waldman 2004). Strong selective pressure is thought to drive reduction in genetic variation and overall fitness. Reduced fecundity, growth rate, and survival are considered classical fitness costs, but a broader interpretation could also include differential sensitivity to other stressors, both chemical and physical. These types of costs may be better described as tradeoffs, because altered sensitivity may actually be tied directly to the mechanism of adaptation and these tradeoffs could also be beneficial (e.g. cross-resistance in insects to multiple insecticides).

Tradeoffs associated with resistance have been described in several fish populations adapted to aryl hydrocarbon contamination. Tomcod in the PCB-
contaminated portion of the Hudson River exhibited reduced abundance and a truncated age structure (Wirgin and Waldman 2004). Killifish in New Bedford Harbor were observed to have elevated levels of parasitic infection compared to reference fish (Cohen 2002), although they did not exhibit increased susceptibility to bacterial challenge (Nacci et al. 2009). In addition, investigation of reproductive costs found no correlation with contamination (Black et al. 1998). A variety of tradeoffs have been described in Elizabeth River killifish including altered immune function and increased disease susceptibility (Faisal et al. 1991; Frederick et al. 2007; Meyer and Di Giulio 2003; Meyer et al. 2005; Weeks et al. 1988), increased sensitivity of lab-reared offspring to PAH-mediated phototoxicity and hypoxia, and reduced survival and growth of lab-reared offspring in clean conditions (Meyer and Di Giulio 2003). While the aforementioned studies’ authors described these results as examples of fitness costs, many of these results could also be evidence of direct or secondary toxic effects.

Very few studies in vertebrates have investigated the effect of contaminant adaptation on response to other chemical stressors, especially highly-divergent classes of contaminants. In addition to the aforementioned study of PAH-mediated sensitivity to phototoxicity, there is some evidence that mosquitofish adapted to exposure to toxaphene and endrin were also resistant to other organochlorine pesticides and to some herbicides (Culley and Ferguson 1969; Fabacher and Chambers 1974). The ability of fish populations to adapt to chronic anthropogenic contamination and for this adaptation to drive changes in response to multiple classes of xenobiotics has interesting implications from both an evolutionary and regulatory perspective. If organisms can adapt to chronic pollution, some might argue that regulatory limits could be set higher. Conversely, the strength of pollution-driven selection that engenders
changes in xenobiotic response on a population level clearly demonstrates the ability of complex mixtures of contaminants to alter population genetics in a relatively rapid and drastic fashion.
Figure 12: Response of King’s Creek and Elizabeth River larvae exposed to chlorpyrifos.

Average percent mortality (±SEM) of King’s Creek (black bars) and Elizabeth River (white bars) 5 dph larvae exposed for 24 h to 5 µg/L chlorpyrifos (CPF) or 10 µg/L chlorpyrifos, alone or in combination with 1 µg/L β-naphthoflavone (BNF). Bars not marked by the same letter are significantly different at p<0.05 (ANOVA, Tukey-adjusted LSMeans). n≥45 per treatment group.
Figure 13: Response of King’s Creek and Elizabeth River larvae exposed to permethrin.

Average percent mortality (±SEM) of King’s Creek (black bars) and Elizabeth River (white bars) 5 dph larvae exposed for 24 h to 400 µg/L permethrin (PRM) or 10 µg/L permethrin, alone or in combination with 1 µg/L β-naphthoflavone (BNF). Bars not marked by the same letter are significantly different at p<0.05 (ANOVA, Tukey-adjusted LSMeans). n≥45 per treatment group.
Figure 14: Response of King’s Creek and Elizabeth River larvae exposed to fenvalerate.

Average percent mortality (±SEM) of King’s Creek (black bars) and Elizabeth River (white bars) 5 dph larvae exposed for 24 h to 10 µg/L fenvalerate (FNV) or 25 µg/L fenvalerate, alone or in combination with 1 µg/L β-naphthoflavone (BNF). Bars not marked by the same letter are significantly different at p<0.05 (ANOVA, Tukey-adjusted LSMeans). n≥45 per treatment group.
Figure 15: Response of King’s Creek and Elizabeth River larvae exposed to carbaryl.

Average percent mortality (±SEM) of King’s Creek (black bars) and Elizabeth River (white bars) 5 dph larvae exposed for 24 h to 1 mg/L carbaryl (CRB) or 10 mg/L carbaryl, alone or in combination with 1 µg/L β-naphthoflavone (BNF). Bars not marked by the same letter are significantly different at p<0.05 (ANOVA, Tukey-adjusted LSMean). n≥45 per treatment group.
Figure 16: Ethoxyresorufin-o-deethylase (EROD) and deformity response of King’s Creek and Elizabeth River embryos exposed to carbaryl.

Response of King’s Creek (black bars and solid line) and Elizabeth River (white bars and dashed line) embryos exposed to 1 mg/L carbaryl or 10 mg/L carbaryl from 24 to 144 hours post fertilization (hpf). CYP1 activity was measured by the in ovo ethoxyresorufin-o-deethylase (EROD) assay at 96 hpf; lines show average EROD activity (±SEM) expressed as percent of King’s Creek DMSO-dosed control EROD activity. Cardiac deformities were scored blind on a three point scale (0 = normal heart, 1 = mild deformity, 2 = severe deformity) at 144 hpf; bars show average deformity score (±SEM). Bars marked by * are significantly different from the corresponding DMSO-dosed control at p<0.05 (ANOVA, Dunnett’s adjustment). EROD points marked by # are significantly different at p<0.05 (ANOVA, Dunnett’s adjustment). n=30 per treatment group.
5. Compound- and mixture-specific differences in resistance to aryl hydrocarbons among Fundulus heteroclitus subpopulations throughout the Elizabeth River estuary (Virginia, USA)

This chapter is a collaborative effort of Bryan W. Clark, Ellen M. Cooper, Heather M. Stapleton, and Richard T. Di Giulio.

5.1 Introduction

The Elizabeth River (Virginia, USA) is a highly industrialized and urbanized estuary in the southern portion of the Chesapeake Bay watershed. Human activities have greatly influenced contaminant inputs to the entire Elizabeth River estuary (Walker et al. 2004). In particular, several former wood treatment facilities have contaminated significant portions of the river and sediments with creosote. Creosote is a complex mixture consisting primarily of unsubstituted polycyclic aromatic hydrocarbons (PAHs), as well as heterocyclic and phenolic PAHs. At the Atlantic Wood Industries Superfund site, total PAH concentrations of 100-500 µg/g dry sediment are commonly reported (Mulvey et al. 2002; Mulvey et al. 2003; Vogelbein and Unger 2003; Walker et al. 2004).

PAHs are generated by incomplete combustion of organic compounds and can enter the environment through natural sources such as forest fires or through anthropogenic activities such as fossil fuel use. Furthermore, PAHs are ubiquitous and appear to be increasing in the environment, tracking human population growth and utilization of fossil fuels (Van Metre and Mahler 2005; Van Metre et al. 2000). Estuarine habitats are particularly vulnerable to PAH contamination; exposures occur from a
variety of sources, including oil shipping and refining, industrial outfalls, wastewater discharges, urban runoff, and atmospheric deposition (Latimer and Zheng 2003).

Classically, PAHs are known as carcinogenic, immunosuppressive, and non-specific narcotic toxicants contaminants. Some are agonists for the aryl hydrocarbon receptor (AHR), while others are antagonistic or do not appear to have great affinity for the receptor (Billiard et al. 2004; Billiard et al. 2002; Denison and Nagy 2003). In addition to the well-established modes of PAH action, environmentally-relevant concentrations of some PAHs can cause early life stage toxicity and teratogenic effects in fish. In these cases, PAH toxicity often manifests as cranio-facial and cardiac malformations, and pericardial and yolk-sac effusion similar to the “blue-sac syndrome” observed with the related and highly-studied planar halogenated aromatic hydrocarbons (pHAHs; e.g. 2,3,7,8 tetrachlorodibenzo-p-dioxin (TCDD)) (Hahn 2002; Prasch et al. 2003a; Toomey et al. 2001).

*Fundulus heteroclitus* (the Atlantic killifish or mummichog; hereafter referred to as killifish), is a small teleost fish found in the Elizabeth River and in estuarine ecosystems along the Atlantic coast of North America from Newfoundland to Florida (Shute 1980). Killifish are one of the most abundant intertidal fishes and are a major component of estuarine food webs (Kneib 1986b; Meredith and Lotrich 1979; Teo and Able 2003a; Yozzo et al. 1994). Despite wide distribution, individual killifish have relatively small home ranges (Lotrich 1975; Skinner et al. 2005); their high site fidelity and relatively limited migration make them ideal for studying the impacts of local contamination and other stressors (reviewed in Burnett et al. 2007).

The killifish population inhabiting the Atlantic Wood Industries Superfund Site is subject to chronic exposure to highly PAH-contaminated sediments, but has developed a
remarkable resistance to the acute toxicity and teratogenic effects caused by aryl hydrocarbons and Elizabeth River sediments (Meyer and Di Giulio 2002; Meyer et al. 2002; Ownby et al. 2002). Ownby et al. (2002) compared tolerance caused by PAH-contaminated sediments in killifish embryos from populations inhabiting four sites on the Elizabeth River, including the Atlantic Wood site, and a York River, VA reference population. Exposed embryos from the reference population suffered from a variety of cardiac abnormalities, but embryos from Atlantic Wood parents showed a near-complete resistance to these effects. Embryos from other Elizabeth River populations had an intermediate degree of tolerance to the same effects. The level of resistance was roughly associated with contaminant levels at the sites of collection, perhaps reflecting population exposure history.

In addition to resisting toxicity, Atlantic Wood killifish are recalcitrant to induction of cytochrome P450 (CYP1) metabolic enzymes by aryl hydrocarbon receptor (AHR) agonists such as certain PAHs and PCB-126 (3, 3', 4, 4', 5-pentachlorobiphenyl) (Meyer and Di Giulio 2002; Meyer et al. 2002; Van Veld and Westbrook 1995; L.P. Wills et al. 2010). Recalcitrance to CYP1 induction is also concomitant with marked resistance to the toxic effects of dioxin-like compounds (DLCs) in several other fish populations from polluted sites (Bello et al. 2001; Nacci et al. 2002b; Powell et al. 2000; Prince and Cooper 1995b; Roy et al. 2002). CYP1 induction is generally used as a marker of AHR pathway activation, so recalcitrance to CYP1 induction may indicate that suppression of AHR pathway activity is an important component of the aryl hydrocarbon-resistant phenotype. Furthermore, gene knockdown demonstrated that the teratogenic effects of aryl hydrocarbons in killifish and other fish are mediated at least in part through the AHR pathway (Billiard et al. 2006; Clark et al. 2010; Prasch et al. 2003b). Ownby and co-
workers did not investigate CYP1 induction in their experiments; with the exception of the Atlantic Wood subpopulation, the role that AHR pathway suppression played in the resistance of individual Elizabeth River subpopulations was unknown.

The goal of the current study was to compare the resistance of killifish subpopulations from throughout the Elizabeth River estuary to CYP1 induction and cardiac teratogenesis generated by aryl hydrocarbons, both individually and in mixtures. Embryos were exposed to individual PAHs, PCB-126, a simple PAH mixture, and two complex PAH mixtures.

5.2 Materials and methods

5.2.1 Fish

Adult killifish were collected with wire mesh minnow traps from five sites in the Elizabeth River (Virginia, USA) (Figure 1). The Atlantic Wood site is located at the Atlantic Wood Industries Superfund Site on the Southern Branch (36°48'27.2" N, 76°17'38.1" W). The Scuffeltown Creek site (36°48'33.9" N, 76°17'04.1" W) is located directly across the river, about 0.8 km from the Atlantic Wood site in a city recreation area. The Jones Creek site (36°48'05.5" N, 76°16'43.8" W) is located about two km south (upriver) of the Atlantic Wood site in the upstream end of a small tributary that empties into the southern branch in between the Atlantic Wood site and another former creosote facility (Republic Creosoting). Previous work utilized killifish collected closer to the mouth of Jones Creek (Mulvey et al. 2002; Mulvey et al. 2003; Ownby et al. 2002). The Pescara Creek site (36°50’02.7” N, 76°16’38.4” W) is on the Eastern Branch, approximately 5.6 km by river from the Atlantic Wood site. It is located near a shipyard
and in an area that may have been the historical site of a wood treatment facility 
(Michael Unger, Virginia Institute of Marine Science, personal communication). The 
Mains Creek site is located approximately 8 km south of the Atlantic Wood site, 
upstream of the major sites of creosote contamination and close to the junction with the 
Intracoastal Waterway (36°45’13.5” N, 76°24’58.9” W). Killifish from a well-studied 
reference population were collected at King’s Creek, a relatively-uncontaminated 
tributary of the Severn River which feeds into the lower Chesapeake Bay in southeastern 
Virginia (37°18’16.2”N, 76°24’58.9’W). In the laboratory, fish were maintained in 20‰ 
artificial sea water (ASW; Instant Ocean, Foster & Smith, Rhinelander, WI, USA) at 23-
25 °C, with a 14:10 light:dark cycle, and were fed pelleted fish feed (Aquamax ® 
Fingerling Starter 300, PMI Nutritional International, LLC, Brentwood, MO, USA). Eggs 
were collected by manual spawning of females and fertilized \textit{in vitro} by expressing 
sperm from males directly into a beaker containing eggs in ASW. Eggs were set aside 
for a minimum of one hour for fertilization, then washed briefly with 0. 3% hydrogen 
peroxide in ASW. Adult care and reproductive techniques were non-invasive and 
approved by the Duke University Institutional Animal Care & Use Committee (A234-07- 
08).

5.2.2 Chemicals and dosing

\(\beta\)-naphthoflavone (BNF), ethoxyresorufin, and dimethyl sulfoxide (DMSO) were 
purchased from Sigma-Aldrich (St. Louis, MO, USA). Benzo[k]fluoranthene (BkF), 
benzo[a]pyrene (BaP), and fluoranthene (Fl) were purchased from Sigma-Aldrich (St. 
Louis, MO, USA) or from Absolute Standards, Inc. (Hamden, CT, USA) and 3,3’,4,4’,5-
pentachlorobiphenyl (PCB-126) was purchased from AccuStandard (New Haven, CT, 
USA). Coal tar (standard reference material 1597a), a standard mixture of PAHs
consisting of 4363.83 mg total PAHs/L, was purchased from the National Institute of Standards and Technology (Gaithersburg, MD, USA). Coal tar came dissolved in toluene; it was dried by nitrogen evaporation and then redissolved in DMSO. Chemical stocks were prepared by dissolving the appropriate mass of BNF, BkF, BaP, Fl, or PCB-126 in DMSO. Elizabeth River sediment extract (ERSE) was prepared from sediment collected along two roughly east to west transects in the ditches adjacent to the bridge on the north side of the Atlantic Wood Superfund Site. Around 2 kg of sediment were collected from each of three points along the two transects and stored in glass jars in the lab at 4 °C for approximately 30 days. For extraction, approximately equal volumes of sediment and deionized water were combined and shaken vigorously for 1 minute, then centrifuged at 10000 g for 25 minutes. The supernatant from multiple extractions was decanted, combined and mixed, then frozen at -80 °C in individual aliquots.

In the first experiment, embryos were exposed to BNF, BkF, PCB-126, a mixture of BaP and Fl, ERSE, or coal tar; EROD response and cardiac teratogenesis were assessed. The exposure concentrations were 10 µg/L BNF, 300 µg/L BkF, 1 µg/L PCB-126, and 100 µg/L BaP plus 500 µg/L Fl. The ERSE concentration was 10% (v/v) and the coal tar concentration was 0.005% (v/v). A second experiment was conducted to compare EROD response in embryos exposed to equimolar (3 nM) doses of BNF, BaP, BkF, and PCB-126. For all experiments, embryos were dosed individually with 10 mL of dosing solution in 20-mL glass scintillation vials (VWR, West Chester, PA) beginning at 24 hpf. In all experiments, control embryos were exposed to DMSO at a concentration (v/v) equal to that in the dosed groups; DMSO concentrations were maintained at <0.03% across all treatments. All dosing solutions were made in 20‰ ASW. In addition, all dosing solutions contained 21 µg/L ethoxyresorufin, a substrate for the
ethoxyresorufin-o-deethylase (EROD) assay. Embryos were maintained in dosing solution in the incubator at 27 °C from 24 hours post fertilization (hpf) until the appropriate time for the given analysis (96 hpf for EROD, 144 hpf for deformity assessment). EROD data were not collected for the BaP and Fl mixture exposure because the dose of Fl used reduces CYP1 activity to low levels even in highly inducible fish. All exposures consisted of at least three experimental replicates with n ≥ 10 embryos per treatment group per experiment with two exceptions; the equimolar exposures and the BaP plus Fl exposures consisted of three experimental replicates with n ≥ 8 embryos per treatment group per experiment.

5.2.3 EROD assay

CYP1 activity was measured via the in ovo EROD assay modified from Nacci et al. (1998). In brief, embryos are exposed to ethoxyresorufin with dosing solutions, as described in section 2.2. CYP1s mediate cleavage of the ethoxy group yielding the fluorescent product resorufin, which collects in the urinary bladder of the embryo. At 96 hpf, the fluorescence due to resorufin in the urinary bladder was visualized using fluorescent microscopy (50X magnification, rhodamine red filter set; Axioskop, Zeiss, Thornwood, NY, USA). EROD activity was measured as intensity of fluorescence within the bladder normalized to intensity within a region outside the bladder and quantified using IPLab software (BD Biosciences, Rockville, MD, USA). All EROD values are expressed as percent of the King’s Creek (reference) population DMSO dosed control group response unless otherwise noted.
5.2.4 Deformity assessment

Cardiac teratogenesis was scored blind at 144 hpf under light microscopy. Deformity severity was scored as a 0 (normal), 1 (moderate deformity), or 2 (severe deformity) as described previously (Clark et al. 2010; Matson et al. 2008a). Cardiac abnormalities primarily manifested as heart elongation, improper atrial-ventricular alignment, and pericardial edema. Embryos scored as a 1 on the deformity scale have a range of heart abnormalities ranging from moderate changes in atrial-ventricular alignment to misalignment of the chambers accompanied by elongation of the atrium and sinus venosus (the inflow vessel of the heart). All embryos scored as a 1 maintained some blood flow observable by light microscopy. Embryos scored as a 2 on the deformity scale exhibited severe atrial-ventricular misalignment and elongation of the heart or complete alteration of the heart to a single, elongated tube. Many of the individuals scored as a 2 did not have any apparent blood flow, but some exhibited severely reduced blood flow.

5.2.5 Determination of PAHs in sediments

Wet sediment (approximately 0.6 g) was ground with Na₂SO₄, spiked with a surrogate standard mix containing deuterated 2-methylnaphthalene, fluorene, fluoranthene and perylene, and extracted in 50/50 dichloromethane (DCM) and hexane (v/v) with an accelerated solvent extractor (ASE 200, Dionex, Sunnyvale, CA). Extracts were concentrated with rapid evaporation under N₂ (Turbo Vap, Caliper LifeSciences, Hopkinton, MA, USA) to approximately 0.5 mL, and cleaned up with column chromatography on Na₂SO₄ and 6% deactivated alumina (4 g) eluting with 50 mL 50/50 DCM/hexane. Purified extracts were again concentrated under N₂ with HCl-cleaned
copper turnings to remove sulfur. PAHs were analyzed using a gas chromatograph and mass spectrometer (Agilent GC 6890N, MS 5975, Newark, DE) in electron impact mode using selected ion monitoring and splitless injection (250°C). Analytes were separated on a DB-5 column (30 m, 250 µm nominal diameter, 0.25 µm film thickness; J&W Scientific) using an oven temperature program with a thermal gradient (40°C for 0.6 min, increase to 280°C over 14.6 min, hold at 280°C for 24 min). Prior to analysis, extracts were spiked with an internal standard mix for quantitation, which contained deuterated naphthalene, phenanthrene, pyrene and benzo[a]pyrene. Recoveries of surrogate standards were 68-103% with the exception of 2-methylnaphthalene, for which recoveries were near 36%. Sediment moisture content was measured gravimetrically by weighing approximately 1.3 g wet sediment before and after drying at 105°C for 16 hours. Moisture content was calculated as (moist weight – dry weight)/dry weight, and used to correct PAH concentrations to dry weight.

5.2.6 Data analysis

All analyses were performed using JMP 8.0 (SAS Institute Inc, Cary, NC, USA). For all analyses, the individual embryo was the unit of replication. Non-parametric deformity and EROD data were rank-transformed and were analyzed by analysis of variance (ANOVA) followed by least square means (LSMeans) procedures. As stated previously, experiments were replicated a minimum of three times; no differences between experimental replicates were observed for any test. For post hoc comparisons, Tukey-adjusted pairwise comparisons were conducted to determine differences between groups. Statistical significance was accepted at $p \leq 0.05$ for all tests.
5.3 Results

5.3.1 PAHs in sediments

The total sediment burden of the targeted PAHs (ng/g dry sediment) is shown in Table 1. Atlantic Wood sediments had by far the highest PAH burden whereas Scuffeltown Creek, Pescara Creek, and Jones Creek had significant, but lower PAH burdens. The King’s Creek (reference) site had a lower PAH burden than all of the Elizabeth River sites except for the Mains Creek site. The concentrations (ng/g dry sediment) of individual measured PAHs at each of the five Elizabeth River sites and the King’s Creek site are displayed in Figure 2. The PAH profile was relatively similar among the sites, although the profile of the King’s Creek and Mains Creek sites was shifted toward lower molecular weight PAHs. The PAH profile at the Pescara Creek site was shifted toward higher molecular weight PAHs.

5.3.2 Response of subpopulations to individual aryl hydrocarbons

The mean deformity score and EROD activity induced by individual aryl hydrocarbons in the five Elizabeth River subpopulations and the reference population are shown in Figure 3. The model PAH BNF (10 µg/L) induced a mild degree of cardiac deformity (0.64±0.08) and a strong EROD response (3030±270%) in the susceptible embryos from the King’s Creek reference population but not in the highly resistant Atlantic Wood population (Figure 3A). In addition, the Scuffeltown Creek and Pescara Creek subpopulations were resistant to teratogenesis caused by BNF. The Jones Creek subpopulation exhibited a slight elevation in mean deformity score (0.25±0.09), but this was not statistically different from DMSO exposure (p=0.8952). The EROD responses of the Scuffeltown Creek and Jones Creek subpopulations were similar to that of the highly
resistant Atlantic Wood subpopulation, whereas EROD was clearly induced above control (674±140%; \( p=0.0052 \)) in the Pescara Creek subpopulation. Finally, the Mains Creek subpopulation exhibited a mean deformity score that was not statistically different from that of the reference population (0.47±0.09; \( p=0.6790 \)); however, it was also not statistically different from the score of DMSO-dosed Mains Creek embryos (\( p=0.0542 \)).

The Mains Creek EROD response was statistically elevated above DMSO-dosed controls (1950±210%; \( p<0.0001 \)), and was intermediate between that of the other Elizabeth River subpopulations and the reference population.

BkF (300 µg/L) induced substantial cardiac teratogenesis (mean deformity score of 1.2±0.09) and a strong EROD response (1890±160%) in reference embryos (Figure 3B). Atlantic Wood embryos were resistant to BkF-induced teratogenesis, but did exhibit a low EROD response. However, it was not statistically different from controls (254±30%; \( p=0.4924 \)). In contrast to the Atlantic Wood subpopulation, all other Elizabeth River subpopulations exhibited at least some induction of EROD response that was statistically greater than control (\( p<0.05 \)). Several subpopulations also exhibited significant cardiac teratogenesis due to BkF. The response of the Scuffeltown Creek and Jones Creek subpopulations was similar. BkF induced an EROD response of 627±60% in Scuffeltown Creek embryos and 630±50% in Jones Creek embryos, which was statistically greater than controls for both populations (\( p<0.0001 \), both subpopulations). The mean deformity scores were slightly elevated in both Scuffeltown Creek (0.33±0.10) and Jones Creek (0.28±0.09) but neither were significantly different from their controls (\( p=0.3229 \) and \( p=0.8244 \), respectively). The mean deformity score of the Pescara Creek subpopulation was slightly higher but also not statistically different from its control (0.44±0.10, \( p=0.1525 \)). The EROD response of Pescara Creek embryos
was significantly greater than controls (1120±83%; \( p < 0.0001 \)). As with BNF, BkF induced an elevated EROD response (1560±130%) and a mean deformity score (0.87±0.09) in the Mains Creek subpopulation that were significantly elevated above DMSO-dosed embryos (\( p < 0.0001 \) for both).

Exposure to PCB-126 (1 µg/L) induced a strong EROD response (1790±170%) and severe cardiac deformities (mean deformity score of 1.5±0.12) in reference embryos, but embryos from the Atlantic Wood subpopulation were highly resistant (Figure 3C). Embryos from the Scuffeltown Creek and Jones Creek subpopulations were also highly resistant to both EROD induction and cardiac deformity induced by PCB-126. The Pescara Creek subpopulation did not exhibit an increase in mean deformity score compared to controls, but EROD activity was significantly induced (475±110%; \( p < 0.0001 \)). Finally, the Mains Creek subpopulation exhibited EROD induction (1230±110%) that was about two-thirds of the response of reference fish. Although the mean deformity score of PCB-126 exposed Mains Creek embryos was elevated (0.44±0.12), it was not statistically different from DMSO-dosed controls (\( p = 0.2876 \)) and it was much lower than the response of reference fish.

5.3.3 Response of subpopulations to PAH mixtures

The mean deformity score and EROD activity due to exposure of the five Elizabeth River subpopulations and the reference populations to PAH mixtures are shown in Figure 4. A mixture of 100 µg/L BaP and 500 µg/L Fl caused severe cardiac teratogenesis (mean deformity score of 1.44±0.18) in reference embryos (Figure 4A; EROD measurements were not made for BaP and Fl mixture). The mean deformity score was not elevated above the respective DMSO-dosed control in any of the
Elizabeth River subpopulations exposed to the BaP and Fl mixture, except the Jones Creek subpopulation (mean deformity score of 0.47±0.13; $p=0.0391$).

Coal tar (0.005% v/v) was a very potent teratogen in reference embryos (Figure 4B). Coal tar dosed reference embryos had a mean deformity score of 1.89±0.11 and EROD activity of 908±130%. Despite the potency, coal tar did not induce deformities in Atlantic Wood embryos. EROD activity was significantly induced in Atlantic Wood embryos (207±29%; $p=0.0452$). Coal tar also did not cause deformities in Scuffeltown Creek embryos, although EROD activity was significantly induced (502±44%; $p<0.0001$). The Jones Creek subpopulation exhibited an intermediate deformity response (0.60±0.13) and EROD activity (346±25%) that were significantly greater than controls ($p \leq 0.0001$, for both). The Pescara Creek subpopulation also exhibited an intermediate deformity response (0.60±0.13) and EROD response (863±80%) that were significantly greater than controls ($p=0.0011$ and $p<0.0001$, respectively). Mains Creek embryos exhibited a mean deformity score of 1.33±0.13 and an EROD response of 834±93% which were significantly greater than controls ($p<0.0001$ for both). Although the response of the Mains Creek subpopulation to coal tar was somewhat similar to that of the reference population, the deformity score was significantly lower than that of reference fish ($p<0.0001$).

Much like coal tar, ERSE (10% v/v) induced severe cardiac deformities in reference embryos (Figure 3C). Reference embryos exposed to ERSE exhibited a mean deformity score of 1.80±0.13 and an EROD activity of 742±94%. ERSE did not cause deformities in Atlantic Wood embryos. EROD activity was somewhat induced in Atlantic Wood embryos (197±37%), but was not statistically different from control ($p=0.0545$). ERSE also did not cause deformities in Scuffeltown Creek embryos,
although EROD activity was significantly induced (480±53%; p<0.0001). The Jones Creek subpopulation exhibited an intermediate deformity response (0.57±14) and EROD response (325±30%) that were significantly greater than controls (p=0.0016 and p<0.0001, respectively). The Pescara Creek subpopulation was responsive to ERSE; the mean deformity score was 1.13±0.13 and their EROD response was 882±104%, both of which were significantly greater than controls (p<0.0001). The Mains Creek subpopulation was highly-response to ERSE. Mains Creek embryos exhibited a mean deformity score of 1.43±0.13 and an EROD response of 776±78% that were significantly different from controls (p<0.0001). Although lower, the Mains Creek deformity response was not statistically different from that of reference embryos (p=0.3326).

5.3.4 Response of subpopulations to equimolar concentrations of individual aryl hydrocarbons

To better compare the AHR pathway responsiveness of each subpopulation to different agonists, EROD activity was assessed in response to equimolar (3 nM) concentrations of BaP, PCB-126, BNF, and BkF (Figure 5). The reference population exhibited the highest EROD response to BaP (631±66%), PCB-126 (822±130%), BNF (1440±83%), and BkF (1310±95%). The Atlantic Wood subpopulation exhibited very little response (<40% of KC DMSO response for all exposures). The Scuffeltown Creek subpopulation had a very similar EROD response to that of the highly resistant Atlantic Wood population. The Jones Creek subpopulation exhibited low response to most exposures. The Jones Creek EROD response (261±59%) to BkF exposure appeared elevated but it was not statistically different from the DMSO-dosed control (p=0.4013). The Pescara Creek subpopulation exhibited elevated EROD responses to PCB-126 (180±55%) and BNF (139±48%), but neither were statistically different from control
In addition, the Pescara Creek subpopulation EROD response to BkF (490±60%) was significantly greater than control (p<0.0001). The Mains Creek subpopulation response was similar to the other Elizabeth River subpopulations for BaP, but its EROD response was statistically elevated for exposure to PCB-126 (657±150%; p<0.0001), BNF (720±160%; p<0.0001), and BkF (691±91%; p<0.0001).

5.4 Discussion

The Elizabeth River killifish investigated in the current study exhibited subpopulation-specific patterns of response to aryl hydrocarbons and PAH mixtures. Furthermore, even subpopulations from sites with relatively low sediment PAH concentrations exhibited strong resistance to some compounds. These varied responses among subpopulations demonstrated that while the adapted phenotype is found across a great distance within the estuary (~20 river km), the underlying nature of the adaptation is not completely uniform from site to site.

5.4.1 Variation in adaptive response of Elizabeth River subpopulations

The response of Elizabeth River killifish to aryl hydrocarbons, both singly and in mixtures, varied greatly among subpopulations. In addition, the pattern of response was often specific to an individual subpopulation. To allow better comparison of the patterns of aryl hydrocarbon response, all of the deformity scores for each subpopulation are compiled in Figure 6. The reference population was obviously the most sensitive to cardiac teratogenesis across all contaminants, and the Atlantic Wood subpopulation the least sensitive. Overall, the Jones Creek and Scuffeltown Creek subpopulations
demonstrated similar levels of cardiac teratogenesis to the highly-adapted Atlantic Wood subpopulation, although the Jones Creek subpopulation was more sensitive to the complex PAH mixtures (ERSE and coal tar). On the other hand, the Pescara Creek subpopulation was relatively resistant to cardiac teratogenesis generated by all of the individual aryl hydrocarbons (BNF, BkF, and PCB-126) and the simple mixture of BaP and Fl, but was more sensitive to the complex mixtures. It is also noteworthy that the Pescara Creek subpopulation was more sensitive to ERSE than coal tar; each of the other subpopulations showed a consistent response to the two complex mixtures. Finally, the Mains Creek subpopulation was nearly as susceptible as reference fish to cardiac teratogenesis generated by all of the exposures tested, with the dramatic exception of their resistance to PCB-126 and the BaP and Fl mixture.

Notably, all of the Elizabeth River subpopulations are quite resistant to cardiac teratogenesis generated by PCB-126 and the simple mixture of BaP and Fl. Interestingly, several of the Elizabeth River subpopulations show a greater deformity response due to BkF than PCB-126, despite the fact that BkF is a common component of PAH contamination in the Elizabeth River. This lack of sensitivity to PCB-126 has been reported in previous studies of the Atlantic Wood subpopulation (Meyer and Di Giulio 2002). In fact, Nacci et al. (2010) demonstrated that Atlantic Wood killifish were even less sensitive to PCB-126 than other adapted killifish from sites with much greater PCB contamination than in the Elizabeth River.

In addition to varying in susceptibility to cardiac teratogenesis, the subpopulations varied in their AHR pathway response as measured by EROD activity (compiled in Figure 7). To compare overall magnitude of response, Figure 7A shows EROD responses of each subpopulation normalized to the same point (King’s Creek
DMSO response). As seen for cardiac teratogenesis, the reference population showed the largest EROD responses and the Atlantic Wood subpopulation showed the lowest EROD responses. The Jones Creek and Scuffeltown Creek EROD responses were similar to those of the Atlantic Wood subpopulation, while the Mains Creek EROD responses were similar to those of the reference population. The Pescara Creek subpopulation demonstrated an intermediate magnitude of EROD response.

Interestingly, several of the subpopulations exhibit intermediate EROD response when exposed to aryl hydrocarbons that do not induce cardiac deformities in those subpopulations. For example, EROD activity was strongly induced in Mains Creek embryos exposed to PCB-126, despite their resistance to PCB-induced teratogenesis. This disconnect in some subpopulations between resistance to AHR pathway activation and resistance to cardiac teratogenesis is made even more apparent by visualizing each subpopulation's fold change or relative inducibility of EROD activity (Figure 7B). The fold change in EROD activity remains low for the Atlantic Wood subpopulation, but several of the other subpopulations demonstrate a fold change in EROD response that is very similar to that of the reference population. This is particularly noteworthy for BkF, which induced a fold change in EROD activity in Scuffeltown Creek, Jones Creek, and Pescara Creek embryos that was almost identical to that of reference embryos. However, all three Elizabeth River subpopulations were quite resistant to cardiac teratogenesis generated by BkF. Likewise, the fold change in PCB-induced EROD activity for Pescara Creek and Mains Creek embryos was similar to that induced in reference embryos, yet both Elizabeth River subpopulations were resistant to PCB-induced cardiac teratogenesis. Additionally, the fold change in EROD response generated by ERSE and coal tar was disproportionately high in Scuffeltown Creek
embryos; Scuffeltown Creek embryos were nearly as resistant to cardiac teratogenesis generated by ERSE and coal tar as were Atlantic Wood fish. Finally, the fold change in EROD activity of the Pescara Creek subpopulation was greater than or very similar to that of reference fish for all exposures. This seems to indicate that the Pescara Creek subpopulation possesses an AHR pathway that is just as inducible as that of reference fish, but that their basal (and perhaps maximal) EROD response is lower.

A number of interesting conclusions can be drawn from the varied patterns of AHR pathway activation and resistance to cardiac teratogenesis exhibited by the Elizabeth River subpopulations. First, while it is clear that adaptation to PAHs occurs throughout the Elizabeth River estuary, it is also clear that the underlying nature of the adaptation is not identical throughout the estuary. If the adaptation were simple and identical among subpopulations, one would expect to see a pattern of resistance that perhaps varied only in magnitude across all exposure types. Instead, some subpopulations (i.e. Mains Creek and Pescara Creek) exhibited a high degree of AHR pathway activation for all exposures and others for only some. Likewise, some subpopulations were highly resistant to cardiac teratogenesis generated by all aryl hydrocarbons, while others (i.e. Jones Creek and Pescara Creek) were highly resistant to cardiac teratogenesis due to some exposures but only moderately resistant to others. As described previously, aryl hydrocarbon-generated cardiac teratogenesis in fish is mediated at least in part through the AHR pathway (Billiard et al. 2006; Clark et al. 2010; Prasch et al. 2003b) and the AHR is a likely target for development of resistance in multiple fish populations adapted to DLCs (reviewed in Wirgin and Waldman 2004). However, the fact that some subpopulations exhibit resistance to teratogenicity while still displaying a robust AHR pathway response demonstrates that suppression of the AHR
pathway alone is not responsible for resistance to toxicity in those groups. Overall, this variability in subpopulation response suggests that resistance to aryl hydrocarbons in killifish from the Elizabeth River is conferred by multiple genes.

Evolutionary theory generally predicts that adaptation of a population to a stressor would be polygenic, but in the process of resistance to xenobiotics this is actually somewhat rare (Coustau et al. 2000; Macnair 1991; Reznick and Ghalambor 2001). In fact, Macnair (1991) predicted polygenic adaptation to be probable only if the distance the mean response of a population (i.e. susceptibility to a toxicant) must move to confer survival is not substantially larger than the mean response of the original population. This seems to explain why many of the cases of chemical resistance in plants and insects are conferred by "mutations of major effect" (Coustau et al. 2000). Therefore, the severity of toxicity of the Elizabeth River environment to naïve fish and the degree of tolerance exhibited by the Elizabeth River killifish would lead to a prediction that monogenic adaptation is the only way to achieve such a dramatic change in tolerance.

However, previous work has identified alterations in several xenobiotic metabolism and excretion pathways associated with resistance in Atlantic Wood killifish. These include elevated levels of glutathione S-transferase (Armknecht et al. 1998), hepatic P-glycoprotein (Cooper et al. 1999), and UDP-glucuronosyl transferase (UGT) and sulfotransferase (Gaworecki et al. 2004). Work in our lab determined that Atlantic Wood killifish also have upregulated antioxidant defenses (Bacanskas et al. 2004; Meyer et al. 2003a). It is important to note that the current study was conducted with F1 embryos, demonstrating heritable patterns of response. However, heritability of most of
the adaptive responses just described has not been demonstrated, although upregulation of antioxidant defenses was at least in part heritable.

It is possible that the Elizabeth River killifish may be exhibiting something intermediate between the monogenic and polygenic extremes. For example, butterflies that mimic other species were shown to have achieved their initial similarity through a single gene change, which was later enhanced by polygenic modifiers (Sheppard et al. 1985; Turner et al. 1979). Perhaps Elizabeth River killifish have undergone a major change (i.e. AHR pathway suppression) accompanied or followed by such modifiers. It has been postulated that over the course of adaptation to acute stress, a single major gene change might eventually be replaced by multiple changes with lesser fitness costs (Guillemaud et al. 1998; Labbe et al. 2009; Taylor and Feyereisen 1996). However, because heritability of the adaptations other than AHR pathway suppression is unclear, it is possible that the modifiers are actually acclamatory rather responses than genetic changes.

5.4.2 Association of aryl hydrocarbon response with PAH contamination level and proximity to the Atlantic Wood subpopulation

From the current data it is clear that PAH contamination has had an estuary-wide effect on killifish subpopulations. However, it is not clear how the PAH contamination is driving adaptive changes among the subpopulations. One possibility is that the lower levels of PAHs found outside of the major contamination sites are sufficient to drive adaptation of individual isolated subpopulations of killifish. Another possibility is that adapted subpopulations in the most contaminated areas are acting as sources of adaptive genetic material for other subpopulations.
In general, the overall responsiveness of the subpopulations studied here followed the order King’s Creek > Mains Creek > Pescara Creek > Jones Creek > Scuffeltown Creek > Atlantic Wood. This pattern was roughly the inverse of the total PAH levels found at the sites, which followed the order Mains Creek < King’s Creek < Jones Creek < Pescara Creek = Scuffeltown Creek << Atlantic Wood. However, PAH level does not seem to be fully explanatory. For example, the total PAHs (4493±557 ng/g dry sediment) measured at Pescara Creek were very similar to those measured at the Scuffeltown Creek site (6328±1238 ng/g dry sediment), yet Pescara Creek killifish were one of the more responsive subpopulations. One possible explanation is that the sediment contamination at the Pescara Creek site may be from an older source than at other sites (Michael Unger, Virginia Institute of Marine Science, personal communication), perhaps allowing for greater weathering of the PAHs or making them less bioavailable to drive adaptation. The former possibility is supported by examination of the PAH profile in Figure 2, which shows a greater relative burden of the higher molecular weight PAHs. If this were the case, the Pescara Creek subpopulation might represent a subpopulation “recovering” from adaptation to PAH contamination. However, a shift toward a greater relative burden of higher molecular weight PAHs would also represent enrichment in PAHs that are AHR agonists; this would be expected to make the sediments more toxic unless bioavailability was decreasing concurrently. In contrast to the Pescara Creek subpopulation, the Jones Creek subpopulation was subject to lower total PAH contamination and yet was overall quite resistant to teratogenesis generated by aryl hydrocarbons. Interestingly, Ownby et al. (2002) also found that a killifish subpopulation collected at the mouth of Jones Creek, approximately 900 meters from our collection site, was much more resistant to cardiac teratogenesis
than another subpopulation collected in an area with higher total PAHs in sediments. The most striking example of a disassociation between sediment contamination and resistance to aryl hydrocarbons was exhibited by the Mains Creek subpopulation. Mains Creek killifish were resident in an area with lower measured sediment total PAHs than the reference site, yet were highly-resistant to some of the aryl hydrocarbon exposures tested herein.

Mulvey and coworkers used allozyme frequency (Mulvey et al. 2002) and mitochondrial DNA haplotype (Mulvey et al. 2003) to investigate the genetic structure of subpopulations from throughout the Elizabeth River estuary, including those from the Atlantic Wood, Scuffeltown Creek, and Pescara Creek sites studied here. They also examined fish from Jones Creek (collected in the same sites described by Ownby et al. 2002). They found that genetic distance was significantly correlated with PAH contamination, but not with geographic distance. This is somewhat at odds with the results of the current study. Because it seems highly likely that resistance to cardiac teratogenesis is a major component of adaptation to PAHs, the gene or genes underlying this resistance are probable units of selection. However, in this study the pattern of resistance phenotypes, likely reflecting underlying genotypes, did not correlate very well with contamination level.

As stated previously, one possible explanation for poor correlation of resistance to contaminant level is that the highly-resistant Atlantic Wood subpopulation acts as a source of resistance genes for other subpopulations. This would be logical with the proximate, highly-resistant but relatively less-contaminated Scuffeltown Creek and Jones Creek subpopulations. The varied aryl hydrocarbon response of the Mains Creek subpopulation might also provide evidence in support of a movement of resistance
genes. Mains Creek killifish responded to most aryl hydrocarbons in a very similar fashion to the reference population, as might be expected for a subpopulation exposed to the lowest PAH levels we measured in the Elizabeth River. However, unlike fish from the reference site, they were highly resistant to teratogenesis from PCB-126 and the mixture of BaP and Fl. This could indicate that the Mains Creek subpopulation has some components, but not others, of a multigenic PAH-adaptation present in the Elizabeth River estuary. Mulvey and coworkers did not find evidence of this kind of gene flow, but they did find that migration among localities in the Elizabeth River was 9.6 juveniles and 17.5 adults per generation. This is more than sufficient to move traits among the sites. However, even if this migration were sufficient to distribute the genes underlying resistance throughout the estuary, they would only remain in the individual subpopulations if they conveyed a competitive benefit or at least did not hinder performance. There is evidence that Atlantic Wood fish do not survive as well under clean conditions (Meyer and Di Giulio 2003), perhaps indicating that migrants from a highly-adapted subpopulation would be at a competitive disadvantage in a less-contaminated locale.

Reznick and Ghalambor (2001) reviewed examples of micro-evolution and described the conditions favoring its occurrence. One was the colonization of a new landscape accompanied by the opportunity for rapid population growth. The severe contamination at a Superfund site creates just such an opportunity for an organism able to develop tolerance to these conditions and killifish seem to be particularly adept at this. Another condition favoring micro-evolution was local adaptation within portions of a metapopulation in a heterogeneous environment. They describe this second case as being exemplified by the way anthropogenic contamination creates "new and often
hostile patches of habitat within a landscape.” This would appear to be a perfect description of the situation faced by the killifish metapopulation of the Elizabeth River estuary. Interestingly, gene-flow among subpopulations within a metapopulation can have a homogenizing affect; this was observed for Elizabeth River subpopulations (Mulvey et al. 2002; Mulvey et al. 2003). This concept is actually used to combat insecticide resistance by creation of refugia in the process of integrated pest management (Kinnison and Hairston 2007). However, gene flow among Elizabeth River subpopulations does not seem to be reducing resistance, even in some subpopulations exposed to much lower levels of PAHs than in the Atlantic Wood Superfund site. As discussed previously, this could indicate that the adaptation is useful even for killifish faced with lower levels of PAH contamination, or that the adaptation does not have deleterious consequences for killifish in those cleaner conditions.

5.4.3 Importance of estuary-wide contaminant influence

The occurrence of spatially-extensive resistance to contaminants demonstrates one way in which anthropogenic contamination can exert influence across an entire ecosystem. In addition to the current work, several studies have described the spatial distribution of resistance to contaminants in fish. Yuan et al. (2006) found that Atlantic tomcod (*Microgadus tomcod*) collected in sites spanning 144 km of the Hudson River are resistant to CYP induction by PCBs and polychlorinated dibenzo-*p*-dioxins. Nearly 320 km of the Hudson River is part of a Superfund site, with PCB contamination originating upstream. Fernandez et al. (2004) showed that the degree of PCB burden of tomcod was associated with proximity to the original PCB sources. Despite apparent differences in exposure history, tomcod from throughout the estuary did not vary significantly in their degree of resistance (Yuan et al. 2006). This is likely because
tomcod move throughout the Hudson River during their life cycle and represent a single population. Unlike with the Hudson River tomcod, PCB tolerance of killifish was predicted by the level of sediment PCBs at the site of collection in populations distributed over greater than 100 km (Nacci et al. 2002a). This study appears to demonstrate a case where spatial distribution of contamination drove landscape level changes in populations, rather than movement of adapted individuals.

Regardless of whether landscape level changes in population parameters are driven directly by contamination or by movement of adaptive genes throughout the ecosystem, the fact that anthropogenic contamination exerts effects at this scale may have major consequences. As stated previously, killifish are widely abundant and a major component of estuarine food webs (Teo and Able 2003a; Yozzo et al. 1994). They are omnivorous feeders; reported food sources include plant detritus, macroalgae, grass shrimp, crabs, annelids, and other fish (Able et al. 2007; Allen et al. 1994; Kneib 1986b; Kneib and Stiven 1978; McMahon et al. 2005). In turn, killifish are prey for birds, fishes, and invertebrates (Kneib 1982; Nemerson and Able 2003; Post 2008). For these reasons, alterations of multiple killifish subpopulations could have consequences for the entire Elizabeth River ecosystem.

It is generally accepted that adaptation to a stressor could be accompanied by concurrent fitness costs to populations (Kinnison and Hairston 2007; Wirgin and Waldman 2004). Strong selective pressure is thought to drive reduction in genetic variation and overall fitness, although this has not been demonstrated in the adapted populations described herein (McMillan et al. 2006; Mulvey et al. 2002; Mulvey et al. 2003). Reduced fecundity, growth rate, and survival are also considered classical
fitness costs, and a broader interpretation could also include greater sensitivity to other stressors, both chemical and physical.

Tradeoffs associated with resistance have also been described in the fish populations adapted to aryl hydrocarbon contamination. Tomcod in the Hudson River exhibited reduced abundance and a truncated age structure (Wirgin and Waldman 2004). Killifish in New Bedford Harbor had elevated levels of parasitic infection (Cohen 2002), but no reproductive costs were correlated with contamination (Black et al. 1998). A variety of tradeoffs and altered contaminant sensitivity have been described in Elizabeth River killifish including widespread occurrence of hepatic lesions (Van Veld et al. 1991), altered immune function and increased disease susceptibility (Faisal et al. 1991; Frederick et al. 2007; Meyer and Di Giulio 2003; Meyer et al. 2005; Weeks et al. 1988), increased sensitivity of lab-reared offspring to PAH-mediated phototoxicity and hypoxia, reduced survival and growth of lab-reared offspring in clean conditions (Meyer and Di Giulio 2003), and altered response to other contaminants (Chapter 4). While these results may be examples of fitness costs, many of these results could also be evidence of direct or secondary toxic effects.

5.5 Conclusion

The current study has revealed a diverse pattern of resistance to aryl hydrocarbon-generated AHR pathway activation and cardiac teratogenesis among killifish subpopulations from throughout the Elizabeth River estuary. The results demonstrate that PAH contamination is affecting fish populations across a wide geographic area, either through widespread sediment contamination or through
movement of adaptive genes from subpopulations resident in highly-contaminated sites. Furthermore, the fact that subpopulations exist that are resistance to the toxicity caused by certain aryl hydrocarbons but not to AHR pathway activation suggests that aryl hydrocarbon adaptation in Elizabeth River killifish is brought about by multiple adaptive changes.
<table>
<thead>
<tr>
<th>Site</th>
<th>Total selected PAHs (ng/g dry sediment)</th>
</tr>
</thead>
<tbody>
<tr>
<td>King’s Creek (reference)</td>
<td>526 ± 624</td>
</tr>
<tr>
<td>Atlantic Wood</td>
<td>122,665 ± 16,854</td>
</tr>
<tr>
<td>Scuffeltown Creek</td>
<td>6,328 ± 1,253</td>
</tr>
<tr>
<td>Jones Creek</td>
<td>1,910 ± 518</td>
</tr>
<tr>
<td>Pescara Creek</td>
<td>4,493 ± 557</td>
</tr>
<tr>
<td>Mains Creek</td>
<td>186 ± 201</td>
</tr>
</tbody>
</table>
Figure 17: Location of King’s Creek and Elizabeth River collection sites.

The smaller map shows the lower Chesapeake Bay with the location of the King’s Creek (reference) site and the Elizabeth River. The expanded map shows the location (black dots) of the five Elizabeth River collection sites. Circles mark the approximate location of three former creosote facilities that have contributed major contamination to the river.
Figure 18: Individual PAHs measured at five Elizabeth River sites and King's Creek (reference).

Concentration (ng PAH/g dry sediment) of selected PAHs at each of five sites in the Elizabeth River and the King's Creek site (reference). Note that the horizontal axes indicating concentrations vary across sites.
Figure 19: Ethoxyresorufin-o-deethylase (EROD) response and mean deformity score of five Elizabeth River killifish subpopulations and a reference population (King’s Creek) exposed to individual aryl hydrocarbons.

Mean deformity score (shown in bars and on the left vertical axis) and ethoxyresorufin-o-deethylase (EROD) response (shown in lines and on the right vertical axis) of embryos from five Elizabeth River subpopulations and the reference population (King’s Creek) exposed to A) 10 µg/L β-naphthoflavone (BNF), B) 300 µg/L benzo[k]fluoranthene (BkF), or C) 1 µg/L 3,3',4,4',5-pentachlorobiphenyl (PCB-126). Embryos were exposed individually at 24 hours post fertilization (hpf); EROD was measured at 96 hpf and deformities assessed at 144 hpf. White bars and dashed lines represent DMSO-dosed groups and grey bars and solid lines represent aryl hydrocarbon-dosed groups. Error bars represent ±SEM. Bars not marked by the same letter are significantly different at $p<0.05$ (ANOVA, Tukey-adjusted LSMeans). EROD values marked by * are significantly different from the population-matched DMSO-dosed control at $p<0.05$ (ANOVA, Tukey-adjusted LSMeans). $n \geq 30$ individuals per treatment group.
Figure 20: Ethoxyresorufin-o-deethylase (EROD) response and mean deformity score of five Elizabeth River killifish subpopulations and a reference population (King’s Creek) exposed to PAH mixtures.

Mean deformity score (shown in bars and on the left vertical axis) and ethoxyresorufin-o-deethylase (EROD) response (shown in lines and on the right vertical axis) of embryos from five Elizabeth River subpopulations and the reference population (King’s Creek) exposed to A) 100 µg/L benzo[a]pyrene (BaP) plus 500 µg/L fluoranthene (Fl), B) 0.005% (v/v) coal tar or C) 10% (v/v) Elizabeth River sediment extract (ERSE). Embryos were exposed individually at 24 hours post fertilization (hpf); EROD was measured at 96 hpf and deformities assessed at 144 hpf. White bars and dashed lines represent DMSO-dosed groups and grey bars and solid lines represent aryl hydrocarbon mixture-dosed groups. Error bars represent ±SEM. Bars not marked by the same letter are significantly different at $p<0.05$ (ANOVA, Tukey-adjusted LSMeans). EROD values marked by * are significantly different from the population-matched DMSO-dosed control at $p<0.05$ (ANOVA, Tukey-adjusted LSMeans). $n\geq30$ individuals per treatment group except for BaP plus Fl where $n\geq24$ individuals per group.
Figure 21: Ethoxyresorufin-o-deethylase (EROD) response of five Elizabeth River killifish subpopulations and the reference population (King's Creek) exposed to 3 nM benzo[a]pyrene, 3,3',4,4',5-pentachlorobiphenyl, β-naphthoflavone, or benzo[k]fluoranthene.

Ethoxyresorufin-o-deethylase (EROD) response of embryos from five Elizabeth River subpopulations and the reference population (King’s Creek) exposed to dimethyl sulfoxide control (DMSO, dark grey bars), benzo[a]pyrene (BaP; hatched bars), 3,3',4,4',5-pentachlorobiphenyl (PCB-126; black bars), β-naphthoflavone (BNF; white bars), or benzo[k]fluoranthene (BkF, light grey bars). Embryos were exposed individually at 24 hours post fertilization (hpf) and EROD activity was measured at 96 hpf. Error bars represent ±SEM. Bars not marked by the same letter are significantly different at $p<0.05$ (ANOVA, Tukey-adjusted LSMeans). n≥30 individuals for all treatment groups.
Figure 22: Compilation of deformity response of five Elizabeth River killifish subpopulations and the reference population (King’s Creek) across all aryl hydrocarbon exposures.

Mean deformity score of embryos from five Elizabeth River subpopulations and the reference population (King’s Creek) exposed to 10 µg/L β-naphthoflavone (BNF), 300 µg/L benzo[k]fluoranthene (BkF), 1 µg/L 3,3′,4,4′,5-pentachlorobiphenyl (PCB-126), 100 µg/L benzo[a]pyrene (BaP) plus 500 µg/L fluoranthene (Fl), 0.005% (v/v) coal tar, or 10% (v/v) Elizabeth River sediment extract (ERSE).
Figure 23: Compilation of ethoxyresorufin-o-deethylase (EROD) response of five Elizabeth River killifish subpopulations and the reference population (King’s Creek) across aryl hydrocarbon exposures.

Ethoxyresorufin-o-deethylase (EROD) response of embryos from five Elizabeth River subpopulations and the reference population (King’s Creek) exposed to 10 µg/L β-naphthoflavone (BNF), 300 µg/L benzo[k]fluoranthe (BkF), 1 µg/L 3,3′,4,4′,5-pentachlorobiphenyl (PCB-126), 0.005% (v/v) coal tar, or 10% (v/v) Elizabeth River sediment extract (ERSE). EROD response is expressed as A) % KC DMSO, normalizing all responses to one baseline (reference population control response) and B) average fold change, demonstrating relative induction compared to each individual population’s baseline.
6. Heritable changes in CYP1 activity and mRNA expression associated with resistance to aryl hydrocarbon-induced teratogenesis in Fundulus heteroclitus embryos

This chapter is a collaborative effort of Bryan W. Clark, Audrey J. Bone, and Richard T. Di Giulio.

6.1 Introduction

As with any stressor, chronic pollutant stress has the potential to drive an adaptive response in exposed populations. Multiple populations of fish living in polluted estuaries in eastern North America have been shown to be remarkably resistant to the toxic effects of the contaminants they face and several have likely developed heritable adaptations (reviewed by Wirgin and Waldman 2004). In particular, several populations of fish have been identified that are resistant to the toxic effects of various aryl hydrocarbons, including polychlorinated biphenyls (PCBs), dioxins, and polycyclic aromatic hydrocarbons (PAHs) (Bello et al. 2001; Meyer and Di Giulio 2002; Nacci et al. 1999; Ownby et al. 2002; Prince and Cooper 1995b). One example, a population of Fundulus heteroclitus (the Atlantic killifish or mummichog, hereafter referred to as killifish) is found in the Elizabeth River (Virginia, USA) in the southern portion of the Chesapeake Bay watershed. These killifish inhabit an inlet adjacent to the Atlantic Wood Industries Superfund site, an area heavily-contaminated with creosote from former wood-treatment operations (Mulvey et al. 2002; Walker et al. 2004). Creosote is a complex mixture consisting primarily of unsubstituted polycyclic aromatic hydrocarbons (PAHs), as well as heterocyclic and phenolic PAHs.
PAHs are ubiquitous contaminants generated by incomplete combustion of organic compounds. They can enter the environment through natural sources such as forest fires and through a wide variety of anthropogenic activities, especially through the use of fossil fuels. PAHs are increasing in the environment, tracking human population growth and fossil fuel consumption (Van Metre and Mahler 2005; Van Metre et al. 2000). Estuarine habitats are particularly vulnerable to PAH contamination via industrial outfalls, oil shipping and refining, wastewater discharges, urban runoff, and atmospheric deposition (Latimer and Zheng 2003).

Classically, PAHs are known as carcinogenic, immunosuppressive, and as non-specific narcotic toxicants. In addition to these well-established toxicities, recent work has shown that some PAHs cause early life stage toxicity and teratogenesis in fish. Many PAHs are agonists for the aryl hydrocarbon receptor (AHR), but others are antagonistic or do not appear to have great affinity for the receptor (Billiard et al. 2004; Billiard et al. 2002; Denison and Nagy 2003). Various PAHs have been shown to cause developmental toxicity in both an AHR-independent (Incardona et al. 2006b; Incardona et al. 2005; Incardona et al. 2004) and AHR-dependent manner (Billiard et al. 2006; Clark et al. 2010; Incardona et al. 2006b). In many cases, PAH teratogenesis manifests as cranio-facial and cardiac malformations, reminiscent of the “blue-sac syndrome” observed with the related and highly-studied planar halogenated aromatic hydrocarbons (pHAHs; e.g. 2,3,7,8 tetrachlorodibenzo-p-dioxin (TCDD)) (Hahn 2002; Prasch et al. 2003a; Toomey et al. 2001). While initially it was noted that killifish inhabiting the Atlantic Wood Superfund site had high rates of liver lesions (Vogelbein et al. 1990), it became apparent that the population had developed remarkable resistance to the acute effects and teratogenesis caused by aryl hydrocarbons and Elizabeth River sediments.
Perhaps the most dramatic difference between Elizabeth River killifish and naïve fish is their recalcitrance to induction of cytochrome P450 (CYP) metabolic enzymes by AHR agonists (Meyer and Di Giulio 2002; Meyer et al. 2002; Van Veld and Westbrook 1995). Lack of CYP induction is generally a marker of down-regulation of the AHR pathway. In the Elizabeth River killifish and in other fish populations exposed over multiple generations to aryl hydrocarbon pollution, recalcitrance to CYP induction is correlated with marked resistance to the toxic effects of the contaminants (Bello et al. 2001; Nacci et al. 2002; Powell et al. 2000; Prince and Cooper 1995b; Roy et al. 2002). In addition, Elizabeth River killifish exhibit potentially adaptive alterations in antioxidant defenses (Bacanskas et al. 2004; Meyer et al. 2003a) and several xenobiotic metabolism and excretion pathways, including elevated levels of glutathione S-transferase (Armknecht et al. 1998), hepatic P-glycoprotein (Cooper et al. 1999), UDP-glucuronosyl transferase (UGT), and sulfotransferase (Gaworecki et al. 2004). It is possible that alterations in AHR pathway response, antioxidant defense, and xenobiotic metabolism all play important roles in the adaptation of Elizabeth River killifish.

However, heritability of the various traits and even the resistance itself has been inconsistently demonstrated to carry through multiple generations in the absence of the chemical stressors. Ownby et al. (2002) showed that both F1 embryos and F2 embryos from laboratory-reared F1 adults were resistant to teratogenesis due to Elizabeth River sediments. Likewise, Nacci et al. (2010) found that Elizabeth River killifish were resistant to induction of CYP activity and early life stage toxicity caused by PCB-126 (3,3’,4,4’,5-pentachlorobiphenyl). In contrast, investigation by Meyer and co-workers
found more complicated patterns of heritability. They found that toxicity resistance was less marked in the F2 generation, although still evident (Meyer and Di Giulio 2003). In addition, they found that the recalcitrance to CYP induction faded somewhat in later generations (Meyer and Di Giulio 2002; Meyer and Di Giulio 2003; Meyer et al. 2002). It is notable that some data obtained by Meyer and colleagues support a conclusion of genetic heritability, but other data do not. In general, the strongest evidence for full genetic heritability was obtained for resistance to teratogenesis in embryos, while studies of heritable resistance in larvae and adults yielded mixed results. Refractory CYP response and resistance to toxicity tended to fade with age, perhaps indicating that components of the adaptation are developmental stage specific.

The patterns of heritability of various adaptive traits in Elizabeth River killifish and their lab-reared offspring are summarized in Table 1. To date, it is unclear in some cases what the role of each alteration is in tolerance to Elizabeth River conditions, which are the result of acclimation, and which are genetically-heritable adaptations. Understanding the heritability of the resistance and its underlying components can help us better understand the nature of population-level responses to chronic exposure to PAH mixtures. Furthermore, elucidating if the adaptation is conferred through genetically-heritable mechanisms will aid in identifying the important mechanistic components of resistance to aryl hydrocarbons in Elizabeth River killifish.

In the current study we compared the response to various aryl hydrocarbons of Elizabeth River F1 embryos (offspring of wild-caught parents), Elizabeth River F2 embryos (offspring of lab-reared F1 adults), and embryos of fish from a reference population. Embryos were exposed to benzo[a]pyrene (BaP), benzo[k]fluoranthene (BkF), PCB-126, and a mixture of BkF and fluoranthene (Fl). Resistance to aryl
hydrocarbon-mediated induction of CYP activity, CYP mRNA expression, and cardiac teratogenesis was determined. This study focused on these endpoints for several reasons. Due to the probability of close contact with contaminated sediments and the potential for increased sensitivity in early life stages, effects on embryos are of particular importance. Furthermore, it is likely that heritable PAH adaptation is driven by acute toxicity and early life stage effects that prevent survival to reproduction. This investigation focused on CYP induction because of the aforementioned importance of CYP and the AHR pathway in the toxicity of and resistance to many aryl hydrocarbons.

6.2 Materials and methods

6.2.1 Fish

Adult killifish from the PAH-adapted Elizabeth River population were collected with wire mesh minnow traps at the Atlantic Wood Industries Superfund Site (36°48’27.2” N, 76°17’38.1” W). Adult killifish from a reference population were collected from King’s Creek, a relatively uncontaminated tributary of the Severn River in Virginia (37°18’16.2”N, 76° 24’58.9”W). In the laboratory, adults were maintained in flow-through systems consisting of a series of 30-L or 40-L tanks containing 20‰ artificial sea water (ASW; Instant Ocean, Foster & Smith, Rhinelander, WI, USA). The system was maintained at 23-25 °C on a 14:10 light:dark cycle. Adults were fed pelleted feed ad libidum (Aquamax ® Fingerling Starter 300, PMI Nutritional International, LLC, Brentwood, MO, USA). Eggs were obtained by manual spawning of females and fertilized in vitro by expressing sperm from males into a beaker containing eggs in ASW.
Following spawning, embryos were set aside for a minimum of one hour to allow fertilization, then washed briefly with 0.3% hydrogen peroxide solution.

To obtain F1 Elizabeth River adults, mixed breedings of >100 females and >20 males were conducted. This resulted in several thousand eggs and approximately 1000 were maintained in petri dishes (VWR International, West Chester, PA, USA) lined with absorbent filter paper (No. 3MM chromatography paper, Whatman International Ltd., Maidstone, England). Enough ASW was added to the dishes to keep the eggs moist but not completely submerged. They were maintained in an incubator for 12-14 days at 27 °C. For hatching, more ASW was added to the petri dishes, the absorbent paper was removed, and the dishes were gently rocked in a shaker. After hatching, larvae were maintained in 2-L beakers of ASW in an incubator at 27 °C and fed Artemia nauplii. Larvae were maintained in beakers for several weeks, then moved to a 19-liter aquarium in the same room as the adult colonies (23-25 °C on a 14:10 light:dark cycle) for several months. Finally, juveniles were moved into dedicated tanks in the flow-through system described previously. The Elizabeth River F1 generation fish began reproducing approximately 6 months after hatching. The experiments described in the current paper were conducted with F2 embryos obtained while the Elizabeth River F1 fish were 1-2 years old. All care, reproductive, and rearing techniques were non-invasive and approved by the Duke University Institutional Animal Care & Use Committee (A234-07-08).

6.2.2. Chemicals

Ethoxyresorufin, and dimethyl sulfoxide (DMSO) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Benzo[k]fluoranthene (BkF), benzo[a]pyrene (BaP), and fluoranthene (Fl) were purchased from Absolute Standards, Inc. (Hamden, CT, USA)
and 3,3',4,4',5-pentachlorobiphenyl (PCB-126) was purchased from AccuStandard (New Haven, CT, USA). Chemical stocks were prepared by dissolving the appropriate mass of BkF, BaP, Fl, or PCB-126 in DMSO.

### 6.2.3 Dosing

Adult King’s Creek (reference), Elizabeth River F1s, and Elizabeth River F2s were spawned as described previously. Embryos were dosed individually in 20-mL glass scintillation vials (VWR, West Chester, PA) beginning at 24 hours post fertilization (hpf). Embryos were exposed in 10 mL of dosing solution (20‰ ASW) containing the following: 100 µg/L BaP, 20 µg/L BkF plus 100 µg/L Fl, 1 µg/L PCB-126, or 300 µg/L BkF. Control embryos were exposed to DMSO at a concentration (v/v) equal to that in the dosed group; DMSO concentrations were held at <0.03% across all treatments. All exposure groups also received 21 µg/L ethoxyresorufin. Embryos in the dosing solution were maintained in at 27 °C from 24 hpf. CYP1 activity was measured using the in ovo EROD (ethoxyresorufin-o-deethylase) assay at 96 hpf (section 2.4), and cardiac deformities were assessed at 144 hpf (section 2.5). After deformity screening, embryos exposed to DMSO, BkF, or PCB-126 were flash frozen in liquid nitrogen and stored at -80 °C for later mRNA analysis. This timepoint was chosen for mRNA analysis to allow comparison to previous work with Elizabeth River F1 embryos (L.P. Wills et al. 2010). All exposures consisted of at least three experimental replicates with n ≥ 10 embryos per treatment group unless otherwise noted.

### 6.2.4 EROD assay

CYP1 activity was measured via the in ovo EROD assay modified from Nacci et al. (1998), which uses co-exposure to ethoxyresorufin and the dosing solutions.
Resorufin, the fluorescent product of CYP1 activity on ethoxyresorufin, collects in the urinary bladder of the embryo. At 96 hpf, this fluorescence was visualized using fluorescent microscopy (50X magnification, rhodamine red filter set; Axioskop, Zeiss, Thornwood, NY, USA). EROD activity was measured as the intensity of fluorescence within the bladder normalized to the intensity within a region outside the bladder and quantified using IPLab software (BD Biosciences, Rockville, MD, USA). All EROD values are expressed as percent of the King’s Creek (reference) population DMSO-dosed control group response.

6.2.5 Deformity assessment

Embryos were scored for cardiac deformities at 144 hpf. Deformity assessment was performed blind using a scale shown in detail previously (Clark et al. 2010; Matson et al. 2008a). The scale consists of three scores categorized as normal (0), mild deformities (1), and severe (2) deformities. Embryos receiving a score of 0 had hearts with a normal appearance including properly aligned and sized chambers, no visible pericardial edema, and unrestricted blood flow. Embryos receiving a score of 1 had slightly elongated hearts, with generally distinct but misaligned chambers, and visible pericardial edema. Embryos with a score of 2 had greatly elongated hearts often with no identifiable chambers and an extremely reduced or complete absence of blood flow.

6.2.6 Quantitative real-time PCR

Paired embryos were thawed on ice, homogenized with RNA-Bee for 30 seconds, and mRNA was extracted by modified phenol-chloroform extraction according to the RNA-Bee protocol (Tel-Test, Inc., Friendswood, Texas, USA). RNA quantity and quality was analyzed using a NanoDrop ND-1000 (NanoDrop Technologies, Wilmington,
DE, USA). Using the Omniscript cDNA synthesis kit for Reverse Transcription (Qiagen, Valencia, CA, USA), cDNA was prepared according to manufacturer’s instructions using 500 ng of RNA, random hexamers, and RNase inhibitor, and carried out for 1 h at 37 °C in a Biometra T1 thermocycler (Göttingen, Germany).

QPCR was performed in a 25 μL reaction containing 200 nM of each primer, 12.5 μL of 2X SYBR Green PCR Master Mix (Applied Biosystems, Carlsbad, CA, USA), 9.5 μL dH₂O, and 4 ng cDNA template. The β-actin (F – 5’-ACCACACATTCTCATACACTCGGG-3’, R – 5’-CGCCTCCCTTCATCGTTCCAGTTT-3’), CYP1A (F – 5’-AAGAATGGAGGACACTGGATGACC-3’, R – 5’-AGATTACAGGACAACACGACAGCG-3’), and CYP1B1 (F – 5’-CCAAAGAATACACAGAGGCAACGG-3’, R – 5’-ATGAAGGCATCCAGGTAAGGCAT-3’) primers used were previously reported by Wills et al. (2010) and the CYP1C1 (F – 5’-TCTGGACGCCTTCATCTACGA-3’, R – 5’-GTGACGTCCGATGTGGTTGA-3’) primers reported by Wang et al. (2006). The reactions were carried out on an Applied Biosystems 7300 Real-Time PCR system with the following profile: 10 min at 95 °C and 40 cycles of 15 s at °C followed by 1 min at 60 °C. Dissociation curves were calculated for each sample at the end of the run to confirm formation of a single product. Each sample was run in duplicate wells, a minimum of six biological replicates (consisting of paired individuals) was analyzed per experiment, and embryos from duplicate experiments were analyzed. Data analyses were performed using ABI PRISM 7300 Sequence Detection System Software (Applied Biosystems). Expression was calculated using relative quantification by the $2^{-\Delta\Delta Ct}$. Target gene expression was normalized to β-actin and then compared to the appropriate control to determine average fold induction.
6.2.7 Statistical analyses

All analyses were performed using JMP 8.0 (SAS Institute Inc, Cary, NC, USA). For all EROD and deformity analyses, the individual was the unit of replication. To measure mRNA, two individuals were pooled. Therefore, a pair of embryos was the unit of replication. EROD and deformity data were rank-transformed and analyzed by non-parametric analysis of variance (ANOVA), followed by least square means (LSMeans) procedures. As stated previously, experiments were replicated a minimum of three times; no differences between experimental replicates were observed for any test. For post hoc comparisons Tukey-adjusted pairwise comparisons were conducted to determine differences between all groups. Statistical significance was accepted at $p \leq 0.05$ for all tests.

6.3 Results

6.3.1 Heritability of resistance to cardiac teratogenesis due to aryl hydrocarbons

Compared to King’s Creek (reference) embryos, both Elizabeth River F1 and F2 embryos were highly resistant to the teratogenic effects of each of the aryl hydrocarbon exposures (Figure 1). The mean deformity score of King’s Creek embryos was significantly elevated above that of controls (0.04±0.03) for exposure to BkF (1.0±0.05; $p<0.0001$), PCB-126 (1.3±0.10; $p<0.0001$), and the mixture of BkF and Fl (1.5±0.10; $p<0.0001$). In contrast, Elizabeth River F1 embryos exhibited no differences from controls (0.0±0.0) in mean deformity score for exposure to BkF (0.0±0.0, $p=1.000$), PCB-126 (0.02±0.02, $p=1.000$), and the mixture of BkF and Fl (0.0±0.0, $p=1.000$). Likewise, Elizabeth River F2 embryos exhibited no statistical differences from controls (0.02±0.02)
in mean deformity score for exposure to BkF (0.11±0.05, \( p=0.9733 \)), PCB-126 (0.04±0.02, \( p=1.000 \)), and the mixture of BkF and Fl (0.08±0.06, \( p=0.9995 \)). The deformity scores of Elizabeth River F1 and F2 embryos were not statistically different for any exposure tested (all \( p>0.9 \)).

6.3.2 Heritability of resistance to induction of CYP1 mRNA expression by aryl hydrocarbons

The BkF-induced and PCB-126-induced expression of CYP1 mRNA are shown in Figure 2. The fold change in expression of all three CYPs in BkF-dosed King’s Creek embryos was significantly elevated above controls (\( p<0.05 \)). In contrast, expression of CYP1A, CYP1B1 and CYP1C1 mRNA was not elevated above DMSO-dosed controls in F1 embryos (\( p<0.05 \)). Although expression of CYP1A, CYP1B1, and CYP1C1 mRNA was elevated in BkF-dosed Elizabeth River F2 embryos, no statistically significant differences from control were observed for any of the three genes (\( p>0.05 \)). The BkF-induced expression of CYP1A mRNA was significantly lower compared to King’s Creek embryos for both Elizabeth River F1 (\( p=0.0022 \)) and Elizabeth River F2 embryos (\( p=0.0028 \)). However, the BkF-induced expression of CYP1B1 and CYP1C1 mRNA were only statistically different from that of King’s Creek embryos for Elizabeth River F1 embryos (\( p=0.0135 \) and \( p=0.0007 \), respectively). Finally, there were no statistically significant differences between Elizabeth River F1 and F2 embryos BkF-induced expression of CYP1A (\( p=0.3655 \)), CYP1B1 (\( p=0.2680 \)), or CYP1C1 (\( p=0.2568 \)).

PCB-126 increased the expression of CYP1A, CYP1B1, and CYP1C1 mRNA in King’s Creek embryos (\( p<0.05 \)) but not Elizabeth River F1 or F2 embryos (\( p<0.05 \)). In Elizabeth River F1s, expression was significantly lower than that of King’s Creek embryos for CYP1A (\( p<0.0001 \)), CYP1B1 (\( p=0.0002 \)), and CYP1C1 (\( p=0.0044 \)).
Likewise, the level of induction in Elizabeth River F2s was significantly lower than that of King’s Creek for CYP1A ($p<0.0001$), CYP1B1 ($p=0.0059$), and CYP1C1 ($p=0.0190$). PCB-126 induced higher expression of all three genes in Elizabeth River F2 embryos than Elizabeth River F1 embryos, but this was not statistically significant for expression of CYP1A ($p=0.6967$), CYP1B1 ($p=0.5299$), or CYP1C1 ($p=0.4857$).

### 6.3.3 Heritability of resistance to induction of CYP1 activity by aryl hydrocarbons

CYP activity (as measured by EROD response) exhibited a similar pattern to that observed for mRNA expression (Figure 3). Aryl hydrocarbon exposure consistently induced CYP1 activity in the King’s Creek (reference) embryos. The EROD response for King’s Creek embryos was 1118±85% for BaP, 407±33% for the mixture of BkF and Fl, and 3215±350% for PCB-126. For all exposures of King’s Creek embryos, EROD activity was statistically different from DMSO-dosed controls ($p<0.0001$). In contrast, the F1 Elizabeth River embryos exhibited very little increase in EROD activity with any exposure. The EROD response of Elizabeth River F1 embryos was not significantly different from DMSO-dosed F1s (34±2.7%) for exposure to BaP (32±2.9%; $p=1.000$), the mixture of BkF and Fl (70±6.7%; $p=1.000$), or PCB-126 (51±4.6%; $p=1.000$). For Elizabeth River F2 embryos, EROD activity was significantly different from control response (35±2.6%) for exposure to BaP (204±53%; $p=0.0302$), BkF and Fl (139±18%; $p<0.0001$), and PCB-126 (532±150%; $p=0.0294$). Furthermore, the EROD response of Elizabeth River F2s was significantly different from that of Elizabeth River F1s for BaP ($p=0.0302$), BkF and Fl ($p=0.0233$), and PCB-126 ($p=0.0253$), but not for DMSO alone ($p=1.000$). However, the response was also much lower than that of the King’s Creek
embryos. Both Elizabeth River F1 and F2 embryos exhibited statistically lower EROD activity than King’s Creek embryos for all treatments ($p<0.0001$).

6.4 Discussion

Resistance of Elizabeth River killifish embryos to cardiac teratogenesis and AHR pathway activation by aryl hydrocarbons was found to be heritable for two laboratory-raised generations, suggesting a genetically inherited adaptive response. However, Elizabeth River F2s demonstrated less resistance to induction of EROD activity, perhaps indicating that some part of the resistance is not inherited genetically.

6.4.1 Resistance to cardiac teratogenesis

In the current study, Elizabeth River F1 and F2 embryos were highly resistant to aryl hydrocarbon exposures that caused severe cardiac teratogenesis in King’s Creek reference embryos. This agrees with much of the previous work investigating resistance of Elizabeth River embryos to aryl hydrocarbons. Ownby et al. (2002) showed that F2 offspring of laboratory-reared F1 Elizabeth River killifish from the Atlantic Wood site were highly resistant to Elizabeth River sediments. Previous work in our laboratory also showed that Elizabeth River F1 and F2 embryos were resistant to Elizabeth River pore water generated developmental abnormalities and mortality observed in reference embryos (Meyer and Di Giulio 2003; Meyer et al. 2002). In addition, a recent study showed that both F1 and F2 Elizabeth River killifish were even more resistant to PCB-126 toxicity than adapted killifish from sites in Newark Bay, NJ and New Bedford Harbor, MA where contamination is dominated by PCBs and dioxins (Nacci et al. 2010). In contrast, Meyer and Di Giulio (2002) found that F1 Elizabeth River embryos were highly
resistant to cardiac teratogenesis generated by PCB-126 exposure, but at the highest
doses resistance by F3 embryos (F2 embryos were not studied) was intermediate
between that of F1 embryos and susceptible reference embryos (Meyer and Di Giulio
2002). It is not clear why this study showed a loss of toxicity resistance in F3 embryos
whereas all other studies, including the current study, found resistance to toxicity to be
inherited for at least two laboratory-reared generations. However, the heritability of
resistance to embryonic toxicity in Elizabeth River killifish has been fairly consistently
observed across studies. This suggests that resistance to embryonic toxicity is a major
driving force in adaptation to contaminated sediments in the Elizabeth River habitat.
Because of close contact with contaminated sediments and increased sensitivity in early
life stages, it is likely that heritable PAH adaptation is driven by acute toxicity and early
life stage effects that prevent survival to reproduction, rather than chronic effects
associated with PAH exposures, such as carcinogenesis.

6.4.2 Resistance to induction of CYP1 mRNA expression and EROD activity

Elizabeth River F1 and F2 embryos were resistant to induction of CYP mRNA
expression by both BkF and PCB-126. This agrees with and extends previous work
demonstrating that Elizabeth River F1 embryos were resistant to induction of CYP1A,
CYP1B1, and CYP1C1 mRNA expression by multiple compounds, including BkF and
PCB-126 (L.P. Wills et al. 2010). As with EROD activity (discussed subsequently),
suppression of CYP mRNA expression observed in the current study was greater in
Elizabeth River F1s than Elizabeth River F2s. In fact, expression of CYP1B and
CYP1C1 mRNA by BkF-dosed Elizabeth River F1 embryos was not statistically different
from that of King’s Creek embryos. Overall, these data and work by Wills et al. (2010)
show that mRNA expression of multiple CYPs is suppressed in Elizabeth River F1 and F2 embryos, suggesting at least partially heritable suppression via a shared regulator such as the AHR. Interestingly, the suppression of CYP expression in Elizabeth River F2 embryos appears to be stronger for exposure to PCB-126 than for BkF.

For all exposures in the current study, CYP1 activity (measured by the EROD assay) was highly suppressed in both Elizabeth River F1 and F2 embryos compared to King’s Creek embryos. However, similar to CYP mRNA expression, the response of Elizabeth River F2 embryos was consistently higher than that of Elizabeth River F1 embryos. This consistent pattern suggests that although the resistance to induction of CYP activity may be genetically heritable, it is not brought about solely by genetically inherited mechanisms.

In previous studies in our laboratory, F1 Elizabeth River embryos were highly resistant to induction of CYP1 activity generated by PCB-126 exposure (Meyer and Di Giulio 2002). In addition, Elizabeth River F1 embryos were recalcitrant to induction of CYP1A by the AHR agonist-type PAHs 3-MC and β-naphthoflavone (BNF). However in these studies, the resistance was not consistently heritable to subsequent generations. PCB-126-induced EROD activity in F3 embryos (F2 were not tested) returned to levels similar to those of reference embryos. Likewise, recalcitrance to CYP induction was largely lost in F3 embryos and F2 larvae dosed with 3-MC, BNF, or sediment pore water (Meyer and Di Giulio 2003; Meyer et al. 2002). In addition, hepatic EROD activities in adult Elizabeth River F1s and F2s exposed to Elizabeth River sediments were found to be similar to those of adult reference fish. This is contrasts with the degree of resistance to CYP induction by Elizabeth River F2 embryos observed in the current study. Furthermore, Nacci et al. (2010) found that Elizabeth River F2 individuals were highly
resistant to induction of EROD activity by PCB-126. They did not test Elizabeth River F1 individuals, so it is unknown if they would have observed a difference between F1 and F2 response similar to that seen in the current study. However, they were unable to calculate an exact EC$_{50}$ for F2 EROD response because there was no change in response even at 200 μg/L of PCB-126.

The current work and studies by Meyer and colleagues provide evidence that resistance to CYP induction is heritable but may not be fully genetic. Meyer and coworkers proposed that this might be achieved through epigenetic regulation of CYP1A. However, Timme-Laragy et al. (2005) found no difference between the methylation status of CpG sites in the CYP1A promoter of Elizabeth River and reference fish. As discussed previously, suppression of mRNA expression of multiple CYPs indicates suppression via a shared regulator. This suggests that it is more useful to look at epigenetic regulation of a factor upstream of CYP, such as the AHR.

It is difficult to reconcile the results of Meyer and coworkers with those of the current study and Nacci et al. (2010), although it is notable that some data obtained by Meyer and coworkers did support a conclusion of genetic heritability. As stated previously, the strongest evidence for full genetic heritability was obtained for resistance to teratogenesis in embryos, while studies of heritable resistance in larvae and adults yielded mixed results. Refractory CYP response and resistance to toxicity tended to fade with age, perhaps indicating that components of the adaptation were developmental stage specific. Interestingly, hybrid embryos generated by crossing Elizabeth River fish of either sex with reference fish demonstrated a BNF-induced EROD response intermediate between those of reference and Elizabeth River embryos (Meyer et al. 2002). Furthermore, the response of the two hybrid lines was nearly indistinguishable,
regardless of the sex of the parent from the Elizabeth River population. These results seem to be more consistent with a hypothesis of genetically heritable resistance, transmitted by both male and female Elizabeth River fish.

It is noteworthy that most of the elevated EROD response of Elizabeth River F2 embryos observed was due to a subset of highly-responsive individuals, as shown in Figure 3. Little evidence of such a group was observed for Elizabeth River F1 embryos. Meyer and Di Giulio (2003) discussed the possibility that because resistant fish were less fit under clean conditions, the laboratory population could have undergone “reverse” selection, yielding less resistant offspring in later generations. However, it is hard to imagine that the adaptive response could be selected against in only one generation, especially when the observed response of Elizabeth River F1 embryos does not appear to yield much variation on which to select. Another possibility is that some components of the resistance are fully heritable, while others are not. Elizabeth River F1 embryos might have all components of the resistance, both genetically heritable and non-genetically heritable, so their response is very low. In contrast, some Elizabeth River F2 individuals may have lost some non-genetically heritable components of resistance, yielding a subset of higher-responding individuals. If this is the case, it is interesting that loss of some of these components did not affect the overall resistance to toxicity of Elizabeth River F2 embryos.

6.4.3 Role of AHR pathway in resistance of Elizabeth River killifish to aryl hydrocarbons

As described previously, the AHR pathway plays a pivotal role in the toxicity of many aryl hydrocarbons, including some PAHs, and is a likely target for resistance in aryl hydrocarbon-adapted fish populations (reviewed in Wirgin and Waldman 2004).
Furthermore, aryl hydrocarbon-generated cardiac teratogenesis in fish is mediated at least in part through the AHR pathway (Billiard et al. 2006; Clark et al. 2010; Incardona et al. 2006b; Prasch et al. 2003b) so it seems likely that down-regulation of the AHR pathway is an important target for adaptation of killifish to the PAH-contaminated Elizabeth River habitat. However, resistance to activation of the AHR pathway (as measured by CYP mRNA expression, protein, and enzyme activity) was not consistently heritable past the F1 generation in many studies by Meyer and coworkers previously discussed. There are several conclusions that could be drawn from these studies and the incomplete resistance to CYP induction of Elizabeth River F2 embryos observed in the current study. One possibility is that suppression of AHR pathway activity is not fully genetically heritable. In addition, induction of CYP family members has been shown to be regulated by the constitutive androstane receptor, the pregnane X receptor, the retinoic acid receptor, and the peroxisome proliferators-activated receptor (Xu et al. 2005, Monostory and Pascussi 2008); therefore non-genetically heritable alteration of any of these pathways could also play a role in the observed pattern of heritability of CYP suppression. Alternatively, the degree of AHR pathway suppression could be consistent in F1 and F2 embryos, but the up-regulation of other protective factors observed in Elizabeth River killifish might fade in laboratory-reared generations. As stated previously, feral Elizabeth River killifish have elevated levels of glutathione S-transferase (Armknecht et al. 1998), hepatic P-glycoprotein (Cooper et al. 1999), UDP-glucuronosyl transferase (UGT), and sulfotransferase (Gaworecki et al. 2004). Many of these factors aid in enhancing removal of xenobiotics from the body, which could reduce the amount of chemical that interacts with the AHR in the first place. It is possible that factors such as these are non-genetically heritable and their loss in Elizabeth River F2
embryos results in the apparent increase observed in AHR pathway activation. To our knowledge, heritability of these protective factors has not been reported.

In any case, a complete lack of CYP activity and suppression of the AHR pathway do not appear to be required for strong resistance to aryl hydrocarbons at the doses tested. Similarly, investigation of killifish from throughout the Elizabeth River found that some subpopulations were highly resistant to teratogenesis while still demonstrating significant induction of CYP activity (Chapter 6). These experiments clearly demonstrate that while suppression of the AHR pathway may play a major role in resistance of Elizabeth River killifish to aryl hydrocarbons, it is not the only important factor. This suggests rather that multiple alterations play an important role in PAH-adaptation.

### 6.5 Conclusion

This study confirmed that both resistance to cardiac teratogenesis and induction of CYP were heritable for two generations in the absence of PAH stress. However, the results suggest that suppression of the AHR pathway response is not fully heritable. There is still much to be determined about the mechanisms by which resistance has occurred, although these results support the conclusion that heritable resistance is conferred by multiple alterations in adapted fish. The existence of genetically-heritable resistance to pollutants in this and other fish populations demonstrates the potential influence of anthropogenic contamination at a population and perhaps evolutionary scale.
Table 2: Patterns of heritability of various adaptive parameters in Elizabeth River killifish compared to reference fish.

<table>
<thead>
<tr>
<th>Stage</th>
<th>Generation</th>
<th>Response of Elizabeth River killifish</th>
<th>&quot;Strength&quot; of heritability</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Embryo</strong></td>
<td>F1, F2</td>
<td>Resistant to toxicity and EROD induction due to PCB-126</td>
<td>++ + +</td>
<td>Nacci et al. 2010</td>
</tr>
<tr>
<td></td>
<td>F1</td>
<td>Recalcitrant to CYP mRNA induction by BaP, BkF, &amp; PCB-126</td>
<td>+</td>
<td>Wills et al. 2010</td>
</tr>
<tr>
<td></td>
<td>F1</td>
<td>Resistant to cardiac teratogenesis &amp; EROD induction from BaP/F1 mixture</td>
<td>+</td>
<td>Wills et al. 2009</td>
</tr>
<tr>
<td></td>
<td>F1, F2</td>
<td>Resistant to cardiac abnormalities from ER sediments</td>
<td>++ +</td>
<td>Ownby et al. 2002</td>
</tr>
<tr>
<td></td>
<td>F1, F2</td>
<td>Fewer developmental abnormalities with exposure to ERSE</td>
<td>++ +</td>
<td>Meyer &amp; Di Giulio 2003</td>
</tr>
<tr>
<td></td>
<td>F1, F2</td>
<td>Lack of EROD induction by 3-MC in F1, but not F2</td>
<td>+ / -</td>
<td>Meyer et al. 2002</td>
</tr>
<tr>
<td></td>
<td>F1, F3</td>
<td>Lack of EROD induction by BNF in F1, but not F3; hybrids (regardless of sex of ER parent) had low, but intermediate EROD</td>
<td>++</td>
<td>Meyer et al. 2002</td>
</tr>
<tr>
<td></td>
<td>F1, F3</td>
<td>Resistant to CYP1A activity &amp; protein induction by PCB-126 in F1, not F3</td>
<td>+ / -</td>
<td>Meyer &amp; Di Giulio 2002</td>
</tr>
<tr>
<td></td>
<td>F1, F3</td>
<td>Resistant to teratogenesis from PCB-126; at highest dose F1 resistant, F3 intermediate</td>
<td>++</td>
<td>Meyer &amp; Di Giulio 2002</td>
</tr>
<tr>
<td></td>
<td>F1</td>
<td>Recalcitrant to induction of mitochondrial CYP by BaP, BkF</td>
<td>+</td>
<td>Jung et al. 2010</td>
</tr>
<tr>
<td></td>
<td>F1</td>
<td>Higher basal mtDNA &amp; nDNA damage, resistant to induction of DNA damage by BaP</td>
<td>+</td>
<td>Jung et al. 2009</td>
</tr>
<tr>
<td></td>
<td>F1, F2</td>
<td>Reduced induction of EROD activity by BNF in F1; F2 intermediate</td>
<td>++</td>
<td>Meyer et al. 2002</td>
</tr>
<tr>
<td></td>
<td>F1, F2</td>
<td>Increased survival in t-BOOH exposure (F1 and F2 same)</td>
<td>++</td>
<td>Meyer et al. 2003b</td>
</tr>
<tr>
<td></td>
<td>F1, F2</td>
<td>Elevated TOSC (F1 and F2 same)</td>
<td>++</td>
<td>Meyer et al. 2003b</td>
</tr>
<tr>
<td></td>
<td>F2</td>
<td>Slightly elevated total GSH</td>
<td>+</td>
<td>Meyer et al. 2003b</td>
</tr>
<tr>
<td></td>
<td>F1</td>
<td>Elevated MnSOD protein, lack of induction by ERSE</td>
<td>+</td>
<td>Meyer et al. 2003b</td>
</tr>
<tr>
<td></td>
<td>F1, F2</td>
<td>Increased survival in ERSE (F2 intermediate to ref and F1)</td>
<td>++</td>
<td>Meyer &amp; Di Giulio 2003</td>
</tr>
<tr>
<td></td>
<td>F1</td>
<td>Hybrids (regardless of sex of ER parent) show intermediate tolerance to ERSE</td>
<td>++</td>
<td>Meyer &amp; Di Giulio 2003</td>
</tr>
</tbody>
</table>
### Table 2 (continued)

<table>
<thead>
<tr>
<th>Stage</th>
<th>Generation</th>
<th>Response of Elizabeth River killifish</th>
<th>“Strength” of heritability</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Juvenile</td>
<td>F1</td>
<td>Elevated total GSH in liver, no difference in gill</td>
<td>+</td>
<td>Meyer et al. 2003b</td>
</tr>
<tr>
<td></td>
<td>F1, F2</td>
<td>Reduced EROD activity in F1 (3-year old) and F2 (2-year old) exposed to ERSE (F1 not different on day 2, but different on day 5)</td>
<td>+</td>
<td>Meyer et al. 2002</td>
</tr>
<tr>
<td>Adult</td>
<td>F1, F2</td>
<td>Total GSH (basal and induction by ERSE) roughly same as reference</td>
<td>- -</td>
<td>Meyer et al. 2003b</td>
</tr>
<tr>
<td></td>
<td>F1, F2</td>
<td>No difference from reference in glutamate cysteine ligase activity</td>
<td>- -</td>
<td>Meyer et al. 2003b</td>
</tr>
<tr>
<td></td>
<td>F1, F2</td>
<td>Basal level and induction of GPx activity roughly same as reference</td>
<td>- -</td>
<td>Meyer et al. 2003b</td>
</tr>
<tr>
<td></td>
<td>F1, F2</td>
<td>Reduced induction of glutathione reductase activity</td>
<td>-</td>
<td>Meyer et al. 2003b</td>
</tr>
</tbody>
</table>

Figure 24: Mean deformity score of F1 and F2 Elizabeth River and King’s Creek killifish embryos exposed to aryl hydrocarbons.

Mean deformity score (±SEM) of King’s Creek (black bars), Elizabeth River F1 (white bars), and Elizabeth River F2 (grey bars) embryos exposed to 300 µg/L benzo[k]fluoranthene (BkF), 1 µg/L 3,3',4,4',5-pentachlorobiphenyl (PCB-126), or a mixture of 20 µg/L BkF and 100 µg/L fluoranthene (Fl). Values marked by * are significantly different from the DMSO-dosed population-matched control group at $p<0.05$ (ANOVA, Tukey-adjusted LSMeans). $n \geq 24$ individuals per treatment group.
A) CYP1A

B) CYP1B1

C) CYP1C1
Figure 25: Mean CYP mRNA expression of F1 and F2 Elizabeth River and King's Creek killifish embryos exposed to benzo[k]fluoranthene (BkF) or 3,3’4,4’,5-pentachlorobiphenyl (PCB-126).

mRNA expression of A) CYP1A, B) CYP1B1, C) CYP1C1 of King's Creek (black bars), Elizabeth River F1 (white bars), and Elizabeth River F2 (grey bars) embryos exposed to 300 µg/L benzo[k]fluoranthene (BkF) and 1 µg/L 3,3’4,4’,5-pentachlorobiphenyl (PCB-126). Values marked by * are significantly different from the DMSO-dosed population-matched control group at \( p < 0.05 \) (ANOVA, Tukey-adjusted LSMMeans). All other statistically different post hoc pairwise comparisons are labeled on the figure (ANOVA, Tukey-adjusted LSMMeans). \( n \geq 30 \) individuals per treatment group.
Figure 26: Mean ethoxyresorufin-o-deethylase (EROD) activity of F1 and F2 Elizabeth River and King’s Creek killifish embryos exposed to aryl hydrocarbons.

Mean percent of KC DMSO EROD activity (represented by lines, error bars are SEM) for King’s Creek (circles), Elizabeth River F1 (squares), and Elizabeth River F2 (triangles) embryos exposed to A) DMSO, B) 100 µg/L benzo[a]pyrene (BaP), C) a mixture of 20 µg/L BkF and 100 µg/L fluoranthene (Fl), or D) 1 µg/L 3,3’,4,4’,5-pentachlorobiphenyl (PCB-126). Within a given panel, groups not marked by the same letter are statistically different at $p < 0.05$ (ANOVA, Tukey-adjusted LSMeans). $n \geq 24$ individuals per treatment group.
7. Conclusion

7.1 Summary

In this dissertation I attempted to understand the mechanisms by which Elizabeth River killifish resist the toxicity of complex mixtures of PAHs and to investigate some of the consequences and tradeoffs of this adaptation. The mechanistic work focused primarily on delineating the potential role that suppression of the AHR pathway response could play in adaptation to PAHs and PCB-126. The primary focus of my work investigating tradeoffs was on the response of PAH-adapted killifish to a second, largely unrelated class of contaminants. However, hypotheses about response to other contaminants were generated with the mechanism of PAH adaptation in Elizabeth River killifish in mind.

In order to investigate the role of specific genes in the developmental toxicity of PAHs, a large portion of my initial time in the lab was focused on development of the morpholino gene knockdown technique for use in killifish (Chapter 2). There were significant barriers to transfer of the technique to killifish, but we demonstrated that these issues could be overcome. Although the primary contribution of the work in Chapter 2 was the successful demonstration of the use of the morpholino technique in killifish, the work also corroborated previous results demonstrating the synergistic effect of CYP inhibition on PAH embryo-toxicity (Billiard et al. 2006; Wassenberg and Di Giulio 2004a). The use of this technique was further explored in Chapter 3 by utilization of a splice-junction morpholino.
Next I investigated the ability of AHR down-regulation to provide the resistance to PAHs and PCB-126 observed in Elizabeth River killifish (Chapter 3). Using susceptible reference site fish, we showed that knockdown of AHR2 (but not AHR1) protected from the cardiac teratogenesis generated by several aryl hydrocarbons. The results confirmed that suppression of the AHR was a viable candidate for the mechanism by which Elizabeth River killifish circumvent PAH toxicity. However, knockdown of the AHR was not fully protective. While it is possible that this is a function of the incomplete knockdown afforded by morpholinos, it is also possible that AHR suppression alone is not sufficient for protection from the severe toxicity of these exposures. Additionally, the results of Chapters 2 and 3 are important steps forward in the demonstration of the utility of molecular techniques in "non-traditional" models, although this term is rather humorous considering that killifish have been studied in environmental biology for well over 100 years.

In Chapter 4, we explored whether adaptation to PAHs had tradeoffs for the response of Elizabeth River killifish to other anthropogenic contaminants. Although this work was designed to show the potential for fitness costs of PAH adaptation, it was also designed to yield further understanding of the role of AHR pathway suppression in the xenobiotic resistance of Elizabeth River killifish. Neurotoxic pesticides were chosen partly because their toxicity could be compared in rapid and straightforward fashion, but primarily because we could make simple hypotheses about the role that recalcitrance to CYP induction would play in the toxicity of those pesticides. We found that Elizabeth River killifish were less susceptible to a variety of chemical challenges than reference fish, even when their lack of CYP response might be expected to be detrimental. This was a clear indication that although the recalcitrant CYP response of Elizabeth River
killifish could play a role in response to other xenobiotics, it was likely that other protective factors were of great importance too.

In Chapter 5, we compared the resistance of subpopulations of killifish from throughout the Elizabeth River estuary that were resident in sites with various levels of sediment PAH contamination. Both resistance to AHR pathway activation (as measured by CYP activity) and resistance to cardiac teratogenesis were assessed in response to a variety of aryl hydrocarbons, alone and in mixtures. We found that resistance to aryl hydrocarbons was spread throughout the Elizabeth River estuary, even in sites where the sediment contamination was relatively low, demonstrating that locally-severe pollution has an effect at the landscape and metapopulation scale. Furthermore, the subpopulations exhibited a complex pattern of response, which contributed clues to the mechanistic questions pursued in earlier chapters of this dissertation. The degree of resistance of a given subpopulation relative to the others was often dependent on the particular exposure with which they were challenged. Additionally, some subpopulations demonstrated strong resistance to the teratogenic effects of the exposures despite showing significant activation of their AHR pathway response. These data provided strong evidence that the suppression of the AHR pathway response is not solely responsible for providing PAH resistance in Elizabeth River killifish, but that the adaptation is polygenic.

Finally, we investigated the heritability of the aryl hydrocarbon resistance of Elizabeth River killifish (Chapter 6). Previous work suggested that the response was not fully genetically-heritable (Meyer et al. 2002). In order to better understand whether we were searching for a mechanism of adaptation or a mechanism of acclimation, it was important to further explore the heritability of the response. Comparison of the response
of F1 and F2 Elizabeth River killifish to reference killifish demonstrated that resistance to cardiac teratogenesis was completely heritable in F2s. Likewise, recalcitrance to induction of CYP mRNA expression and activity was also demonstrated in F2s. However, for each measure there was a subset of high-responders in F2 embryos that was not apparent in F1s. One conclusion that could be drawn from this result is that suppression of the AHR pathway response is not fully genetically-heritable. Alternately, heritability of other protective factors (such as Pgp or GSTs) could be partially lost in the F2s; this would allow the suppressed AHR pathway to “see” more of the aryl hydrocarbons to which the embryo is exposed and would then yield the increased response observed in some F2 embryos. Interestingly, the response of F2 embryos (strong resistance to cardiac teratogenesis, incomplete recalcitrance to CYP induction) mirrors the response of some subpopulations observed in Chapter 5.

7.2 Implications

The existence of contaminant-adapted killifish populations provides a unique angle from which to study the impacts of chronic pollutant exposure. Wild populations such as the Elizabeth River killifish are by definition environmentally-relevant, genetically-diverse models for the study of the effects of complex mixtures of xenobiotics and have much to offer that cannot be obtained from traditional laboratory models. They provide a demonstration of gene-environment interactions that can educate us about the way in which chronic contaminant exposure drives changes at an organismal, population, and perhaps even evolutionary scale.
7.2.1 Mechanisms of PAH toxicity

Mechanistically, much is unknown about how PAH mixtures exert the developmental toxicity we have described. However, a process akin to “reverse-engineering” of the adaptation of Elizabeth River killifish has aided in generating testable hypotheses about the mechanisms. In that sense, the adapted population aids us in following a more focused candidate gene approach, as opposed to genome-wide surveys for targets of toxicity. The first example of this was the use of chemical CYP inhibitor co-exposure to mimic the observed suppression of CYP in Elizabeth River killifish (Wassenberg and Di Giulio 2004a). While the work actually yielded unexpected results, it provided major evidence that we should reconsider how we look at exposure to PAH mixtures.

The results in this dissertation also have implications for our understanding of the mechanisms by which PAHs exert toxicity. In Chapters 2 and 3, much of the work confirmed that the roles of CYP1A and the AHR in PAH toxicity demonstrated in zebrafish were consistent in killifish. While the results were not unexpected, they were important for furthering our understanding of how the Elizabeth River killifish adapted to PAHs. However, we also found that AHR knockdown was not completely protective from PAH toxicity. This theme was consistent throughout the later chapters of the dissertation. So what are the mechanistic implications of these findings? One possibility is that not all of the embryo-toxicity of PAH mixtures is mediated through the AHR, suggesting that we should continue to pursue other targets. Another intriguing possibility is that the majority of the toxicity is mediated through the AHR, but that Elizabeth River killifish are unable to suppress AHR activity to sufficient levels to fully protect from the toxicity. One reason that they may be unable to completely suppress AHR activity is that
complete suppression may actually be detrimental. There is evidence that the AHR pathway plays an important role in normal development processes (e.g. Gonzalez et al. 1995; Schmidt et al. 1996). It is possible that one way that PAHs and DLCs cause toxicity is inappropriate stimulation of the AHR and interference with these normal pathways. The need of Elizabeth River killifish to develop additional protective mechanisms may indicate a delicate balancing act between preventing AHR-mediated toxicity and maintaining AHR activity at a level that supports normal development. On the surface, it would seem that a simpler approach would be to modify the AHR in such a way that it is insensitive to xenobiotics, but still able to sense endogenous ligands. However, the AHR is a promiscuous receptor, so it may be that this sort of modification is not easily achieved while maintaining normal function. Furthermore, Elizabeth River killifish are exposed to a diverse mixture of compounds, which also might preclude this sort of a modification.

7.2.2 Adaptation and evolution

Micro-evolution is defined as evolution within or among populations, whereas macro-evolution is essentially the formation of new species (Hendry and Kinnison 2001). The adapted killifish populations provide unique insights into the processes underlying adaptation and micro-evolution. Chemical selection is in many ways more directed and more specific than other stressors requiring adaptation, facilitating assessment of the process.

One of the most interesting findings to arise from this and other work on Elizabeth River killifish is that adaptation to PAH contamination appears to be conferred by multiple adaptive changes. Evolutionary theory generally predicts that adaptation of a population to a stressor would be polygenic, but in the process of resistance to
xenobiotics this is actually somewhat rare (Coustau et al. 2000; Macnair 1991; Reznick and Ghalambor 2001). In fact, Macnair (1991) predicted polygenic adaptation to be probable only if the distance the mean response of a population (i.e. susceptibility to a toxicant) must move to confer survival is not substantially larger than the mean response of the original population. This seems to explain why many of the cases of chemical resistance in plants and insects are conferred by “mutations of major effect” (Coustau et al. 2000). Therefore, the severity of toxicity of the Elizabeth River environment to naïve fish and the degree of tolerance exhibited by the Elizabeth River killifish would lead to a prediction that monogenic adaptation is the only way to achieve such a dramatic change in tolerance. This seems to be at odds with observations of multiple protective factors exhibited by Elizabeth River killifish, although it is worth noting that the genetic heritability of most of these factors is unknown. It is possible that the Elizabeth River fish may be exhibiting something in between the two extremes. For example, butterflies that mimic other species were shown to have achieved their initial similarity through a single gene change, which was later enhanced by polygenic modifiers (Sheppard et al. 1985; Turner et al. 1979). Perhaps Elizabeth River killifish have undergone a major change (i.e. AHR pathway suppression) accompanied or followed by such modifiers. It has been postulated that over the course of adaptation to acute stress, a single major gene change might eventually be replaced by multiple changes with lesser fitness costs (Guillemaud et al. 1998; Labbe et al. 2009; Taylor and Feyereisen 1996). However, because heritability of the adaptations other than AHR pathway suppression is unclear, it is possible that the modifiers are actually acclamatory rather responses than genetic changes.
Reznick and Ghalambor (2001) reviewed examples of micro-evolution and described the conditions favoring its occurrence. One was the colonization of a new landscape accompanied by the opportunity for rapid population growth. The severe contamination at a Superfund site creates just such an opportunity for an organism able to develop tolerance to these conditions and killifish seem to be particularly adept at this. Another condition favoring micro-evolution was local adaptation within portions of a metapopulation in a heterogeneous environment. They describe this second case as being exemplified by the way anthropogenic contamination creates “new and often hostile patches of habitat within a landscape.” This would appear to be a perfect description of the situation faced by the killifish metapopulation of the Elizabeth River estuary. Interestingly, gene-flow among subpopulations within a metapopulation can have a homogenizing affect; this was observed for Elizabeth River subpopulations (Mulvey et al. 2002; Mulvey et al. 2003). This concept is actually used to combat insecticide resistance by creation of refugia in the process of integrated pest management (Kinnison and Hairston 2007). However, gene flow among Elizabeth River subpopulations does not seem to be reducing resistance, even in some subpopulations exposed to much lower levels of PAHs than in the Atlantic Wood Superfund site. As discussed previously, this could indicate that the adaptation is useful even for killifish faced with lower levels of PAH contamination, or that the adaptation does not have deleterious consequences for killifish in those cleaner conditions.

Finally, the existence of multiple populations of killifish independently resistant to similar types of contamination provides an interesting test of convergent evolution. An ongoing debate in the study of evolution is whether patterns of evolution are repeatable. A deterministic view of evolution states that similar selective pressures would lead to
similar adaptive solutions, whereas another view is that contingencies (or major chance events) are so important in the evolution of life that the same outcomes would be unlikely (Hendry and Kinnison 2001). However, Hendry and Kinnison (2001) point out that in cases of micro-evolution, similar selective pressures tend to result in extremely similar adaptations. Although the mechanisms by which the different killifish populations are adapted to aryl hydrocarbon contamination are not completely known, there seems to be significant evidence that the populations followed the pattern of convergent or parallel adaptation. This may indicate that the killifish are particularly suited to adaptation to this type of contamination or that the AHR or other protective factors are kind of “adaptively labile” loci that are particularly suited to modification in the face of great stress.

7.2.3 Ecological risk assessment

Currently, ecological risk assessment is still based primarily on comparison of toxicity and exposure at an organism/species level. However, the ability of contamination to drive changes at the population, community, and ecosystem level – all of which were demonstrated or implied in the current work – suggests that a significant type of risk to the environment is not well-addressed. There are numerous implications of the existence of populations locally-adapted to contaminants. On one hand, it may seem that the risk due to the contaminants is not so bad because adapted populations are demonstrating tolerance to the conditions. However, simple assessment of the community would likely demonstrate that while the killifish are thriving, few other organisms are. Furthermore, the fact that the organisms simply survive may be misleading due to the potential fitness costs and tradeoffs associated with development of resistance. Medina et al. (2007) point out that risk assessment of a pollutant-affected
group or ecosystem is time and space dependent. If selection drives changes in traits, the distribution of these traits before and after a polluting event is obviously different. Therefore, use of wild populations from contaminated environments would potentially over- or under-estimate a species’ “normal” sensitivity.

The survival and relative success of the Elizabeth River killifish in severely-contaminated conditions likely represents the movement of that community to an alternative stable state. If an organism is a major component of a given ecosystem, as are killifish, and adaptation occurs with associated costs, overall ecosystem function could be reset to a new state (Clements and Rohr 2009; Medina et al. 2007). Depending on the magnitude of the threshold crossed to achieve a new stable state, it is possible that such a community or ecosystem could take a very long time to recover (Clements and Rohr 2009). This has major implications for the health of the Elizabeth River estuary and also for the process of remediation. Even after the clean-up of Superfund sites on the Elizabeth River it may take a long time for the ecosystems to approach prior function. In one example, Levinton et al. (2003) showed the rapid return of susceptibility of oligochaetes to metals after clean-up of a Superfund site, perhaps showing a path to system recovery. Conversely, tolerant species of zooplankton have been shown to establish well enough to out-compete recolonization by sensitive species in acidified lakes after removal of the stressor (Frost et al. 2006).

Although the dramatic effects observed in populations adapted to chronic exposure to severe contaminant mixtures are rare, it is highly likely that there are many more subtle versions occurring. Less dramatic effects of a similar type are much harder to detect, but could still have significant long term effects on ecosystem structure and function.
7.3 Future directions

As with any study, there are numerous questions that have arisen from my dissertation work that would be interesting to pursue in the future. There is still much work to be done to understand the mechanisms of PAH toxicity, the consequences of chronic exposure to PAH mixtures, and the process and costs of PAH adaptation.

In Chapter 3 we clearly showed that blockade of the AHR could protect embryos from aryl hydrocarbon generated cardiac teratogenesis. To demonstrate that AHR down-regulation is playing a role in the adaptation, we are currently pursuing several approaches to enhance AHR activity in the resistant embryos (described in the Appendix). In addition, it might be informative to sequence the AHRs of a number of fish from the Elizabeth River and the reference population. From this we might be able to identify single nucleotide polymorphisms (SNPs) or a suite of SNPs that are differentially prevalent between populations. With luck a SNP or set of SNPs could be identified in the ligand-binding pocket of the AHR, perhaps providing differential sensitivity. For example, a few altered nucleotides in the binding domain of the mouse AHR conveyed drastic differences in sensitivity to TCDD (Chang et al. 1993; Ema et al. 1994; Poland et al. 1994). A similar approach has been attempted with PCB-adapted killifish from New Bedford Harbor, MA. However, no truly explanatory differences were identified for sequencing of either AHR1 (Hahn et al. 2004) or AHR2 (M. Hahn, personal communication). If this route were pursued, it would be important to sequence the 5’ regulatory sequences in addition. Recent work has demonstrated that 5’ regulatory elements could play a major role in adaptive variation in gene expression (Wray 2007).
In fact, it has already been shown that such a change is a component of the adaptation of killifish to the thermal cline of their range along the Atlantic coast (Schulte et al. 2000). However, previous work in our lab did not indicate that there were differences in basal expression of the AHR or any of its pathway components, at least in adult fish (Meyer et al. 2003b). At any rate, if a polymorphic gene(s) of interest were identified, it would open up a very interesting avenue of exploration investigating the movement of the polymorphism amongst the Elizabeth River subpopulations. This work would be greatly supported by the susceptibility and AHR pathway activation data described in Chapter 5.

As described previously, suppression of the AHR pathway does not appear to be the only important component of the adaptation and it would also be interesting to further investigate these other protective factors. One major step that would be useful would be to determine the heritability of the elevation in Phase II (e.g. GST) and Phase III (Pgp) enzymes. To my knowledge, outside of some of the antioxidant defense capabilities, none of the other factors have been investigated in F2 fish or beyond. This is crucial to learn if they are important components of the heritable adaptation. It would then be interesting to continue to probe the mechanism of the observed cross-resistance to insecticides. One possibility would be to use co-exposure to chemical inhibitors of Pgp (i.e. verapamil and cyclosporin A) or GST (i.e. ethacrynic acid). Another approach would be to establish the degree of differential toxicity in embryos, and then use a morpholino approach to knock down the protective factors. Finally, we showed in Chapter 4 that the weak AHR agonist carbaryl could cause cardiac teratogenesis similar to that observed with PAH and DLC exposure. This would appear to support the argument that the toxicity is generated by improper stimulation of the AHR, no matter what kind of compound provides that stimulation. To further test this hypothesis, we could expose
fish to other weak agonists in high doses as we did with carbaryl. However, there is a more interesting avenue. One hypothesis for how the mixtures of AHR agonist PAHs (i.e. benzo[a]pyrene or benzo[k]fluoranthene) and CYP inhibitor PAHs (i.e. fluoranthene) are synergistically toxic is that the CYP inhibitors extend the persistence of the AHR agonists, making them more “dioxin-like.” In other words, CYP inhibition serves to overcome weaker binding by virtue of extended persistence. The same approach could be used for other weak agonists, and we’ve begun testing some of these mixtures (data to be shown by another student sometime in the future).

Clean-up work has now begun at Money Point (another Superfund site on the Elizabeth River) and is planned for the Atlantic Wood Superfund site. In addition, a new bridge that is being built at the Atlantic Wood site is likely to greatly disturb sediments. It will be very interesting to monitor the effect of the ongoing clean-up process on the recovery of killifish populations in the Elizabeth River. There are numerous interesting questions that can be addressed because of the solid body of data extant from prior to clean-up. Will the population recover sensitivity? Will the killifish population grow? How much time will it take or what level of PAHs in sediment will need to be achieved? In section 7.2.2, I discussed the fact that Elizabeth River killifish were differentially capable of colonizing the disturbed habitat of the severely contaminated Superfund site. As the sites are cleaned, it will be interesting to see if other species of small fish return to compete with killifish. Although we have not assessed the community structure at the Atlantic Wood site, I can report anecdotally that we very rarely catch anything other than killifish. In contrast, we regularly catch multiple species at the reference site, including the closely related striped killifish (*Fundulus majalis*) and sheepshead minnows (*Cyprinodon variegatus*). In order to better prepare to assess the recovery of the Atlantic
Wood killifish population after remediation, it would probably be useful to characterize the current demographic structure of the population.

### 7.4 Conclusion

Overall, the results of this dissertation have furthered our understanding of the nature and consequences of adaptation to chronic PAH contamination by Elizabeth River killifish. We demonstrated that suppression of the AHR pathway has the potential to be a major contributor to the PAH resistance. However, results of every study consistently suggested that AHR pathway suppression was likely not the only important mechanism contributing to resistance. Furthermore, we have shown that adaptation to severe contamination of the Elizabeth River has the potential for significant consequences for the response of adapted killifish to other contaminants. Finally, we have shown that this severe contamination has significant heritable effects that influence killifish subpopulations throughout a large spatial scale. Hopefully, these data are useful for understanding some of the consequences of chronic exposure to a complex contaminant mixture, both for individuals and for populations.
Appendix A. Enhancement of AHR pathway activity in Elizabeth River killifish.

The objective of ongoing work is to enhance the activity of the AHR pathway in the Elizabeth River killifish to determine if this can overcome their resistance to aryl hydrocarbons. Preliminary results of several approaches are described.

A.1 Morpholino knockdown of the aryl hydrocarbon receptor repressor (AHRR)

The aryl hydrocarbon receptor repressor (AHRR), as described previously, is a factor that down-regulates activity of the AHR pathway. As such, prevention of its translation could be a useful mechanism for increasing activation of the AHR pathway in Elizabeth River killifish. To test this, embryos from both the King’s Creek population and the Elizabeth River population were injected with a morpholino targeting killifish AHRR in the same manner as described in Chapters 2 and 3. Two to five nL of morpholino were injected at a concentration of either 125 µM or 250 µM. At 24 hours post fertilization (hpf) embryos were exposed to DMSO or A) a mixture of 20 µg/L benzo[k]fluoranthene (BkF) and 100 µg/L fluoranthene (Fl) or B) 1 µg/L 3,3’,4,4’,5-pentachlorobiphenyl (PCB-126). Injection of AHRR morpholino in King’s Creek embryos appeared to be detrimental, even in DMSO-dosed individuals (Figure 27). It is not clear if this is because it was causing over-activation of the AHR pathway or if it was simply toxic. There did appear to be some exacerbation of PCB-126 toxicity in King’s Creek embryos with the higher dose of AHRR morpholino. Injection of AHRR morpholino did not generally appear to be toxic in DMSO-dosed Elizabeth River embryos, but may have
increased toxicity slightly in embryos exposed to both PCB-126 and the mixture of BkF and Fl.

Figure 27: Mean deformity score of AHRR morpholino injected embryos from King’s Creek and Elizabeth River populations dosed with A) 20 µg/L BkF and 100 µg/L Fl or B) 1 µg/L PCB-126.

A.2 Inhibition of the 26S proteasome

One of the ways in which the magnitude of AHR pathway response is regulated is through proteasomal degradation of the AHR (Ma and Baldwin 2000; Pollenz 2002; Pollenz and Buggy 2006). This occurs at the 26S proteasome. MG132 (Z-Leu-Leu-Leu-al), an inhibitor of the 26S proteasome, was used to inhibit the degradation of the AHR
and increase AHR pathway activity. In the first experiment, King’s Creek embryos were exposed to DMSO or MG132 (1, 10, or 50 µM) at 6 hpf. The embryos were again exposed to the same concentration of MG132 at 24 hpf, along with 100 µg/L BkF and 21 µg/L ethoxyresorufin. At 96 hpf, ethoxyresorufin-o-deethylase (EROD) activity was determined as described previously. Exposure to MG132 in this fashion did increase the EROD activity measured in King’s Creek embryos (Figure 28). In a second experiment, embryos were only exposed to MG132 at 6 hpf. This regime did not yield any increase in EROD activity, suggesting that any effects of the proteasome inhibitor were short-lived (data not shown).

![Figure 28](image.png)

**Figure 28:** Effect of exposure to 1, 10, or 50 µM MG132 at 6 and 24 hpf on EROD activity induced by 100 µg/L BkF in King’s Creek embryos.
A.3 Over-expression of the AHR

Another mechanism for increasing the activity of the AHR pathway in Elizabeth River embryos is to over-express the receptor. Work is currently underway for this approach. Plasmids containing AHR1 and AHR2 were given to us by Mark Hahn. From these plasmids capped and tagged RNA was prepared using the mMessage mMACHINE Ultra kit (Ambion, Austin, TX, USA). Embryos were injected with AHR2 RNA (100 pg in ER, 100 or 200 pg in KC) at the single cell stage. Thus far, it is not clear if the injections were successful, or if injection of this mass of RNA is sufficient to cause an effect (Figure 29). The dose of 100 pg RNA was based on similar experiments in zebrafish (Kyle Erwin, personal communication), but a larger dose-response will be tested in future experiments. To better demonstrate successful over-expression, a version of each AHR is being prepared with a fluorescent tag. This will enable simple visualization that expression of the protein has occurred.

Figure 29: Effect of injection of AHR2 RNA on EROD activity induced by 1 µg/L PCB-126
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Biography

Bryan William Clark was born in Ames, Iowa on September 5, 1979 to Cheryl Linda Clark and William Richard Clark. He received a Bachelor of Science degree, with Distinction, in Chemistry from Iowa State University (Go Cyclones!) in 2001 and a Master of Science in Toxicology from the same university in 2004. His thesis title was *The environmental fate and effects of Bacillus thuringiensis (Bt) proteins in soil.*

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Research Excellence Award – given to top 10% of graduate researchers, Iowa State University, 2004

1st place, Best Student Poster, Ozark-Prairie Region, SETAC, 2003

2nd place, Best Student Poster, Ozark-Prairie Region, SETAC, 2002

Dow-Goetz Scholarship, Iowa State University Chemistry Department, 1997-2001

Chemistry Undergraduate Academic Achievement Award, 1st 1998-2000, 2nd 2001

Felton Memorial Scholarship, Iowa State University Chemistry Department, 1999