Molecular Mechanisms of Polycyclic Aromatic Hydrocarbon-induced Teratogenesis in

Zebrafish (*Danio rerio*)

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

2011
ABSTRACT
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Abstract

Polycyclic aromatic hydrocarbons (PAHs) are ubiquitous environmental contaminants formed from the incomplete combustion of fossil fuels and are found in the environment as complex mixtures. PAHs are developmentally toxic to fish, causing yolk sac edema, hemorrhaging, craniofacial malformations and cardiac defects including impaired heart looping, elongated heart, decreased blood flow, and pericardial effusion. Previous research has shown that many of the toxic effects of PAHs are mediated through the aryl hydrocarbon receptor (AHR), which upregulates phase I and II metabolic genes, but the underlying mechanisms of PAH-induced toxicity are not yet known. The primary goal of this dissertation was to better understand the molecular mechanisms by which PAH mixtures cause developmental toxicity in fish. To this end, the zebrafish (Danio rerio) was used as a developmental model. Simple mixtures consisting of a PAH that is an AHR agonist (benzo[a]pyrene or benzo[k]fluoranthene) and a PAH that is a cytochrome P450 1 (CYP1) inhibitor (fluoranthene) were used in these experiments along with the dioxin-like compound 3,3',4,4',5-pentachlorobiphenyl (PCB-126). Morpholino gene knockdown was used to examine the role of specific genes in response to PAHs, gene expression changes in response to PAH exposures were examined via QPCR, quantification of pericardial effusion was used as a metric for cardiac toxicity, and CYP1 activity was measured as an indication of AHR pathway
induction. First, PAH mixtures consisting of an AHR agonist (BkF) and a CYP1 inhibitor (FL) induced cardiac toxicity that was preceded by upregulation of CYP1 and redox-responsive gene expression, and these effects were dependent upon the AHR2. Second, knockdown of glutathione s-transferase pi class 2 (GSTp2), part of phase II metabolism, exacerbated PAH-induced toxicity but did not affect PCB-126-induced toxicity. Third, knockdown of another isoform of the AHR, AHR1, exacerbated PAH- and PCB-126-induced toxicity and increased CYP1 activity but did not affect CYP expression in response to these agonists. Simultaneous knockdown of AHR1A and AHR2 did not exacerbate nor ameliorate PAH-induced toxicity but did prevent PCB-126-induced toxicity. Fourth, to examine AHR2-dependent and AHR2-independent gene induction in zebrafish hearts in response to PAHs, microarrays were used. Gene expression changes caused by PAHs were largely AHR2-dependent and consisted of genes involved in cell adhesion, oxidation-reduction, and TGF-β signaling processes as well as genes involved in heart structure and function. These findings help to elucidate how PAHs elicit deformities during development and highlight differences between PAHs and other AHR agonists. Additionally, these experiments have identified other genes in addition to AHR2 that are involved in mediating or responding to the toxicity of PAHs.
Dedication

To my remarkable parents. This work and everything else in my life would not be possible without your love, encouragement, and faith in me.
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Acknowledgements

There are so many people that have guided me, challenged me, helped me, and been there for me during this process. I have so much for which to thank each and every one of you.

Dr. Rich Di Giulio, you are the greatest advisor a graduate student could have. Thank you for letting me find my own way, being constantly supportive, always having an open door and open ear, and occasionally, an open bottle of delicious wine. I know that I have become and will continue to be a better scientist and person because of your guidance. Thank you for the honor and joy of spending my graduate career in your lab.

Thank you to my committee, Drs. David Hinton, Heather Stapleton, Joel Meyer, and Margaret Kirby for supporting me, challenging me, and helping me to make sense of data and think about the big picture. Thank you to Dr. Kirby and the rest of the Kirby lab for allowing me to use their fish and equipment for the first (and second) attempt at the microarray experiment. I owe a special thanks to Dr. Meyer for helping me stay sane through the countless disasters of the microarray experiment when I often felt helpless and hopeless.

I began my time in the Di Giulio lab with great mentors. Dr. Lauren P. (Battle) Wills, your smile and laughter, positive disposition, and MJ singing made graduate school not so intimidating. Dr. Alicia Timme-Laragy, this project literally would not have been
possible without you and your hard work. Thank you for taking me under your wing, teaching me the joys of zebrafish imaging and QPCR, and laying the groundwork for my research. Dr. Cole Matson, thank you for always having time to discuss ideas, help with experimental design, statistics, and fish care, and share endless information about tuataras. Dr. Dawoon Jung, your amazing work ethic is matched by your kindness and positivity. I could never have navigated graduate school without you ("Ask Dawoon!").

In addition to wonderful lab mates, I also joined the toxicology program with the best cohort ever. To my lab mate Dr. Bryan Clark, thank you for your help with designing and carrying out experiments, editing large parts of this dissertation, opening me up to new music and ideas, and your honesty and openness. Kyle Erwin, thank you for helping with so many aspects of my zebrafish work, your friendship, and your quest to always know more. Hilary Miller, you stopped being just my colleague a long time ago, you are an amazing friend; thank you for sharing too many good times to count, and always being there during the rough patches, throughout this process. And Eve Marion, thank you for always helping me no matter how silly the request, and for becoming not just my “graduate school Mom” but also a dear friend.

Thank you to the funding sources. Various portions of this work were funded by the NIEHS-supported Duke University Superfund Research Program (P42ES010356) and Duke Integrated Toxicology and Environmental Health Program (T32ES07031).
Thank you to two of the greatest professors and men I have ever had the privilege of learning from - Dr. James Doyle and Dr. Jeffrey McKelvey. You helped to lead me on this crazy path, and I could not be more appreciative for your guidance and friendship.

Most of all, thank you to my amazing family – the one I was born into and the ones I have gained along the way. To my parents, Paul and Cindy Van Tiem – words just aren’t enough. Thank you for giving me a life of never needing but always wanting to be better and for teaching me to question and dream, to always show my love for those most important to me, and to “work hard and always give back”. I love you and I am so very proud to be your daughter. Thank you to my loving, hilarious, goofy, but sometimes serious, “little” brothers, Jonathan and Joseph – you know I love you so much I’ve never wanted little sisters. To my grandparents, Lawrence and Charlaine Ream, thank you for loving me, supporting me, getting excited about my work, bragging to your friends, and giving me a getaway in sunny FL when I need it most. To my cousins by chance, best friends by choice – Ashley and Colleen Van Tiem. You are my rocks. I could not have done this without your love, support, and occasional peer pressure. LYTTMAB. To my sister, Nermeen El Nokali. No matter how our paths have diverged, we are always going in the same direction, a direction I would not be moving in without you. Thank you for your loyalty, laughter, support, love, and sharing in the happiest moments of my life over the past 15 years. You are my role model and my best friend.
Last, though most certainly not least, to my husband-to-be, Samson Garner.

Thank you for loving me, for accepting me, for never giving up on me, and for always supporting me. Thank you for challenging me and taking me out of my comfort zone but always keeping me safe. Your strength, intelligence, humor, unyielding loyalty to friends and family, and absolute selflessness are a daily inspiration to me. I love you. I am so excited and proud to be your wife and cannot wait to experience the rest of my life with you; it’s going to be a grand adventure.
1. Introduction

Aquatic organisms are exposed to a wide range of toxicants and understanding how various compounds interact with one another is of utmost importance for adequate protection of environmental and human health and enacting appropriate remediation efforts of contaminated areas. Various United States Environmental Protection Agency Superfund sites are heavily contaminated with polycyclic aromatic hydrocarbons (PAHs), and other aquatic ecosystems are exposed to increasing levels of PAHs due to increased industrialization and urban run-off (Van Metre and Mahler, 2005). PAHs are present in aquatic systems as complex mixtures and are highly teratogenic to developing fish embryos, causing cardiac, vascular, and craniofacial deformities as well as yolk sac edema and hemorrhaging. While the phenotypic effects of PAHs are well-documented, the exact mechanisms by which their toxicity is manifested still remain unknown. Understanding the mechanisms underlying the toxicity of PAH mixtures is a central theme of this dissertation.

1.1 Polycyclic aromatic hydrocarbons

Polycyclic aromatic hydrocarbons (PAHs) are petrogenic (components of petroleum) or pyrogenic (formed from the incomplete combustion of organic compounds) chemicals that are ubiquitous environmental contaminants. PAHs enter the environment from natural sources such as forest fires and volcanoes but most of
their environmental input is from anthropogenic sources such as the burning of fossil fuels. The concentration of PAHs in the aquatic environment is increasing due to population growth and increased urbanization leading to increased burning of fossil fuels, urban run-off, and atmospheric deposition (Hylland, 2006; Van Metre and Mahler, 2005). PAHs are highly lipophilic and once they enter the environment, they readily partition to particulate matter and sediments in aquatic systems.

PAHs are of environmental and human health concern because many are carcinogenic, mutagenic, teratogenic, immunotoxic, phototoxic, and/or impair endocrine function. Largely due to the carcinogenicity of some PAHs, PAHs as a class are listed as number seven on the U.S. Agency for Toxic Substances and Disease Registry’s 2007 Comprehensive Environmental Response, Compensation, and Liability Act (CERCLA) priority list (ATSDR, 2007). Several PAHs are also listed individually on the list; for example, benzo[a]pyrene (BaP) is currently listed as #9, benzo[b]fluoranthene is #10, dibenzo[a,h]anthracene is #15, benzo[k]fluoranthene (BkF) is #62, and fluoranthene (FL) is #110. PAHs are a diverse group of over 600 congeners, and their toxicity and mechanisms of action are largely dependent upon structure and size. Moreover, PAHs are present in the environment as complex mixtures and therefore, to more accurately predict toxicity and estimate risk, more studies examining the effects of mixtures are necessary.
Certain PAHs, particularly several with greater than four rings, are agonists for the aryl hydrocarbon receptor (AHR; discussed in section 1.2). PAHs and two other classes of compounds that are AHR agonists, dioxins (most notably 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD)) and coplanar polychlorinated biphenyls (PCBs), are well-documented to cause various developmental toxicities in fish including craniofacial malformations, vascular defects in the brain, yolk sac edema, and numerous cardiac defects such as reduced cardiomyocyte number, impaired cardiac looping, an elongated atrium resulting in the “stringy heart phenotype”, pericardial effusion, and decreased blood flow (Dong et al., 2004; Grimes et al., 2008; Incardona et al., 2005; Peterson et al., 1993; Tanguay et al., 2005).

Our laboratory has previously shown that exposure to a PAH that is an AHR agonist and one that is a cytochrome P450 1A inhibitor results in cardiac and craniofacial deformities similar to those caused by TCDD (Billiard et al., 2006; Wassenberg and Di Giulio, 2004). Our laboratory has also shown that individual exposures to the model PAH β-naphthoflavone (BNF) or the strong AHR agonist BkF causes pericardial effusion and elongated hearts in Atlantic killifish embryos (*Fundulus heteroclitus*) (Clark et al., 2010; Matson et al., 2008a). Additionally, individual tricyclic and tetracyclic PAHs and weathered crude oil have also been shown to cause similar cardiac toxicity in zebrafish (Incardona et al., 2004; 2006).
1.2 The aryl hydrocarbon receptor pathway

1.2.1 Basic biology

The aryl hydrocarbon receptor (AHR) is a member of the basic-helix-loop-helix per-ARNT-SIM (bHLH-PAS) family of transcription factors. It is a ligand-activated transcription factor that is constitutively present in the cytoplasm of cells where it is bound by various cofactors and chaperone proteins including XAP2/AIP (X-associated protein 2/AHR-interacting protein also known as AHR-associated protein 9 (ARA9)), two HSP90 (heat shock protein 90) molecules, and the co-chaperone p23 (Hahn, 2002; Petrulis and Perdew, 2002). The functions of these chaperones have received increasing attention in the last decade. XAP2 appears to help stabilize AHR and maintain its location in the cytoplasm by altering the ability of AHR to be recognized by importin β, which is involved in shuttling AHR into the nucleus (Petrulis and Perdew, 2002). The HSP90 dimer appears to be necessary for proper ligand binding conformation of the AHR, and it represses the ability of AHR to dimerize with the aryl hydrocarbon receptor nuclear translocator (ARNT) (Whitelaw et al., 1995). The HSP90-associated co-chaperone p23 also appears to prevent unbound AHR from forming a heterodimer with ARNT and has been hypothesized to enhance ligand-bound AHR movement into the nucleus (Beischlag et al., 2008). However, a recent study has shown that the presence of
p23 is not necessary for ligand binding or upregulation of gene expression by the AHR in mice (Flaveny et al., 2009).

Upon ligand binding, the AHR undergoes a conformational change and translocates into the nucleus where it forms a heterodimer with ARNT, another member of the bHLH-PAS family. Heterodimer formation with ARNT causes the HSP90 proteins to dissociate from AHR. The AHR/ARNT heterodimer then binds to xenobiotic response elements (XRES, also known as dioxin response elements (DREs) or aryl hydrocarbon response elements (AHREs)) in the promoters of various genes. Once bound to XRES, the AHR/ARNT heterodimer recruits various coactivators including SRC-1 (steroid receptor coactivator 1), nuclear receptor coactivator 2 (NCoA-2), p/CIP (p300/CBP cointegrator protein), brahma-related gene 1 (BRG-1), p300, and Rb (retinoblastoma protein) to enhance XRE-driven gene expression (Fujii-Kuriyama and Mimura, 2005; Hankinson, 2005; Merson et al., 2009). A diagram of the AHR pathway is presented in Fig. 1.

Mammals have one AHR while fish have multiple forms. To date, three AHRs (AHR1A, AHR1B, and AHR2) have been identified in zebrafish (Andreasen et al., 2002a; Karchner et al., 2005; Tanguay et al., 1999). Atlantic killifish have two identified AHRs, AHR1 and AHR2 (Karchner et al., 1999), and Atlantic salmon (Salmo salar) have at least six, two AHR1s and four AHR2s (Hansson et al., 2003, 2004). Fish have multiple forms of many genes involved in the AHR pathway likely due to a gene duplication event that
occurred before the divergence of ray- and lobe-finned fishes and was lost during mammalian evolution and another duplication event that occurred after the divergence of ray- and lobe-finned fishes. The AHR1 clade is orthologous to the mammalian AHR, meaning that the two genes descended from a common ancestor and diverged after a speciation event, and AHR2 is a paralog, diverging after the second gene duplication even that occurred in teleost ancestors (Hahn, 2002). Three ARNTs have been identified in mammals and two ARNTs with multiple splice forms have been identified in zebrafish (ARNT1a, b, and c and ARNT2a, b, and c) (Prasch et al., 2006; Tanguay et al., 2000).

The most highly inducible and well-characterized gene that is upregulated by AHR ligand binding is cytochrome P450 1A (CYP1A). CYPs are an incredibly large and diverse group of enzymes; there are at least 81 putatively functional full-length CYP genes in zebrafish and 57 in humans (Nelson, 2009). As a group, CYPs catalyze the oxidative metabolism of a wide range of endogenous compounds, such as steroids, eicosanoids, and bile acids, and exogenous compounds, such as pharmaceuticals and PAHs (Nelson et al., 2004). The CYP1 family of enzymes metabolizes PAHs. Because CYPI activity is so highly induced by PAHs, it is often used as a biomarker of PAH exposure. The intermediate metabolites of CYP1 metabolism are often more hydrophilic than the parent compound and more easily conjugated to phase II enzymes. However, the reactive metabolites formed via CYP activation can be harmful, as is the case for the
BaP metabolite benzo[a]pyrene-7,8-diol-9,10-epoxide (BPDE), which is highly carcinogenic due to its ability to bind to DNA, and certain semi-quinone metabolites that are capable of redox cycling and producing oxidative stress (Miller and Ramos, 2001). Zebrafish have one CYP1A gene (mammals have two, 1A1 and 1A2), CYP1B1, CYP1C1 and 1C2 (which may be specific to fish), and CYP1D1 (Godard et al., 2005; Goldstone et al., 2009). CYP1A, CYP1B1, and CYP1C1 and 1C2 expression are induced by TCDD and their expression is mediated by AHR2 while CYP1D1 is not upregulated by TCDD and does not appear to be regulated by AHR2 (Goldstone et al., 2009; Jönsson et al., 2007).

The classical AHR gene battery in mammals also includes the phase II enzymes aldehyde dehydrogenase 3, (ALDH3), NAD(P)H:quinone oxidoreductase (NQO1), glutathione s-transferases (GSTs), and UDP-glucuronosyltransferases (UDPGTs) (Nebert et al., 2000). ALDH3 oxidizes potentially damaging electrophilic aldehydes to ketones and carboxylic acids. NQO1 catalyzes the reduction of quinones to hydroquinones via a two-electron transfer rather than a one-electron transfer that produces redox cycling semi-quinones, potentially resulting in oxidative stress. GSTs conjugate reactive epoxides and other electrophilic metabolites to glutathione (Hayes et al., 2005). Seven cytosolic classes of GSTs have been identified in mammals. The pi class has been shown to be highly efficient in conjugating carcinogenic BaP metabolites to glutathione (Robertson et al., 1986), and the pi and mu class have the highest activity with reactive
epoxides (Ketterer and Mulder, 1990). UDPGTs conjugate glucuronic acid to reactive metabolites, and in mammals, Ugt1a6 is strongly upregulated by the AHR ligand binding (Buckley and Klaassen, 2009). Conjugation by GSTs or UDPGTs results in a more water soluble compound that is more readily excreted from the cell and the organism.

In addition to inducing phase I and II metabolic genes, the AHR also upregulates the aryl hydrocarbon receptor repressor (AHRR). Like AHR and ARNT, AHRR is a member of the bHLH-PAS protein family, but it lacks a ligand-binding region (Mimura et al., 1999). AHRR is capable of forming a heterodimer with ARNT and then binding to XREs, but its mechanism of repression does not appear to be solely due to competing for XREs with the AHR/ARNT complex (Evans et al., 2008). There appears to be one AHRR in mammals but zebrafish have two, initially identified as AHRR1 and 2, and now known as AHRRa and AHHRb, respectively (Evans et al., 2005; Jenny et al., 2009). The list of XRE-driven genes is certainly not fully populated and the identification of additional XRE-driven genes is an area of extensive research.

1.2.2. Role of the AHR in mediating toxicity

The AHR pathway appears to be highly conserved between mammals and fishes (Hahn, 2002) and responds to xenobiotics such as TCDD, PCBs, and PAHs similarly in mammals and fishes (Billiard et al., 2006; Jonsson et al., 2007; Prasch et al., 2003). The
AHR mediates most, if not all, of the teratogenic effects of TCDD and certain PAHs in mammals. AHR deficient mice are protected from the carcinogenicity of BaP and the developmental toxicity induced by TCDD (Fernandez-Salguero et al., 1996; Shimizu et al., 2000). AHR2 also mediates TCDD toxicity in developing zebrafish; knockdown of AHR2 prevents pericardial effusion, craniofacial malformations, and reduced blood flow in the brain (Dong et al., 2004; Prasch et al., 2003). Heterodimer formation with ARNT1 has been shown to be necessary for TCDD toxicity (Prasch et al., 2006). CYP1A induction appears to have no effect on TCDD toxicity as knockdown of CYP1A did not alter TCDD-induced toxicity in zebrafish (Carney et al., 2004), but CYP1A serves a protective role against the toxicity induced by BNF in zebrafish and killifish embryos (Billiard et al., 2006; Matson et al., 2008a). Both AHRRa and AHRRb also appear to have a protective role. Knockdown of AHRRa results in deformities similar to TCDD and PAHs in untreated embryos and knockdown of both AHRRs exacerbates TCDD-induced deformities (Jenny et al., 2009).

Though the AHR has been shown to mediate many of the effects of TCDD and some PAHs, studies have shown that certain PAHs elicit their toxicity via AHR-independent mechanisms. Knockdown of AHR2 did not protect zebrafish embryos from the pericardial and yolk sac edema and cardiac arrhythmia caused by two tricyclic PAHs, dibenzothiophene and phenanthrene, or the cardiac dysfunction or intracranial hemorrhage caused by weathered crude oil exposure (Incardona et al., 2005). The AHR-
independent mechanisms by which certain PAHs exert their toxicity as well as the identification of other genes that are regulated by the AHR are an area of vigorous research.

1.2.3 The AHR pathway and oxidative stress

One mechanism by which PAHs are hypothesized to cause toxicity is through production of reactive oxygen species (ROS). ROS production is necessary for normal cell function but production of ROS in excess of levels that cellular enzymes are able to neutralize can lead to oxidative stress and cellular damage (Di Giulio and Meyer, 2008). PAH metabolites can cause uncoupling of the mitochondrial electron transport chain and uncoupling of CYP redox reactions (Livingstone, 2001). PAHs can also be metabolized into reactive o-quinone metabolites that produce ROS via redox cycling (Li et al., 2003; Penning et al., 1996).

Some PAHs, such BaP and BNF, are bifunctional inducers. They induce phase I metabolism via binding to the AHR, which binds to and upregulates genes containing XREs, and following their metabolism into electrophilic metabolites, they induce phase II and redox-responsive genes containing antioxidant response elements (AREs) (Nguyen et al., 2003). Signaling through AREs is regulated by the transcription factor NF-E2 p45–related factor 2 (Nrf2) (Itoh et al., 1997). There is increasing evidence of AHR and Nrf2 crosstalk. Mouse Nrf2 contains three XREs in its promoter region, and Nrf2 binds to a functional ARE in the proximal promoter of the AHR in mouse embryonic
fibroblasts (Miao et al., 2005; Shin et al., 2007). Furthermore, Nrf2 and AHR are both required for induction of NQO1, UGT1a6, and GSTα, members of the typical ‘AHR gene battery’, in response to TCDD exposure in mice (Yeager et al., 2009). The pi class GSTs are also regulated by Nrf2 in zebrafish (Suzuki et al., 2005). However, the mechanism by which AHR and Nrf2 interact in zebrafish is still unclear. It is possible that CYP induction by AHR agonists increases oxidative stress, leading to Nrf2 activation and cell signaling or that ligand binding to the AHR causes it to bind to an XRE on Nrf2, thus upregulating its transcription.

1.3 Zebrafish

1.3.1 Basic biology

Zebrafish (Danio rerio; Cyprinidae family) are tropical freshwater fish native to the Ganges River of East India and are now reared in pet stores and laboratories around the world. They have many attractive qualities that make them an excellent laboratory model. As adults, they are approximately 3-4 cm in length, which allows for housing of a large number of individuals in relatively small stand-alone recirculating systems such as the AHAB system (Aquatic Habitats, Apopka, FL) used in our laboratory. Males and females are sexually dimorphic: males are usually smaller than females and are torpedo-shaped with yellow coloring in between the black stripes, while females have a fuller belly and white coloring in between the black stripes. Zebrafish reproduce quickly,
reaching reproductive maturity by approximately three months, breed year-round, can be bred every 4-5 days, and females are capable of producing hundreds of eggs at one time. Embryos develop outside the mother in eggs that have a transparent chorion, allowing for easy and non-invasive in ovo imaging during development. Embryos also develop very quickly. Organogenesis occurs within the first 48 hours post fertilization (hpf) and embryos hatch around 72 hpf (Kimmel et al., 1995).

1.3.2. Use as a developmental model

Zebrafish are a widely used model in biomedical research and more recently, in toxicological research (Epstein and Epstein, 2005; Hill et al., 2005). They are a superb model for studying normal vertebrate development as well as how toxicants impair development, including cardiac development. Zebrafish contain a two-chambered heart but the atrium, ventricle, and bulbus arteriosus are akin to the atria, ventricles, and aorta/pulmonary trunk, respectively, in four-chambered vertebrate hearts (Grimes et al., 2006). The zebrafish heart develops as a linear tube and begins to beat by 24 hpf (Kimmel et al., 1995). The early heart-tube stage of zebrafish shares similar morphology with higher vertebrates such as mice and humans, and the genetic pathways coordinating heart and vascular development are well-conserved (Fishman and Chien, 1997; Stainier, 2001).

Fish are frequently used as models for AHR ligand toxicity research because developing embryos are highly sensitive to TCDD toxicity. Lake trout (Salvelinus
*namaycush*) are the most sensitive (LC$_{50}$ = 47-80 pg/g) (Walker et al., 1991) while zebrafish are the least sensitive (LC$_{50}$ = 2.5 ng/g) of studied fishes (Henry et al., 1997). In comparison, the LC$_{50}$ of the guinea pig, the most sensitive mammal, is 2 ng/g (Poland and Knutson, 1982).

There are myriad genetic tools available for studying developmental toxicity in zebrafish. The zebrafish genome is sequenced, though has not yet been fully annotated. There is a high degree of synteny between the zebrafish and human genome (Barbazuk et al., 2000), and many of the genes involved in the AHR pathway and in PAH toxicity are well-conserved between fish and higher vertebrates (Hahn, 2002). There are numerous mutants and transgenic zebrafish lines available that facilitate imaging during development. For example, AB(*cmlc2::GFP*) zebrafish express green fluorescent protein (GFP) in myocardial cells (Huang et al., 2003), which allows easy visualization of the heart. Fish of another transgenic line, Tg(*cmlc2::dsRed2-nuc*), express red fluorescent protein in myocardial cell nuclei (Rottbauer et al., 2002), which affords the ability to easily count cardiomyocyte number under fluorescent microscopy. Another powerful tool used in zebrafish is gene knockdown via morpholino antisense oligonucleotides. Morpholinos are synthesized to target a specific gene of interest and are easily injected into the yolk of the embryo after fertilization. They function by binding to the targeted mRNA and blocking protein translation. They are stable throughout the four-day window in which our laboratory and others examine the impacts of PAHs on
development (Nasevicius and Eker, 2000). Morpholinos are now extensively used not only to examine the developmental and physiological functions of specific genes but also to determine the role of genes of interest in mediating toxicity to exogenous compounds. Additionally, due to the sequencing of the zebrafish genome, microarrays, whereby the expression of a vast number of genes can be quantified at one time, have gained popularity in the last decade and can be a powerful tool for identifying novel genes and pathways involved in toxicological responses in this model.

1.4 Dissertation Objective and Outline

The primary goal of this dissertation is to identify and better understand the molecular mechanisms by which PAHs induce toxicity in developing fish. One hypothesis of this work is that AHR2 mediates PAH-induced toxicity not only through regulation of known phase I and II metabolic genes but also through regulation of or interaction with redox-responsive genes and other genes not yet known to be associated with the AHR (Chapters 2 and 5). Similarly, I hypothesized that although the AHR2 mediates a large portion of PAH-induced toxicity, genes not controlled by AHR2 play an important role in responding to PAHs (Chapters 3 and 4). To carry out this work, I used two environmentally relevant AHR agonists, BaP and the stronger agonist BkF, and the CYP1 inhibitor FL (Barron et al., 2004; Billiard et al., 2002; Willett et al., 1998). The PCB 3,3′,4,4′,5-pentachlorobiphenyl (PCB-126), a strong AHR agonist, was used as a
representative dioxin-like compound (DLC). PCB-126, like TCDD, is capable of inducing deformities on its own and was used to compare and contrast the effects and molecular mechanisms of PAHs and DLCs. The chemical structures of the compounds used in this dissertation are presented in Fig. 2.

This dissertation is divided into the four following research chapters:

Chapter 2: AHR2 knockdown prevents PAH-mediated cardiac toxicity and XRE- and ARE-associated gene induction in zebrafish (*Danio rerio*)

The ability of a PAH mixture (BkF + FL) to induce cardiac deformities and XRE- and ARE-mediated gene expression was examined. Subsequently, the role of AHR2 in the induction of deformities and gene expression was determined using morpholino gene knockdown of AHR2. I hypothesized that BkF + FL would cause cardiac deformities and that these deformities would be preceded by XRE- and ARE-mediated gene expression and that knockdown of AHR2 would prevent cardiac toxicity and gene expression changes.

Chapter 3: Glutathione s-transferase pi class 2 (GSTp2) protects against the cardiac deformities caused by exposure to PAHs but not PCB-126 in zebrafish embryos

The expression of GSTp2 in response to PAH mixtures and PCB-126 was examined. The impact of GSTp2 knockdown on PAH- and PCB-126-induced cardiac toxicity was examined. I hypothesized that PAH mixtures and PCB-126 would induce
GSTp2 expression and that knockdown of GSTp2 would exacerbate PAH-induced deformities but not PCB-126-induced deformities.

Chapter 4: The role of AHR1 isoforms in PAH- and PCB-126-induced toxicity

The role of the two zebrafish AHR1 isoforms, AHR1A and 1B, in PAH- and PCB-126-induced toxicity was examined. Additionally, the influence of AHR1A knockdown on CYP1 activity and CYP1 gene expression was determined. The effect of simultaneous knockdown of AHR1A and AHR2 on PAH and PCB-126 toxicity was also examined. I hypothesized that AHR1A knockdown would have no effect on PAH- or PCB-126-induced toxicity or gene expression while knockdown of AHR1B would be protective, though not to the same extent as AHR2 knockdown.

Chapter 5: Heart-specific microarray identification of AHR-dependent and AHR-independent genes involved in the synergistic developmental toxicity of PAHs

Microarray analysis was used to examine PAH-induced gene expression in zebrafish hearts at various time points after dosing. Zebrafish were injected with AHR2-morpholino or control-morpholino to examine which genes induced by PAHs are regulated by the AHR. I hypothesized that the majority of gene expression changes that occurred in response to PAH exposure would be regulated by AHR2, but that certain genes would be affected by PAH exposure in an AHR2-independent manner.

Finally, the results of this research, their implications, and future directions are summarized in Chapter 6.
The AHR (aryl hydrocarbon receptor) is constitutively present in the cytoplasm bound by chaperone proteins including two HSP90 molecules (heat shock protein 90), p23, and XAP2 (hepatitis virus B X-associated protein 2). Upon ligand binding, the AHR translocates into the nucleus and forms a heterodimer with ARNT (aryl hydrocarbon receptor nuclear translocator), which causes the dissociation of the chaperone proteins from the AHR. The AHR/ARNT heterodimer binds to XREs (xenobiotic response elements) present in the promoter regions of targeted genes, resulting in upregulation of genes involved in xenobiotic metabolism (e.g., cytochrome p450s (CYPs)) and negative feedback regulation of the AHR (aryl hydrocarbon receptor repressor (AHRR)).
Figure 2: Chemical structures of the AHR agonists BaP, BkF, and PCB-126 and the CYP1 inhibitor FL.
2. AHR2 knockdown prevents PAH-mediated cardiac toxicity and XRE- and ARE-associated gene induction in zebrafish (Danio rerio)

This chapter was published under the same title in Toxicology and Applied Pharmacology, Volume 254, pages 280-287, in the year 2011. The authors are Lindsey A. Van Tiem and Richard T. Di Giulio.

2.1 Introduction

Polycyclic aromatic hydrocarbons (PAHs) are widespread environmental contaminants that are produced predominantly by anthropogenic activities via the incomplete combustion of petroleum products and are also components of crude oil. PAH levels have been increasing in the environment over the last two decades in association with increased urban sprawl and increased use of automobiles (Lima et al., 2003; Van Metre and Mahler, 2005). PAHs also enter into the environment during oil spills such as the Cosco Busan oil spill in the San Francisco Bay in November 2007 and the more recent Deepwater Horizon oil spill in the Gulf of Mexico in the spring and summer of 2010, and PAHs from oil spills may persist in aquatic ecosystems for decades (Diercks et al., 2010; Lemkau et al., 2010; Short et al., 2004). As PAHs have increased in aquatic ecosystems, their effects on fish have begun to receive greater attention. Some PAHs, as well as coplanar polychlorinated biphenyls (PCBs) and dioxins, elicit a range of developmental toxicities, including cardiac deformities, in various fish species (Incardona et al., 2004; Scott and Hodson, 2008; Wassenberg and Di Giulio, 2004). The
developmental cardiac toxicity caused by some PAHs, coplanar PCBs, and dioxins is mediated through the aryl hydrocarbon receptor (AHR) (Schmidt and Bradfield, 1996). Upon ligand-activated binding by an AHR agonist, the AHR dissociates from chaperone proteins in the cytoplasm and is translocated into the nucleus. Once in the nucleus, the AHR dimerizes with the aryl hydrocarbon receptor nuclear translocator (ARNT) and binds to xenobiotic response elements (XREs), causing the upregulation of numerous phase I and II metabolic enzymes, including the cytochrome P450 1 (CYP1) gene family, glutathione s-transferases (GSTs), and NADP(H):oxidoreductase (Denison and Nagy, 2003; Nebert et al., 2000).

Zebrafish (*Danio rerio*) have three characterized AHRs (Karchner et al., 2005; Tanguay et al., 1999), and knockdown of the AHR2 protects against the developmental toxicity of 2,3,7,8-tetrachlorodibenzodioxin (TCDD) (Teraoka et al., 2003), certain low molecular weight PAHs (Incardona et al., 2006), and the model PAHs β-naphthoflavone (BNF) and α-naphthoflavone (ANF) (Billiard et al., 2006). AHR2 knockdown has also been shown to protect against the cardiac toxicity of BNF, 3,3’,4,4’,5-pentachlorobiphenyl (PCB-126), and the PAH benzo[k]fluoranthene (BkF) in killifish, *Fundulus heteroclitus* (Clark et al., 2010). While exposure of fish to certain individual AHR agonists can induce cardiac deformities, co-exposure of an AHR agonist and a CYP inhibitor results in severe, synergistic toxicity (Billiard et al., 2006; Wassenberg and Di Giulio, 2004).
However, the exact molecular mechanisms and cell signaling pathways responsible for PAH-induced developmental toxicity in fish remain unclear.

One possible mechanism by which PAHs elicit toxicity is through the induction of oxidative stress. The formation of reactive oxygen species (ROS) is essential for normal cell function; however, ROS production in excess of levels that cellular enzymes are able to neutralize can lead to oxidative stress and cellular damage (Di Giulio and Meyer, 2008). PAHs can lead to the overproduction of ROS in multiple ways. PAHs may be enzymatically, auto- or photo-metabolized into reactive o-quinone metabolites that produce ROS via redox cycling (Li et al., 2003; Penning et al., 1996). Furthermore, PAH metabolites may also act to increase uncoupling of the mitochondrial electron transport chain and uncoupling of CYP redox reactions (Livingstone, 2001; Winston and Di Giulio, 1991).

Various studies indicate that the toxicity caused by some PAHs, coplanar PCBs, and dioxins may involve oxidative stress. TCDD has been shown to induce H$_2$O$_2$ production in mice mitochondria, an effect that was dependent upon TCDD binding to the AHR (Senft et al., 2002). In scup (Stenotomus chrysops), PCB-126 has been shown to induce ROS production in liver microsomes and cause increased levels of catalase, glutathione peroxidase, glutathione reductase, and superoxide dismutase activities in the liver (Schleizinger and Stegeman, 2001). In addition, killifish from a creosote-contaminated Superfund site have been shown to have elevated hepatic total
glutathione levels (Bacanskas et al., 2004), and their F1 progeny were resistant to the acute toxicity of the model pro-oxidant tert-butyl hydroperoxide (Meyer et al., 2003). The F1 progeny also exhibited higher manganese superoxide dismutase (MnSOD) protein levels, glutathione concentrations, and total oxy-radical scavenging capacity as compared to reference site F1 fish. Furthermore, BNF + ANF co-exposure in zebrafish has been shown to cause an upregulation of various redox-responsive genes (Timme-Laragy et al., 2009).

In this study, we examined the cardiac deformities and phase I and redox-responsive gene expression changes caused by two environmentally relevant PAHs, BkF and fluoranthene (FL), individually and in combination. BkF is a strong AHR agonist (Billiard et al., 2002), and FL is a CYP1 enzyme inhibitor (Willett et al., 2001). We then examined the effect of AHR2 knockdown on cardiac deformities and gene expression induced by PAH exposure.

2.2 Materials and Methods

2.2.1 Fish care

Adult Ekkwill zebrafish (Danio rerio; Ekkwill Waterlife Resources, Ruskin, FL, USA) were maintained at 28°C in a recirculating AHAB system (Aquatic Habitats, Apopka, FL, USA) under a 14:10 h light:dark cycle. Adults were fed brine shrimp and a mix of Ziegler’s Adult Zebrafish Complete Diet (Aquatic Habitats) and Cyclop-eeze
Embryos were collected after natural spawning of adult zebrafish and were maintained in 30% Danieau (Nasevicius and Ekker, 2000) under the same temperature and photoperiod as adults. Adult care and reproductive techniques were non-invasive and approved by the Duke University Institutional Animal Care & Use Committee (A279-08-10).

2.2.2 Chemicals and exposure

Benzo[k]fluoranthene (BkF) and fluoranthene (FL) standards were purchased from AccuStandard (Hamden, CT, USA). Dimethyl sulfoxide (DMSO), 7-ethoxyresorufin (7-ER), and tricaine methanesulfonate (MS-222) were purchased from Sigma-Aldrich (St. Louis, MO, USA). BkF, FL, and 7-ER were dissolved in DMSO, protected from light, and kept at -20°C until use.

At 24 hours post fertilization (hpf), embryos exhibiting normal development were dosed in 7.5 mL 30% Danieau in 20-mL glass scintillation vials, with five embryos per vial and three vials per treatment. Embryos were dosed with DMSO, 50 μg/L BkF, 150 μg/L FL, or a co-exposure of 50 μg/L BkF + 150 μg/L FL. Final DMSO concentration was < 0.03%. For experiments examining CYP1 activity via the 7-ethoxyresorufin-O-deethylase (EROD) assay, 7-ER was added at a final concentration of 21 μg/L at the time of PAH dosing. Deformity assessment and EROD assay were performed at 96 hpf. For gene expression experiments, six vials per treatment with five embryos per vial were
dosed with either DMSO, BkF, FL, or BkF + FL at 24 hpf. At 48 hpf, embryos were dechorionated, pooled in groups of 10 (two vials), and fixed in RNAlater® (Applied Biosystems, Foster City, CA, USA). Samples were stored at -80°C until RNA extraction.

2.2.3 Morpholino injection

A previously designed morpholino (Teraoka et al., 2003) shown to block the translation of zebrafish aryl hydrocarbon receptor 2 (AHR2-mo) was purchased from Gene Tools, LLC (Philomath, OR, USA). The sequence of the AHR2-mo is 5’-TGTACCGATAACCGCGACATGGTT-3’. Gene Tools’ standard control morpholino (5’-CCTCTTACCTCAGTTACAATTATA-3’) was used as a morpholino injection control. The morpholinos were fluorescein-tagged at the 3’ end to monitor injection success and were diluted to 100 µM working stocks in 30% Danieau.

The morpholinos (approximately 3 nL) were injected by hand into the yolk of zebrafish embryos at the 1-4 cell stage using a microinjection system consisting of a Nikon SMZ-1500 zoom stereomicroscope (Nikon Instruments Inc., Lewisville, TX, USA) and an MDI PM 1000 Cell Microinjector (MicroData Instrument Inc., S. Plainfield, NJ, USA). Embryos exhibiting normal development and strong, uniform incorporation of the morpholinos were used for experiments.
2.2.4 Deformity assessment

At 96 hpf, hatched zebrafish embryos were screened for cardiac deformities via measurement of pericardial effusion (Billiard et al., 2006). Fish were rinsed with 30% Danieau and anesthetized with MS-222. Fish were then placed in a left lateral orientation in 3% methylcellulose on depression slides and were imaged under 50x magnification (Zeiss Axioskop, Thornwood, NY, USA). The two-dimensional image of the pericardial area was manually traced and then quantified using IPLab software (Scanalytics Inc., Fairfax, VA, USA). Deformity values are expressed as a percentage of the two-dimensional pericardial area of non-injected (NI) control embryos.

2.2.5 In vivo EROD assay

At the same time as deformity assessment (96 hpf), CYP1 activity was measured via a modified in vivo EROD assay (Billiard et al., 2006; Matson et al., 2008b; Nacci et al., 1998). The CYP1 enzymes metabolize 7-ER into a fluorescent product, resorufin, which accumulates in the gastrointestinal tract in zebrafish (Billiard et al., 2006). Fluorescence was measured under 50x magnification using a rhodamine red filter set (Zeiss Axioskop) and quantified by IPLab software (Scanalytics Inc.). EROD values are expressed as a percentage of the mean fluorescence of NI control embryos.
2.2.6 RNA extraction and reverse transcription

Samples were homogenized with a sterile hand-held homogenizer for 30 s, and RNA was extracted according to the RNA-Bee protocol (Tel-Test Inc., Friendswood, TX, USA). RNA quantity was analyzed spectrophotometrically using a NanoDrop ND-100 (NanoDrop Technologies, Wilmington, DE, USA). cDNA was synthesized using the Omniscript Reverse Transcriptase kit (Qiagen, Inc., Valencia, CA, USA) according to the manufacturer’s instructions with 500 ng RNA, random hexamers, and RNaseOut (Invitrogen, Carlsbad, CA, USA). The reverse transcription reaction was performed in a thermocycler for 1 hour at 37°C, and the resulting cDNA was diluted to a concentration of 2 ng/μL.

2.2.7 QPCR

The expression of the following genes was examined: CYP1A, CYP1B1, CYP1C1, pi class of glutathione s-transferase (GSTp2), the catalytic subunit of glutamate cysteine ligase (GCLc), glutathione peroxidase 1 (GPx1), manganese superoxide dismutase (MnSOD), and copper zinc superoxide dismutase (CuZnSOD). β-actin was used as a housekeeping gene. CYP1A, CYP1B1, and CYP1C1 primers were published previously (Timme-Laragy et al., 2007). GPx1, MnSOD, and CuZnSOD primers were also published previously (Malek et al., 2004). Primers for GCLc and GSTp2 were designed using PrimerQuest software (Integrated DNA Technologies, Inc., www.idtdna.com). β-
actin primers were supplied by Kyle Erwin; the forward primer was published previously (Grimes et al., 2008) and the reverse was designed using PrimerQuest software. Primer sequences are listed in Table 1.

Each 25 μL QPCR reaction consisted of 12.5 μL SYBR Green PCR Master Mix (Applied Biosystems), 9.5 μL dH2O, 200 nM forward and reverse primer, and 2 μL cDNA template. The reaction was carried out using an Applied Biosystems 7300 Real-Time PCR System with the following thermal profile: 10 min at 95°C and 40 replicates of 15 s at 95°C, 1 min at 60°C. A dissociation curve was calculated for each sample at the end of each profile. All samples were run in duplicate and technical replicates were averaged prior to analysis.

The ABI PRISM 7300 Sequence Detection System Software, Version 1.1 (Applied Biosystems) was used to carry out data analysis. The average mRNA fold induction of each target gene was calculated by comparing the C\textsubscript{T} (threshold cycle) of the target gene to that of β-actin according to Livak and Schmittgen (2001). β-actin expression remained constant across treatments. Each experiment consisted of three biological replicates (10 pooled embryos) per treatment and each experiment was replicated at least three times for a final n = 9-12.
2.2.8 Statistical analysis

Statistical analyses were performed using JMP 8.1.1 (SAS Institute Inc., Cary, NC, USA). For deformity and EROD analysis of non-injected (NI) experiments, data were analyzed via one-way analysis of variance (ANOVA) followed by Tukey’s post-hoc test. For morpholino experiments, the data were analyzed via two-way ANOVA to determine an overall effect of the morpholino injection and dose followed by least square means (LSMeans) procedures. Tukey’s post-hoc test was used to determine differences between groups. All experiments were replicated at least three times with at least three samples per treatment per experiment, and no differences between experimental replicates were observed for any test. Data are represented as mean ± standard error of the mean (SEM). Values were considered significantly different at $p \leq 0.05$.

2.3 Results

2.3.1 Deformities and EROD activity

Individually, 50 $\mu$g/L BkF and 150 $\mu$g/L FL did not cause cardiac deformities in zebrafish embryos; embryos exposed to BkF had an average pericardial area of 97±2% and those exposed to FL had an average pericardial area of 105±2% of control pericardial area (Fig. 3). However, exposure to BkF + FL caused significant pericardial effusion (226±11%; $p < 0.0001$) compared to DMSO controls.
Exposure to FL decreased EROD activity to below control levels but the difference was not significant (67±9%; \(p = 0.0940\)) (Fig. 3). BkF exposure caused a large and significant induction of EROD response (635±15%; \(p < 0.0001\)) compared to controls. Exposure to FL with BkF inhibited the increase in EROD activity caused by BkF alone; the EROD activity of the co-exposure was slightly greater than controls (139±10%; \(p = 0.0366\)).

2.3.2 Gene expression

As expected, the three examined CYPs (CYP1A, 1B1, and 1C1) were significantly upregulated by exposure to BkF (Fig. 4). CYP1A mRNA expression was induced 115.2±10.9-fold above control levels, CYP1B1 9.9±0.8-fold, and CYP1C1 15.1±2.6-fold (\(p < 0.0001\), \(p < 0.004\), and \(p < 0.0001\), respectively). FL exposure did not significantly alter CYP1 expression compared to control levels (CYP1A – 3.4±0.8-fold; CYP1B1 - 1.5±0.3-fold; and CYP1C1 - 0.8±0.1-fold, all \(p > 0.05\)). Exposure to BkF + FL caused a significant (\(p < 0.0001\)) and greater than additive induction of CYP1A, CYP1B1, and CYP1C1 of 281.7±29.0-fold, 23.1±3.1-fold, and 65.1±12.1-fold, respectively (Fig. 4).

Exposure to BkF alone also caused significant induction of certain redox-responsive genes. The greatest induction was observed for GSTp2, which was induced 3.1±0.7-fold (\(p = 0.037\)) (Fig. 5). BkF exposure also significantly upregulated GCLc expression (1.9±0.3-fold, \(p = 0.030\)). FL did not induce mRNA expression of GSTp2,
GPx1, or GCLc. Co-exposure to BkF + FL caused a significant and greater than additive upregulation of GSTp2 (11.0±0.9-fold; \( p < 0.0001 \)) and GPx1 (3.3±0.2-fold; \( p < 0.0001 \)). The co-exposure also caused a significant 2.0±0.1-fold induction of GCLc, but this induction was not different than that caused by BkF alone (\( p < 0.005 \) vs. control, \( p = 0.9372 \) vs. BkF). None of the treatments caused a significant change in MnSOD or CuZnSOD expression (data not shown).

2.3.3 Effect of AHR2 knockdown on deformities and EROD activity

AHR2 knockdown via morpholino injection did not alter the average pericardial area of controls, BkF-exposed, or FL-exposed embryos compared to NI controls (Fig. 6). However, AHR2 knockdown in fish co-exposed to BkF + FL resulted in a mean pericardial effusion of 116±5%; this effusion was significantly (\( p < 0.0001 \)) less than the extreme pericardial effusion of NI BkF + FL-exposed fish and not statistically different than NI DMSO fish. Injection of the control morpholino did not cause any differences in deformities, EROD, or gene expression as compared to controls (data not shown).

AHR2 knockdown caused reduced EROD activity compared to NI fish. In fish exposed to BkF, AHR2 knockdown did not completely prevent EROD activity; the fish still had a significant induction of EROD activity (367±19% of NI control activity; \( p < 0.0001 \)), but the induction was significantly less than the NI fish exposed to BkF (\( p < 0.0001 \) vs. NI BkF activity) (Fig. 6). Fish injected with AHR2-mo and co-exposed to BkF +
FL had an average EROD activity of 107±4%, which was not statistically different than NI controls or NI fish co-exposed to BkF + FL.

2.3.4 Effect of AHR2 knockdown on gene expression

AHR2 knockdown reduced the mRNA expression of the three examined CYPs. CYP1A expression in AHR2-mo embryos following exposure to BkF was not different than NI control levels ($p = 0.9795$) and was significantly less than the induction in NI embryos exposed to BkF ($p < 0.0001$ vs. NI BkF) (Fig. 7). After exposure to BkF + FL, CYP1A expression in AHR2-mo embryos was still significantly higher (83.7±10.4-fold) than NI controls ($p < 0.0002$) but was significantly less than the expression in NI embryos exposed to BkF + FL ($p < 0.0001$ vs. NI BkF + FL). AHR2 knockdown prevented the upregulation of CYP1B1 after exposure to BkF individually (2.3±0.3-fold) and BkF + FL (4.8±0.5-fold) that occurred in NI embryos. These small inductions were significantly reduced compared to NI embryos of the same treatment ($p = 0.0026$ vs. NI BkF and $p < 0.0001$ vs. NI BkF + FL, respectively) and were not significantly different than NI control levels. Expression of CYP1C1 in AHR2-mo embryos following BkF exposure (11.4±1.1-fold) was not induced above NI control levels and was not different than induction in NI embryos exposed to BkF ($p = 0.9137$ vs. NI control and $p = 0.9996$ vs. NI BkF). After exposure of AHR2-mo embryos to BkF + FL, CYP1C1 expression (34.4±6.0-fold) was significantly less than expression in NI embryos ($p = 0.0016$ vs. NI BkF + FL) but was still
significantly greater than control levels ($p = 0.0006$). FL exposure did not significantly alter $CYP1A$, $1B1$, or $1C1$ expression in AHR2-mo embryos.

AHR2 knockdown also altered the expression of several redox-responsive genes. The significant induction of $GSTp2$ that occurred in NI embryos after BkF exposure was prevented by AHR2 knockdown (1.0±0.2-fold) (Fig. 8). AHR2 knockdown also prevented the upregulation of $GSTp2$ in embryos exposed to BkF + FL (2.7±0.3-fold). The upregulation of $GCLc$ that occurred after BkF exposure in NI embryos was prevented by AHR2 knockdown (0.8±0.2-fold). In embryos exposed to BkF + FL, AHR2 knockdown also prevented upregulation of $GCLc$ (1.0±0.1-fold) and $GPx1$ (1.2±0.2-fold). As in NI embryos, AHR2 knockdown did not alter $MnSOD$ or $CuZnSOD$ expression (data not shown).

2.4 Discussion

Co-exposure to the strong AHR agonist BkF and the CYP1 inhibitor FL resulted in severe pericardial effusion in zebrafish. This pericardial effusion was accompanied by increased expression in phase I and redox-responsive genes. Some PAHs, including benzo[a]pyrene (BaP) and BNF, are considered bifunctional inducers. They induce phase I metabolism via ligand binding to the AHR, which binds to and upregulates genes containing XREs, most notably, CYPs. They can also upregulate genes following their metabolism into electrophilic metabolites, which can induce phase II and redox-
responsive genes containing antioxidant response elements (AREs) (Prochaska et al., 1985; Rushmore et al., 1991a). It has been shown that gene signaling through AREs is regulated by the transcription factor NF-E2 p45-related factor 2 (Nrf2) (Itoh et al., 1997). It was initially thought that these two mechanisms of induction, AHR-XRE-mediated and Nrf2-ARE-mediated, occurred independently of one another. However, there is evidence for crosstalk between the AHR pathway and Nrf2-mediated oxidative stress pathways: the mouse Nrf2 contains three XREs in its promoter region, and in mouse embryonic fibroblasts, Nrf2 has been shown to bind to a functional ARE in the proximal promoter of the AHR (Miao et al., 2005; Shin et al., 2007). In accordance with AHR binding and phase I metabolic induction, BkF + FL-induced cardiac deformities were indeed preceded by large increases in CYP1A, 1B1, and 1C1 mRNA expression in the present study. Greater than additive induction of these CYPs has also been shown after co-exposure to BNF + ANF; however, ANF alone significantly induces CYP1 expression (Timme-Laragy et al., 2007). The greater than additive induction of the CYPs caused by BkF + FL is unique in that FL by itself does not induce expression of CYP1A, 1B1, or 1C1.

In further support of PAHs as bifunctional inducers, the deformities caused by BkF + FL co-exposure were also preceded by upregulation of GSTp2, GCLc, and GPx1. The greatest induction occurred in GSTp2 expression. GSTp was also the most highly induced after BNF + ANF co-exposure (Timme-Laragy et al., 2009) and BaP + FL co-exposure (Chapter 3). GSTs participate in phase II metabolism, exhibit glutathione
peroxidase activities via catalyzing the reduction of organic hydroperoxides into
alcohols, and are induced in response to pro-oxidants in a variety of organisms
(reviewed in Hayes et al., 2005; Hayes and Pulford, 1995). The pi class GSTs are
particularly efficient in conjugating PAH metabolites to glutathione (Robertson et al.,
1986). It is not currently known whether zebrafish GSTp2 contains XREs or AREs in its
promoter region. However, ARE-like sequences are present in the single human and rat
GSTp, both known as GSTp1, and mouse GSTp1 (2004a; Ikeda et al., 2002). XREs have
also been found in other mammalian GST isoforms (Pimental et al., 1993). Moreover,
Suzuki et al. (2005) found that zebrafish GSTp1, which shares 90.4% identity of the
coding region sequence with GSTp2, contains a proximal ARE-like sequence 50 bp
upstream of the transcription initiation and that Nrf2 activates GSTp1 gene expression
through this element in zebrafish embryos. GCLc, which catalyzes the rate-limiting step
of glutathione synthesis, and GPx1, which reduces hydrogen peroxide into water and
detoxifies lipid peroxides, were also upregulated by BkF alone and BkF + FL co-
exposure. As with GSTp2, the presence of XREs and AREs in these two genes in piscine
species is not yet known. Previous studies have shown that GCLc induction by BNF
occurs via a distal ARE sequence in human HepG2 cells (Mulcahy et al., 1997; Wild et
al., 1998), and human intestinal GPx2 and plasma GPx3 contain an ARE in their
promoter regions (Banning et al., 2005; Bierl et al., 2004). Because antioxidant defense
systems are highly conserved across species, it is plausible that zebrafish redox-responsive genes contain ARE-like sequences and potentially XREs.

Various studies have examined changes in GST and GPx in response to different PAHs in aquatic organisms. However, most studies have examined protein activity and not mRNA expression response and have focused on exposures to a single PAH. In the common goby, Pomatoschistus microps, Vieira et al. (2008) found that GPx activity was induced in response to low doses of BaP (4, 8, and 16 µg/L) and anthracene (4 µg/L), a three-ring PAH. However, GST activity was induced after BaP exposure yet inhibited after anthracene exposure. This difference could be due to the two compounds different affinities for the AHR; BaP is a strong agonist while anthracene is a very weak agonist (Barron et al., 2004). In the same study, exposure to a fuel-oil mixture induced GST activity almost two times higher than the induction caused by BaP. However, because the PAH congeners in the mixture were not examined individually, it is not clear if the PAHs exhibited an additive or synergistic effect on GST activity. Greater than additive induction of GSTp2 was caused by the simple BkF + FL mixture in the present study. Environmentally relevant concentrations of the three-ring PAH phenanthrene were shown to have different effects on GPx activity in different tissues of golden grey mullet (Liza aurata): GPx was inhibited in the gill, unaffected in the kidney, and increased in the liver in a dose-dependent manner (Oliveira et al., 2008), showing that antioxidant defenses within the same organism are variable. In another study, GPx mRNA
expression was induced in the liver of adult polar cod, *Boreogadus saida*, two days after i.p. injection of 6.6, 85, and 378 µg/kg wwt BaP, while GSTp expression was not induced until four days after injection and only after the low and high dose (Nahrgang et al., 2009). The results of these two studies highlight the fine balance in oxidative stress responses. An increase in redox-responsive genes and protein may indicate that an organism will be able to mount a response to a toxicant and overcome its toxicity. However, an organism’s redox-responsive enzymes may become overwhelmed and lead to oxidative stress.

Very few studies have examined GCLc response to PAHs in aquatic species. GCLc mRNA expression was upregulated 1.7-fold in the liver of juvenile largemouth bass (*Micropterus salmoides*) 48 h after i.p. injection of 66 mg/kg BNF (Hughes and Gallagher, 2004), a response that was concomitant with a 9-fold increase in microsomal CYP activity. In a mammalian study, GST-mu and GPx2 were upregulated 20- and 5-fold, respectively, in rat preneoplastic lesions following BNF exposure (Dewa et al., 2008). This same study showed that BNF also increased microsomal ROS production and lipid peroxidation. In another mammalian study, 1,6 BaP-quinone (BPQ) and 1,3-BPQ induced GCLc expression in MCF-10A human mammary epithelial cells, and it was determined that the gene contained an XRE in its promoter region (Burchiel et al., 2007).

MnSOD and CuZnSOD were not upregulated by BkF or FL individually or by BkF + FL co-exposure in zebrafish embryos in our study. Once again, knowledge of
XREs or AREs in fish MnSOD and CuZnSOD is lacking. Human CuZnSOD contains a functional XRE in the 5’ flanking region that can bind and be induced by TCDD (Cho et al., 2001), and an ARE sequence has been identified in the 5’ flanking region of mouse MnSOD (Jones et al., 1995). Though MnSOD and CuZnSOD gene expression were not affected by BkF or FL, other studies have shown that PAHs can induce SOD expression and activity. SOD activity increased significantly after a four-day exposure of gilthead seabream (*Sparus aurata*) to 1.20 μM fluorene but not after exposure to lower doses (Kopecka-Pilarczyk and Correia, 2009). By comparison, the concentrations of BkF and FL used in this study, 0.20 μM and 0.74 μM, respectively, were significantly lower than the dose at which increased SOD activity was observed. In the liver of polar cod exposed to 85 μg/kg BaP, CuZnSOD mRNA expression was upregulated 15-fold, while MnSOD was also induced but to a much lesser extent, approximately 1.5-fold (Nahrgang et al., 2009). Vieira *et al.* (2008) found that SOD activity was induced in the common goby after BaP and anthracene exposure. Our laboratory also previously found that MnSOD and CuZnSOD expression were each upregulated approximately three-fold above control levels at 24 h after exposure to BNF + ANF, but not 72 h after exposure in zebrafish (Timme-Laragy et al., 2009), which is further evidence that in addition to variability in response amongst different tissues, antioxidant responses are also variable in time.
AHR2 knockdown protected embryos from the cardiac deformities induced by BkF + FL. AHR2 knockdown also decreased expression of \textit{CYP1A, 1B1,} and \textit{1C1} compared to NI embryos after BkF + FL exposure. CYP1B1 expression was reduced to control levels in AHR2-mo embryos exposed to BkF and BkF + FL, while \textit{CYP1A} and \textit{1C1} were reduced compared to NI BkF + FL levels but were still significantly greater than control levels. The inability of the AHR2 morpholino to completely prevent \textit{CYP1A} and \textit{1C1} expression may be due to the fact that the morpholino only provides a knockdown, not knockout, of AHR2, and thus, AHR2 ligand binding and signaling events may still occur (Eisen and Smith, 2008; Matson et al., 2008a). Furthermore, the CYPs may be partly controlled via AHR-independent pathways. For example, CYP1A1 and CYP1B1 have been shown to be inducible by PAHs in various tissues of AHR null knockout mice (Kerzee and Ramos, 2001; Nakatsuru et al., 2004). Another explanation may be that zebrafish have two other identified AHR isoforms, AHR1A and AHR1B, and \textit{CYP1A} and \textit{1C1} may be at least partially controlled by one or both of these isoforms. AHR1A is present predominantly in the liver of adult zebrafish and was initially thought to be nonfunctional, as it does not bind TCDD in tissue culture (Andreasen et al., 2002a). However, Incardona \textit{et al.} (2006) showed that embryos injected with AHR1A-mo were protected from the abnormal liver development and pericardial effusion caused by high doses of the four-ringed PAH pyrene. AHR1B was shown to be expressed as early as 24 hpf in zebrafish development and at higher levels than AHR1A. It was shown \textit{in vitro} to
have high affinity binding of TCDD but was not inducible by TCDD (Karchner et al., 2005). The role of zebrafish AHR1A and AHR1B in the toxicity of high molecular weight PAHs, including BkF and mixtures, has not yet been examined.

AHR2 knockdown, which protected against BkF + FL-induced deformities and caused a reduction of CYP1 mRNA expression compared to NI embryos, also prevented the upregulation of GSTp2, GPx1, and GCLc that occurred in NI embryos in response to the BkF + FL co-exposure. The absence of redox-responsive gene expression after BkF + FL co-exposure in AHR2-mo embryos may be due to the lack of metabolism of PAHs in these embryos. When AHR2 is knocked down, there is less receptor present to which the PAHs can bind. This reduction of ligand binding prevents CYP activity, thereby preventing phase I metabolism and the potential formation of redox-cycling quinone metabolites and uncoupling of the electron transport chain and CYP reactions. Furthermore, oxidative stress responses may be a secondary result of the AHR2-mediated developmental toxicity caused by PAHs. Thus, preventing overall PAH toxicity via AHR2 knockdown may prevent oxidative stress. The lack of redox-responsive gene induction in PAH-exposed fish after AHR knockdown provides further evidence of that AHR-XRE-mediated pathways and Nrf2-ARE-mediated pathways are not completely independent of one another.

Various experiments have shown that knockdown or knockout of Nrf2 exacerbates the toxicity of PAHs and ROS (e.g., Timme-Laragy et al., 2009; Zhu et al.,
However, few studies have looked at the effects of AHR knockdown or knockout on oxidative stress parameters and none that we know of have done so in fish. siRNA experiments that eliminated the AHR in human esophageal epithelial cells showed that elimination of the AHR prevented the upregulation of various UDP-glucuronosyltransferases (UGTs), another class of detoxifying enzymes that contain both XREs and AREs, by TCDD (Kalthoff et al., 2010). Furthermore, in wild-type Hepa 1c1c7 cells, BNF and BaP increased H$_2$O$_2$ production and lipid peroxidation, but this increase did not occur in cells lacking the AHR, indicating that the AHR is necessary for production of oxidative stress in these cells (Elbekai et al., 2004). In mouse aortic endothelial cells overexpressing CuZnSOD and/or catalase, knockdown of the AHR via siRNA prevented the BaP-induced increase in GSTp1 mRNA expression and GST activity that occurred in cells treated with control siRNA (Wang et al., 2009). These studies provide evidence that the absence of the AHR in cell culture prevents an oxidative stress response consistent with our results with zebrafish.

In summary, co-exposure of zebrafish embryos to two environmentally relevant PAHs, BkF and FL, resulted in pericardial effusion and upregulation of CYP1A, 1B1, 1C1, and the redox-responsive genes GSTp2, GPx1, and GCLc. The co-exposure did not affect the expression of two other redox-responsive genes, MnSOD and CuZnSOD. AHR2 knockdown prevented the cardiac toxicity and upregulation of the various CYPs and redox-responsive genes caused by BkF + FL co-exposure. AHR2 mediates the
cardiac toxicity of some PAHs in zebrafish and appears to be involved in the oxidative stress response of zebrafish to PAHs. Additional studies are required to clarify the role of the AHR in the oxidative stress response of zebrafish to the embryotoxicity of select PAHs.
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Figure 3: Effect of PAHs on deformities and EROD induction. Embryos were dosed at 24 hpf and scored at 96 hpf. Deformities are represented by bars, and scale is along the left y-axis; values are expressed as percent control (DMSO) pericardial effusion ± SEM (n = 12 per treatment; each n represents the average of five embryos). EROD values are represented by lines, and scale is along the right y-axis; values are expressed as percent DMSO EROD ± SEM (n = 9 per treatment). For EROD comparisons, groups not sharing a common letter are significantly different (p ≤ 0.05; ANOVA, Tukey adjusted LSMeans). For deformity comparisons, an asterisk (*) represents a significant difference from control (p ≤ 0.05; ANOVA, Tukey adjusted LSMeans).
Figure 4: Effect of PAHs on CYP1 mRNA expression

Embryos were dosed at 24 hpf, and expression was measured at 48 hpf. (a) CYP1A; (b) CYP1B1; (c) CYP1C1. Expression is shown as fold induction compared to DMSO controls. n = 9-12 per treatment; each n represents 10 pooled embryos. Groups not sharing a common letter are significantly different (p ≤ 0.05; ANOVA, Tukey adjusted LSMeans).
Figure 5: Effect of PAHs on redox-responsive gene expression. Embryos were dosed at 24 hpf, and expression was measured at 48 hpf. (a) GSTp2; (b) GPx1; (c) GCLc. Expression is shown as fold induction compared to DMSO controls. n = 9-12 per treatment; each n represents 10 pooled embryos. Groups not sharing a common letter are significantly different ($p \leq 0.05$; ANOVA, Tukey adjusted LSMeans).
Figure 6: Effect of PAHs on deformities and EROD induction in non-injected (NI) and AHR2-morpholino-injected (AHR2-mo) embryos.

Embryos were dosed at 24 hpf and scored at 96 hpf. Deformities are represented by bars, and scale is along the left y-axis; values are expressed as percent NI control (DMSO) pericardial effusion ± SEM (n = 12 per treatment; each n represents average of five embryos). EROD values are represented by lines and scale is along the right y-axis; values are expressed as percent NI control EROD ± SEM (n = 9 per treatment). For EROD comparisons, groups not sharing a common letter are significantly different (p ≤ 0.05; ANOVA, Tukey adjusted LSMeans). For deformity comparisons, an asterisk (*) represents a significant difference from control (p ≤ 0.05; ANOVA, Tukey adjusted LSMeans).
Figure 7: Effect of PAHs on CYP1 mRNA expression in non-injected (NI) and AHR2-morpholino-injected (AHR2-mo) embryos.
Embryos were dosed at 24 hpf, and expression was measured at 48 hpf. (a) CYP1A; (b) CYP1B1; (c) CYP1C1. Expression is shown as fold induction compared to NI DMSO controls. n = 9-12 per treatment; each n represents 10 pooled embryos. Groups not sharing a common letter are significantly different (p ≤ 0.05; ANOVA, Tukey adjusted LSMeans).
Figure 8: Effect of PAHs on redox-responsive gene expression in non-injected (NI) and AHR2-morpholino-injected (AHR2-mo) embryos. Embryos were dosed at 24 hpf, and expression was measured at 48 hpf. (a) GSTp2; (b) GPx1; (c) GCLc. Expression is shown as fold induction compared to NI DMSO controls. n = 9-12 per treatment; each n represents 10 pooled embryos. Groups not sharing a common letter are significantly different ($p \leq 0.05$; ANOVA, Tukey adjusted LSMeans).
3. **Glutathione s-transferase pi class 2 (GSTp2) protects against the cardiac deformities caused by exposure to PAHs but not PCB-126 in zebrafish embryos**

This chapter will be submitted for publication with Lindsey A. Van Tiem and Richard T. Di Giulio as authors.

3.1 **Introduction**

Polycyclic aromatic hydrocarbons (PAHs) are ubiquitous environmental contaminants that are often found in aquatic systems as mixtures. Many PAHs are agonists for the aryl hydrocarbon receptor (AHR), a ligand-activated cytosolic transcription factor. Upon ligand binding, the AHR translocates to the nucleus, dimerizes with the aryl hydrocarbon receptor nuclear translocator (ARNT,) and binds to xenobiotic response elements (XREs) on genes encoding various phase I and II enzymes that in turn metabolize PAHs (Schmidt and Bradfield, 1996). The cytochrome P450s (CYPs), particularly CYP1A, are well-characterized phase I enzymes, and phase II enzymes known to be activated by the AHR in mammals include UDP-glucuronosyltransferases (UDPGTs), NAD(P)H:quinone oxidoreductase 1 (NQO1), and glutathione S-transferases (GSTs) (Denison et al., 2002; Nebert et al., 2000).

GSTs are a family of phase II enzymes that detoxify a wide range of toxicants. GSTs function by conjugating electrophilic substrates, both endogenous and exogenous, to reduced glutathione. The conjugates are generally less toxic than the unconjugated metabolite, more water soluble, and thus, more easily excreted from the cell. GSTs also
exhibit glutathione peroxidase activities by catalyzing the reduction of organic hydroperoxides into alcohols, and certain GSTs are induced in response to prooxidants in a variety of organisms (reviewed in Hayes et al., 2005). Seven cytosolic GST enzymes (alpha, mu, pi, sigma, theta, omega, and zeta) have been identified in mammals and are grouped according to substrate specificity, amino acid sequence, and immunological crossreactivity; collectively they may comprise 2–4% of total cytosolic proteins in the liver (Hayes et al., 2005; Schlenk et al., 2008). While relatively little is known about GSTs in fishes, four classes (pi, mu, theta, and alpha) have been identified in salmonids (Donham et al., 2005), and a unique piscine GST, initially characterized in plaice (Pleuronectes platessa) (Leaver et al., 1997), has been designated rho. Isoforms of alpha, mu, omega, pi, rho, theta, and a microsomal GST have been identified in zebrafish (Danio rerio) (Schlenk et al., 2008). The various GST isoforms have been shown to metabolize many environmental pollutants, including pesticides, antibiotics, and PAHs in mammals. Among the GST isoforms, the pi class has been shown to be highly efficient in conjugating carcinogenic benzo[a]pyrene (BaP) metabolites to glutathione (Robertson et al., 1986). In addition, it has been shown in mouse liver that the contribution of GST pi 1 (GSTp1) in detoxifying the carcinogenic metabolite of BaP, (+)-anti-7,8-dihydroxy-9,10-oxy-7,8,9,10-tetrahydrobenzo[a]pyrene, is greater than the contribution of the other GSTs combined (Hu et al., 1997).
PAHs, as well as coplanar polychlorinated biphenyls (PCBs) and dioxins, cause a wide range of toxicities, and fish early life stages are particularly sensitive to the developmental toxicity caused by PAHs. Exposure to 2-4-ringed PAHs has been shown to alter heart morphology, impair heart looping, and cause atrioventricular conduction block in zebrafish (Incardona et al., 2004). Co-exposure to a PAH that is an AHR ligand (such as BaP, benzo[k]fluoranthene (BkF), or the model PAH β-naphthoflavone (BNF)) and one that is a CYP inhibitor (such as fluoranthene (FL) or the model PAH α-naphthoflavone (ANF)) causes severe pericardial effusion and the stringy heart phenotype in Atlantic killifish embryos (*Fundulus heteroclitus* (Wassenberg and Di Giulio, 2004) and zebrafish embryos (Billiard et al., 2006; Van Tiem and Di Giulio, 2011). Our laboratory has previously shown that upregulated glutathione s-transferase pi class 2 (GSTp2) expression precedes the cardiac deformities caused by co-exposure to an AHR ligand and a CYP1 inhibitor in zebrafish (Timme-Laragy et al., 2009; Van Tiem and Di Giulio, 2011).

In this study, we sought to determine the role of zebrafish GSTp2 in mediating the cardiac toxicity caused by PAH co-exposures. We utilized morpholino gene knockdown to determine the effect of GSTp2 knockdown on zebrafish embryos exposed to the AHR agonists BkF and BaP individually and also in combination with the CYP1 inhibitor FL. Additionally, we compared the role of GSTp2 in PAH toxicity with its role in mediating the toxicity caused by exposure to 3,3′,4,4′,5-pentachlorobiphenyl (PCB-
126), a non-ortho-substituted coplanar PCB that is the strongest PCB ligand for the AHR (Tillitt et al., 2008).

3.2 Material and methods

3.2.1 Fish care

Adult EkkWill zebrafish (D. rerio; EkkWill Waterlife Resources, Ruskin, FL, USA) were maintained in a recirculating AHAB system (Aquatic Habitats, Apopka, FL, USA) at 28°C under a 14:10 h light:dark cycle. Adult fish were fed brine shrimp and a mix of Cyclop-eeze (Argent Chemical Laboratories, WA, USA) and Zeigler’s Adult Zebrafish Complete Diet (Aquatic Habitats).

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

3.2.2 Chemicals

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). Dimethyl sulfoxide (DMSO) and tricaine methanesulfonate (MS-222) were purchased from Sigma-Aldrich (St. Louis, MO, USA). BkF, BaP, FL, and PCB-126 stocks were dissolved in DMSO, protected from light, and kept at -20°C until use.

3.2.3 Morpholino injection

Morpholino antisense oligonucleotides were designed and produced by Gene Tools, LLC (Philomath, OR, USA). A splice-junction morpholino targeting the exon 2-intron 2 boundary was designed to knockdown GSTp2 (GSTp2-mo; 5’-ATTTTCATACAAACCTTTGATAGCG-3’). Splice-junction morpholinos cause aberrant splicing of pre-mRNA, most commonly via deletion of the targeted exon or insertion of the targeted intron, and knockdown via splice-junction morpholino can be quantified using PCR. Gene Tools’ standard control morpholino (Co-mo, 5’-CCTCTTACAAACCTTTGATAGCG-3’) was used as a morpholino injection control. Both morpholinos were fluorescein-tagged at the 3’ end to monitor injection success. Morpholinos were diluted to either 100 μM or 250 μM working stocks in 30% Danieau.

Morpholinos (approximately 3 nL injection volume) were injected by hand into the yolk of zebrafish embryos at the 1-4 cell stage using a microinjection system consisting of a Nikon SMZ-1500 zoom stereomicroscope (Nikon Instruments Inc., Lewisville, TX, USA) and an MDI PM 1000 Cell Microinjector (MicroData Instrument
Inc., S. Plainfield, NJ, USA). Embryos exhibiting normal development and strong, uniform incorporation of the morpholinos were used for experiments.

3.2.4 Dosing

3.2.4.1 Dosing for deformities

At 24 hpf, embryos were dosed in 7.5 mL 30% Danieau in 20-mL glass scintillation vials (VWR, West Chester, PA, USA) with five embryos per vial and three vials per treatment. The four dosing regimes were as follows: DMSO, 100 µg/L BaP, 500 µg/L FL, and 100 µg/L BaP + 500 µg/L FL co-exposure; DMSO, 100 µg/L BkF, 100 µg/L FL, and 100 µg/L BkF + 100 µg/L FL co-exposure; DMSO, 10 µg/L BkF, 200 µg/L FL, and 10 µg/L BkF + 200 µg/L FL co-exposure; and DMSO, 1 µg/L PCB-126, and 2 µg/L PCB-126. Final DMSO concentrations were ≤ 0.03% across all treatments. Dosed embryos were maintained in an incubator at 28°C until the time of deformity assessment at 96 hpf.

3.2.4.2 Dosing for reverse transcription PCR and QPCR

To measure GSTp2 expression in response to AHR agonists using real-time quantitative PCR, non-injected (NI) embryos were dosed at 24 hpf in 7.5 mL 30% Danieau in 20-mL glass scintillation vials with five embryos per vial and six vials per treatment. Embryos were dosed with the three following regimes: DMSO, 10 µg/L BkF,
200 μg/L FL, and 10 μg/L BkF + 200 μg/L FL; DMSO, 100 μg/L BaP, 500 μg/L FL, and 100 μg/L BaP + 500 μg/L FL; and DMSO and 1 μg/L PCB-126. To measure GSTp2-mo efficacy via reverse transcription PCR, NI and GSTp2-mo (100 μM) embryos were dosed at the same time and manner with either DMSO or a co-exposure of 10 μg/L BkF + 200 μg/L FL. For QPCR and RT-PCR, embryos were dechorionated at 48 hpf, pooled in groups of ten (two vials), and fixed in RNAlater (Applied Biosystems, Foster City, CA, USA). Samples were stored at -80°C until RNA extraction.

3.2.5 Confirmation of GSTp2 splice-mo efficacy by reverse transcription PCR analysis

Samples were thawed on ice, homogenized with a sterile hand-held homogenizer in RNA-Bee for 30 s, and RNA was extracted according to the RNA-Bee protocol (Tel-Test Inc., Friendswood, TX, USA).

RNA quantity and quality were analyzed spectrophotometrically using a NanoDrop ND-100 (NanoDrop Technologies, Wilmington, DE, USA). cDNA was synthesized using the Omniscript Reverse Transcriptase kit (Qiagen, Inc., Valencia, CA, USA) according to the manufacturer’s instructions with 500 ng RNA, random hexamers, and RNaseOut (Invitrogen, Carlsbad, CA, USA). The reaction was carried out in a Biometra T1 thermocycler (Göttingen, Germany) for 1 h at 37°C.

RT-PCR was performed in a 25-μL reaction containing 62.5 ng template, 12.5 μL AmpliTaq Gold Master Mix (Applied Biosystems), 0.5 μL each 10 μM forward and
reverse primer, and 9 μL distilled H₂O. Thermocycler conditions were as follows: 95°C for 5 min; 35 cycles of 95°C for 15 s, 58°C for 15 s, and 72°C for 4 min; and finally, 72°C for 10 min. All reactions were performed in triplicate. To examine potential deletion of exon 2 or insertion of intron 2 by the GSTp2 splice-mo, the forward primer was located in exon 1 (5'-CAGCAACTTCACAGACCTCGCTTT-3') and the reverse primer was located on exon 3 (5'-ATGTCACCCCTCATCCAGTCTCCT-3'). Primers were designed using PrimerQuest software (Integrated DNA Technologies, Inc., www.idtdna.com). Gel electrophoresis of 10 μL of PCR product was performed using 2% agarose gels stained with SYBR Safe DNA gel stain (Invitrogen). Gels were imaged with an AlphalImager HP (Cell Biosciences, Santa Clara, CA, USA).

3.2.6 QPCR

RNA extractions and cDNA synthesis were performed as described in section 3.2.5. β-actin was used as a housekeeping gene, and β-actin and GSTp2 primers were published previously (β-actin, F: 5’-AAGATCAAGATCATTGCTCC-3’ and R: 5’-CCAGACTCATCGTACTCCT-3’ and GSTp2, F: 5’-TCTGGACTCTTTCCGTCTCTCA-3’ and R: 5’-ATTGACTGTTGGCCGTCGCT-3’) (Grimes et al., 2008; Van Tiem and Di Giulio, 2011). Each 25-μL QPCR reaction consisted of 12.5 μL SYBR Green PCR Master Mix (Applied Biosystems), 9.5 μL dH₂O, 200 nM each forward and reverse primer, and 4 ng cDNA template. The reactions were carried out using an Applied Biosystems 7300
Real-Time PCR System with a thermal profile of 10 min at 95 °C and 40 replicates of 15 s at 95 °C, 1 min at 60 °C. A dissociation curve was calculated for each sample at the end of each profile to confirm formation of a single product during the reaction. All samples were run in duplicate and technical replicates were averaged prior to analysis. The ABI PRISM 7300 Sequence Detection System Software, Version 1.1 (Applied Biosystems) was used to carry out data analysis. The average mRNA fold induction of each target gene was calculated by comparing the Ct (threshold cycle) of the target gene to that of β-actin according to Livak and Schmittgen (2001). β-actin expression remained constant across treatments. Gene expression is expressed as fold-induction relative to DMSO controls.

3.2.7 Deformity assessment

At 96 hpf, hatched zebrafish embryos were screened for cardiac deformities via measurement of pericardial effusion. Embryos were removed from dosing solution, rinsed with 30% Danieau, and anesthetized with MS-222. Fish were then placed on depression slides in the left lateral position in 3% methylcellulose and were imaged using light microscopy under 50x magnification (Zeiss Axioskop, Thornwood, NY, USA). The two-dimensional area of the pericardium was manually traced and then quantified using IPLab software (Scanalytics Inc., Fairfax, VA, USA). Deformity values are expressed as a percentage of the two-dimensional pericardial area of NI control embryos.
3.2.8 **Statistical analysis**

Statistical analyses were performed using JMP 8.1.1 (SAS Institute Inc., Cary, NC, USA). For GSTp2 expression in NI embryos, the data were analyzed via one-way analysis of variance (ANOVA) followed by Tukey’s post-hoc test. Deformity data were analyzed via two-way ANOVA to determine an overall effect of the morpholino injection and dose, followed by least square means (LSMeans) procedures. Tukey’s post-hoc test was used to determine differences between groups. Experiments were replicated at least three times and no differences between experimental replicates were observed. Data are represented as mean ± standard error of the mean (SEM). Values were considered significantly different at \( p \leq 0.05 \).

### 3.3 Results

3.3.1 **GSTp2 mRNA expression in response to PAH and CPB-126 exposure**

GSTp2 mRNA expression was induced by various PAH treatments and PCB-126 exposure. Exposure to 10 \( \mu g/L \) BkF induced GSTp2 expression 2.3±0.3-fold over control levels, and exposure to 10 \( \mu g/L \) BkF + 200 \( \mu g/L \) FL significantly induced GSTp2 expression greater than controls and BkF alone (5.7±0.8-fold, \( p < 0.001 \)) (Fig. 9A). FL alone did not induce GSTp2 expression above control levels. Exposure to 100 \( \mu g/L \) BaP and 100 \( \mu g/L \) BaP + 500 \( \mu g/L \) FL also induced GSTp2 expression. BaP caused a 3.3±0.3-
fold induction ($p = 0.012$) and BaP + FL caused a $7.0 \pm 0.9$-fold induction above controls ($p < 0.01$); at this higher dose, FL still did not significantly induce GSTp2 expression above control levels ($1.4 \pm 0.3$-fold; Fig. 9B). At 1 μg/L, PCB-126 significantly induced GSTp2 mRNA expression above control levels ($2.9 \pm 0.7$-fold, $p = 0.0229$; Fig. 9C).

3.3.2 Confirmation of morpholino efficacy by reverse transcription PCR analysis

Knockdown of GSTp2 was measured via RT-PCR and was demonstrated by comparing amplification of a cDNA fragment in the region targeted by the morpholino, exon 1–3, between NI and GSTp2-mo embryos. As shown in Fig. 10, neither NI nor GSTp2-mo embryos exposed to DMSO exhibited amplification of the GSTp2 cDNA fragment (lanes 2 and 3, respectively). NI embryos exposed to BkF + FL exhibited strong amplification of the GSTp2 cDNA fragment (lane 4). In contrast, GSTp2-mo embryos exposed to BkF + FL exhibited very weak amplification of the GSTp2 cDNA fragment and also exhibited weak amplification of a smaller band, indicating successful knockdown of GSTp2 via deletion of the 36 base pair exon 2 by the splice-junction morpholino (lane 5).

3.3.3 Deformity Assessment

The role of GSTp2 in ameliorating PAH-induced cardiotoxicity was examined via morpholino knockdown of the gene. NI and GSTp2-mo embryos were exposed to
various combinations of an AHR agonist (BkF or BaP) and a CYP1A inhibitor (FL). Two
different morpholino concentrations, 100 μM and 250 μM, were used to determine if a
greater concentration of morpholino would produce a greater effect. As we have shown
in previous studies (Billiard et al., 2006; Van Tiem and Di Giulio, 2011), co-exposure to
an AHR agonist and a CYP1A inhibitor caused cardiac deformities in embryonic
zebrafish in this study. The severity of this cardiac toxicity was dependent upon the
concentrations and types of PAHs used.

3.3.4 Influence of GSTp2 knockdown on deformities caused by BkF + FL co-exposure

Exposure to 100 μg/L BkF or 100 μg/L FL individually did not cause toxicity in NI
embryos compared to controls; NI BkF-exposed embryos had an average pericardial
area of 99±2% and NI FL-exposed embryos had an average pericardial area of 98±3%
control pericardial area (Fig. 11A). Co-exposure to 100 μg/L BkF + 100 μg/L FL induced
moderate but statistically significant pericardial effusion in NI embryos (120±3%; p =
0.0001). Neither concentration of GSTp2-mo caused toxicity in fish exposed to DMSO,
BkF, or FL individually. Both the 100 and 250 μM GSTp2-mo exacerbated the pericardial
effusion caused by 100 μg/L BkF + 100 μg/L exposure, resulting in average pericardial
areas of 171±7% and 167±8%, respectively; these areas were significantly greater than the
areas of NI fish of the same treatment (both p < 0.0001) but not different from one
another. The severity of pericardial effusion in Co-mo injected dosed individuals was not statistically different from that of NI dosed individuals (p = 0.78, data not shown).

GSTp2 knockdown also caused exacerbation of deformities caused by different concentrations of BkF and FL. In NI embryos and embryos injected with 100 μM and 250 μM GSTp2-mo, exposure to 10 μg/L BkF and 200 μg/L FL individually did not result in deformities (Fig. 11B). However, co-exposure to 10 μg/L BkF + 200 μg/L FL caused significant pericardial effusion in NI embryos (145±6%; p < 0.0001). This BkF + FL-induced effusion was exacerbated by injection with both the 100 and 250 μM GSTp2-mo (213±12% and 205±12%, respectively; both p < 0.0001 vs. NI DMSO and NI BkF + FL and not different from one another).

3.3.5 Influence of GSTp2 knockdown on deformities caused by BaP + FL co-exposure

To test if deformity exacerbation by GSTp2 knockdown was unique to BkF + FL co-exposure, we also examined the effects of GSTp2 knockdown on the toxicity caused by co-exposure to FL and another AHR agonist, BaP. As with BkF and FL, exposure to 100 μg/L BaP or 500 μg/L FL alone did not cause pericardial effusion in NI or GSTp2-mo embryos (Fig. 11C). BaP + FL co-exposure caused moderate but significant pericardial effusion in NI embryos compared to NI controls (126±5%; p < 0.001). GSTp2 knockdown significantly exacerbated BaP + FL-induced pericardial effusion; 100 μM GSTp2-mo
embryos had an average pericardial area of 147±6% and 250 μM GSTp2-mo embryos had an average pericardial area of 145±6% (both p < 0.001 vs. NI DMSO and NI BkF + FL).

3.3.6 Influence of GSTp2 knockdown on deformities caused by PCB-126

In addition to PAH co-exposures, we exposed NI and GSTp2-mo injected embryos to the strong AHR agonist, PCB-126. In NI embryos, 1 μg/L PCB-126 resulted in a slight but significant increase in pericardial effusion compared to controls (120±4%; p = 0.02; Fig. 12). In contrast to the interaction with PAH exposures, knockdown of GSTp2 with either concentration of morpholino did not exacerbate the deformities caused by 1 μg/L PCB-126 (124±5% in 100 μM GSTp2-mo embryos and 132±11% in 250 μM GSTp2-mo embryos; not significantly different from treated NI embryos or one another). NI embryos exposed to a higher concentration of 2 μg/L PCB-126 had a pericardial area of 178±11% NI control area (p < 0.001 vs. NI DMSO). Once again, GSTp2 knockdown with either concentration of morpholino did not exacerbate the deformities caused by 2 μg/L PCB-126; 100 μM GSTp2-mo embryos had an average pericardial area of 172±9% and 250 μM GSTp2-mo embryos had an average pericardial area of 179±14% (not significantly different from PCB-126-exposed NI embryos or one another).
3.4 Discussion

Our results show that knockdown of GSTp2 in zebrafish embryos exacerbates the cardiac toxicity caused by co-exposures to a PAH that is an AHR agonist (BkF or BaP) and one that is a CYP1 inhibitor (FL), but does not affect the toxicity caused by the strong AHR agonist PCB-126.

Experiments in mammals have shown that different GST isoforms have specificities for different electrophilic metabolites of exogenous compounds. In particular, the mu and pi class GSTs show the highest activities with epoxides (Kettererer and Mulder, 1990). Because phase I metabolism of PAHs may result in the formation of epoxide metabolites, these two GSTs have received the most attention with regard to PAH toxicity. GSTp activity was shown to be induced approximately two-fold over control levels in the livers of rainbow trout seven days after i.p. injection of BNF (Celander et al., 1993). In a similar study, hepatic GST activity was significantly increased 2.3-fold in adult rainbow trout (Oncorhynchus mykiss) 14 days after i.p. injection of 50 mg/kg body weight BNF (Zhang et al., 1990). In another study utilizing rainbow trout, GSTp expression was induced in juveniles exposed to crude oil or heavy fuel oil for 96 h (Hook et al., 2010). GST mRNA expression was also shown to be induced after a 16-day exposure of juvenile thicklip grey mullet (Chelon labrosus) to weathered heavy fuel oil, a mixture of PAHs (Bilbao et al., 2010). It is likely that the induction of GSTs by AHR ligands occurs either by interaction of the AHR with a
xenobiotic response element (XRE) or antioxidant response element (ARE) present in the GSTs. It is currently unknown if zebrafish GSTp2 contains an XRE or ARE. However, both have been identified in the 5' flanking region of rat GST alpha (Rushmore et al., 1990; 1991b), and rat and mouse GSTp1 also contain ARE-like sequences (Ikeda et al., 2004a; 2002). Moreover, an ARE-like sequence has been identified upstream of the transcription initiation site in zebrafish GSTp1, which shares 90.4% sequence identity of the coding region with GSTp2 (Suzuki et al., 2005). We have also previously shown that knockdown of the AHR2 in zebrafish results in decreased GSTp2 expression in response to 50 µg/L BkF and 50 µg/L BKF + 150 µg/L FL exposure, indicating that PAH induction of GSTp2 likely occurs through the AHR (Chapter 2, Van Tiem and Di Giulio, 2011). GSTp2 induction as well as the deformities caused by PAHs may also be a result of oxidative stress caused by PAHs. However, attempts to rescue PAH-induced deformities in zebrafish embryos with the antioxidant n-acetyl cysteine or exacerbate deformities with buthionine sulfoxide, an inhibitor of the rate-limiting enzyme in GSH synthesis, glutamate-cysteine ligase, proved unsuccessful (Timme-Laragy et al., 2009). Thus, in response to PAHs, the role of GSTp2 in phase II metabolism may be of greater importance than its role in an oxidative stress response. Identification of XREs or AREs in zebrafish GST isoforms will help to elucidate the mechanism by which GSTs are induced by PAHs.
No other studies of which we know have knocked down GSTp in fish. However, \( \text{GstP1/P2}(-/-) \) mice exposed to a single topical application of the carcinogenic PAH 7,12-dimethylbenzanthracene and repeat applications of the tumor promoting agent 12-O-tetradodecanoyl-13-acetate had a 10-fold higher incidence of skin papillomas than \( \text{GstP1/P2}(+/+) \) mice (Henderson et al., 1998), indicating the protective function of GSTp isoforms in mice in response to the carcinogenic activity of PAHs. Even though the mechanisms by which PAHs induce carcinogenesis and developmental toxicity are most likely different, the pi class GSTs appear to serve a protective role in both of these processes, suggesting a common importance for metabolic activation. In another study examining a different GST isoform, \( \text{Gstz1}(-/-) \) mice had reduced liver glutathione levels and increased hepatotoxicity in response to acetaminophen via production of oxidative stress (Blackburn et al., 2006), showing the protective capacity of certain GSTs against drug-induced toxicity via AHR-independent mechanisms.

Because GSTs conjugate reactive epoxides and other electrophilic metabolites, the induction of GST expression and activity is often considered to be part of an adaptive response to toxicants (Hayes et al., 2005). Adult killifish from the Atlantic Wood Superfund site on the Elizabeth River in VA, a site contaminated with high levels of PAHs, were found to have GST levels six-fold higher than those of fish from a control site while fish from an intermediate site had GST levels two-fold higher than those of reference site fish (Armknecht et al., 1998). Similarly, the GST activity in the liver of
brown bullhead catfish (*Ameiurus nebulosus*) from a PCB-contaminated site was three-fold higher than in reference site fish (Otto and Moon, 1996). Our results also indicate that the presence and activation of GSTp2 is a protective response to PAH exposure. However, induction of GSTs does not necessarily equate to protection, as PCB-126 induced GSTp2 expression in our study but GSTp2 does not appear to play a protective role in the cardiac toxicity caused by PCB-126 exposure.

As with PAHs, the inducibility of GSTs by PCBs seems to be species-, compound-, dose-, time-, assay-, and sex-dependent. For example, there was no change in GST activity in rainbow trout embryos after three or seven days of exposure to 1, 10, or 100 µg/L of another coplanar PCB, 3, 3', 4, 4'-tetrachlorobiphenyl (PCB-77) (Koponen et al., 2000). PCB-126, at doses of 10 and 100 µg/kg body weight, was also incapable of inducing GST activity in adult male sea bass (*Dicentrarchus labrax*) (Vaccaro et al., 2005). However, PCBs have been shown to induce GST activity. In an early experiment in juvenile rainbow trout, BNF and Clophen A50, a commercial mixture of PCBs, both induced hepatic GST activity (Andersson et al., 1985). PCB-77 and PCB-126 also induced GST activity in rainbow trout six days after i.p. injection with 0.1 mg/kg or 0.5 mg/kg body weight (Huuskonen et al., 1996). Activity and expression data are not directly comparable, and QPCR is more sensitive than GST activity assays; however, we could not find examples of increased GSTp2 expression in response to PCB-126 in the literature. While PAHs are rapidly metabolized via phase I and II metabolism, this is
not the case for coplanar PCBs, such as PCB-126. Even though co-planar PCBs are capable of inducing GSTs, they are not good substrates for the enzymes. Co-planar PCBs such as PCB-126 are largely resistant to metabolism due to their high degree of chlorination (Safe, 1994). Thus, it was not unexpected that GSTp2 knockdown did not have an effect on the cardiac toxicity caused by PCB-126 in this study.

In conclusion, GSTp2 mRNA expression was induced by individual AHR agonists (BkF, BaP, and PCB-126) and even more highly by co-exposure to BkF + FL or BaP + FL in zebrafish embryos. Knockdown of GSTp2 exacerbated the cardiac toxicity caused by BkF + FL and BaP + FL but did not affect the toxicity induced by PCB-126 exposure. We hypothesize that exacerbation of PAH-induced toxicity occurs in embryos injected with GSTp2-mo because GSTp2 is not present to conjugate reactive PAH metabolites to glutathione, thus not allowing for detoxification via this component of phase II metabolism. These results further suggest that pi class GSTs serve a protective function against the toxicity caused by PAHs in developing zebrafish.
Figure 9: Effect of PAHs and PCB-126 on GSTp2 gene expression. A) BkF and FL. B) BaP and FL. C) PCB-126. Embryos were dosed at 24 hpf, and expression was measured at 48 hpf. Expression is shown as fold induction compared to DMSO controls. n = 9 per treatment; each n represents 10 pooled embryos. Groups not sharing a common letter in each panel are significantly different (p ≤ 0.05; ANOVA, Tukey’s post-hoc test).
Figure 10: Knockdown of GSTp2 via splice junction morpholino.
Embryos were exposed to DMSO or 10 μg/L BkF + 200 μg/L FL at 24 hpf. Representative image is from RT-PCR amplification of 10 pooled embryos per each treatment. Ld = 25 bp DNA ladder. NI embryos exposed to BkF + FL show strong amplification of the GSTp2 exon 1-3 cDNA fragment. BkF + FL-exposed GSTp2-mo embryos exhibit weaker amplification of the GSTp2 exon 1-3 cDNA fragment and exhibit weak amplification of a smaller band, indicating deletion of exon 2 by the GSTp2-mo.
Figure 11: PAH-induced deformities in NI and GSTp2-mo injected embryos. A) 100 μg/L and 100 μg/L FL; B) 10 μg/L BkF and 200 μg/L FL; C) 100 μg/L BaP and 500 μg/L FL. Embryos were dosed at 24 hpf and scored at 96 hpf. NI embryos are represented by black bars, 100 μM GSTp2-mo embryos are represented by grey bars, and 250 μM GSTp2-mo embryos are represented by white bars. Deformities are expressed as percent NI control (DMSO) pericardial effusion ± SEM (n = 12 per treatment; each n represents average of five embryos). Groups not sharing a common letter are significantly different (p ≤ 0.05; ANOVA, Tukey adjusted LSMeans).
Figure 12: Effect of PCB-induced deformities in NI and GSTp2-mo injected embryos.

Embryos were dosed at 24 hpf and scored at 96 hpf. NI embryos are represented by black bars, 100 μM GSTp2-mo embryos are represented by grey bars, and 250 μM GSTp2-mo embryos are represented by white bars. Deformities are expressed as percent NI control (DMSO) pericardial effusion ± SEM (n=12 per treatment; each n represents average of five embryos). Groups not sharing a common letter are significantly different (p ≤ 0.05; ANOVA, Tukey adjusted LSMeans).
4. The role of AHR1 isoforms in PAH- and PCB-126-induced toxicity

This chapter will be submitted for publication with Lindsey A. Van Tiem, Daniel R. Brown, and Richard T. Di Giulio as authors.

4.1 Introduction

Environmental contaminants such as 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD), coplanar non-ortho-substituted polychlorinated biphenyls (PCBs), and certain polycyclic aromatic hydrocarbons (PAHs) are known agonists for the aryl hydrocarbon receptor (AHR). Unlike TCDD and PCBs, the concentration of PAHs in aquatic environments is increasing in the environment due to increased urbanization and run-off (Van Metre and Mahler, 2005). Fish early life stages are particularly sensitive to TCDD, PCB, and PAH toxicity, and the developing heart is a target organ for the toxicity of all three classes of compounds. Early life exposure to these compounds has been shown to result in cardiac deformities including reduced cardiomyocyte number, impaired blood flow, pericardial effusion, and an elongated atrium that results in the “stringy heart phenotype” in various fish species (Carls et al., 2008; Peterson et al., 1993; Scott and Hodson, 2008; Wassenberg and Di Giulio, 2004).
The AHR is a member of the basic-helix-loop-helix per-ARNT-SIM (bHLH-PAS) family of transcription factors. It is constitutively present in the cytoplasm and is bound by various cofactors including HSP90 (heat shock protein 90) and XAP2/AIP (X-associated protein 2/AhR-interacting protein) (Hahn, 2002). Upon ligand binding by an agonist, the AHR translocates into the nucleus and forms a heterodimer with the aryl hydrocarbon receptor nuclear translocator (ARNT), also a member of the bHLH-PAS family. The AHR-ARNT heterodimer then binds to xenobiotic response elements (XREs) and upregulates gene expression (Hankinson, 1995; Schmidt and Bradfield, 1996). Amongst the genes upregulated by the AHR, cytochrome P4501A is well characterized and is highly inducible. The AHR also upregulates other phase I and II metabolic enzymes including various CYPs, aldehyde dehydrogenase 3, NAD(P)H:quinone oxidoreductase (NQO1), glutathione s-transferases (GSTs), and UDP-glucuronosyltransferases (UDPGTs) (Denison and Nagy, 2003; Nebert et al., 2000).

Additionally, the AHR upregulates the aryl hydrocarbon receptor repressor (AHRR), which binds to XREs, potentially competing with AHR and downregulating the pathway (Karchner et al., 2002).

One AHR has been identified in mammals and has been shown to mediate some of the toxic effects of TCDD and PAHs. AHR deficient mice are protected from the developmental toxicity induced by TCDD and the carcinogenicity of benzo[a]pyrene (BaP) (Fernandez-Salgueiro et al., 1996; Shimizu et al., 2000). Three AHRs (AHR1A,
AHR1B, and AHR2) have been identified in zebrafish (*Danio rerio*) (Andreasen et al., 2002a; Karchner et al., 2005; Tanguay et al., 1999). AHR2 has been shown to mediate TCDD, PCB, and PAH toxicity (Billiard et al., 2006; Incardona et al., 2006; Prasch et al., 2003; Van Tiem and Di Giulio, 2011), but the role of the AHR1 isoforms has not yet been elucidated.

The purpose of the current study was to examine the role of AHR1A and AHR1B in mediating PAH and PCB-126 (3,3′,4,4′,5-pentachlorobiphenyl) cardiac toxicity in developing zebrafish. Morpholinos were used to knockdown the various AHRs; deformities, CYP1 activity, and CYP1 gene expression were then examined in response to PAHs and PCB-126. AHR1B knockdown had no effect on PAH- and PCB-126 induced deformities. Surprisingly, AHR1A knockdown exacerbated chemical toxicities and increased CYP1 activity but did not affect CYP1 gene expression. These results suggest that AHR1A may interact with the classical AHR2 pathway in such a way that it serves a protective role when present (such as sequestering ligands away from AHR2 or acting as a repressor of the pathway) or may have a role in the normal development of zebrafish embryos.
4.2 Materials and Methods

4.2.1 Fish Husbandry

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

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

4.2.2 Chemicals and Dosing

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

At 24 hours post fertilization (hpf), embryos exhibiting normal development, and uniform incorporation of the morpholinos for injected embryos, were dosed in 20-mL scintillation vials containing 7.5 mL 30% Danieau. Five embryos were dosed per vial with three vials per treatment in each experiment. After dosing, embryos were placed back into a 28°C incubator and kept on the same photoperiod as adults.

For initial deformity experiments with AHR1A-mo and AHR1B-mo, embryos were exposed to DMSO, 50 μg/L BkF, 150 μg/L FL, 50 μg/L BkF + 150 μg/L FL, and 1 μg/L PCB-126. For subsequent deformity experiments involving AHR1A-mo, embryos were exposed to DMSO as a control and the following doses: 100 μg/L BkF, 100 μg/L FL, and BkF + FL; 50 μg/L BkF, 150 μg/L FL, and 50 μg/L BkF + 150 μg/L FL; 100 μg/L BaP, 500 μg/L FL, and BaP + FL; and 1 μg/L PCB-126. For experiments involving AHR2-mo and AHR1A/2-mo co-knockdown, the same doses were used with the addition of 2 μg/L PCB-126. Final DMSO concentrations were < 0.03% across all treatments for all experiments. For experiments examining CYP1 activity via the 7-ethoxyresorufin-O-deethylase (EROD) assay, embryos were exposed to DMSO, 100 μg/L BaP, 50 and 100 μg/L BkF, and 1 and 2 μg/L PCB-126. The substrate 7-ER was added at a final concentration of 21 μg/L at the time of PAH or PCB-126 dosing.
For RT-PCR and confirmation of morpholino efficacy, embryos were dosed with DMSO or 50 μg/L BkF. For QPCR, embryos were dosed with DMSO as a control and the following individual doses and mixtures: 100 μg/L BaP, 500 μg/L FL, and 100 μg/L BaP + 500 μg/L FL; 50 μg/L BkF, 150 μg/L FL, and 50 μg/L BkF + 150 μg/L FL; and 1 μg/L PCB-126. Embryos were dechorionated at 48 hpf and fixed in RNAlater (Applied Biosystems, Foster City, CA, USA). Samples were stored at -80°C until RNA extraction.

4.2.3 Morpholino Injection

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

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

4.2.4 Deformity Assessment

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

4.2.5 In vivo EROD Assay

In addition to deformity assessment, CYP1 activity was also measured at 96 hpf via a modified in vivo EROD assay (Matson et al., 2008b; Nacci et al., 1998). The 7-ER added at the time of dosing is a substrate for CYP1 metabolism; CYP1s cleave 7-ER into a fluorescent product, resorufin, which accumulates in the gastrointestinal tract in zebrafish and can be measured under fluorescent microscopy (Billiard et al., 2006). Fluorescence was measured under 50x magnification using a rhodamine red filter set (Zeiss Axioskop) and quantified by IPLab software (Scanalytics Inc.). EROD values are expressed as a percentage of the mean fluorescence of NI control embryos.

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

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

RT-PCR was performed in a 25-μL reaction containing 62.5 ng template, 12.5 μL AmpliTaq Gold Master Mix (Applied Biosystems), 9 μL distilled H2O, and 0.5 μL each 10 μM forward and reverse primer. The thermocycler conditions were run according to Incardona et al. (2006) and were as follows: 94°C for 10 min followed by 35 cycles of 94°C for 15 s, 56.7°C for 30 s, 72°C for 45 s, and a final extension step at 72°C for 7 min. Primers spanning AHR1A exon 2 (F: 5’-CGCAAAAGGAGGAAACCTGTC-3’ and R: 5’-CCTGTAGCAAAAATTCCCCCT-3’) were used in the PCR reaction. For confirmation of AHR1B morpholino efficacy, the thermocycler conditions were as follows: 95°C for 5 min; 35 cycles of 95°C for 15 s, 58°C for 15 s, and 72°C for 4 min; and finally, 72°C for 10 min. AHR1B forward primer was 5’-GTACGCGGACGAAAAAGAAG-3’ (located on exon 1) and the reverse primer was 5’-CCTGTGCTTTACTGTGGTGCGTG-3’ (located on exon 3). All reactions were performed in triplicate. AHR1B primers were designed

Gel electrophoresis of 10 µL of PCR product was performed using agarose gels stained with SYBR Safe DNA gel stain (Invitrogen, Carlsbad, CA, USA); 4% agarose gels were used for AHR1A final products and 2% agarose gels were used for AHR1B final products. Gels were imaged with an AlphaImager HP (Cell Biosciences, Santa Clara, CA, USA).

4.2.7 QPCR

RNA extraction and cDNA synthesis were carried out as described above. cDNA was synthesized at a concentration of 25 ng/µL, which was subsequently diluted to 2 ng/µL. Each 25-µL QPCR reaction consisted of 12.5 µL SYBR Green PCR Master Mix (Applied Biosystems), 9.5 µL dH2O, 200 nM each forward and reverse primer, and 4 ng cDNA template. The reactions were run on an Applied Biosystems 7300 Real-Time PCR System with cycling parameters of 10 min at 95°C and 40 replicates of 15 s at 95°C, 1 min at 60°C. Dissociation curves were calculated for each sample at the end of each profile to confirm formation of a single product during the reaction. All samples were run in duplicate and technical replicates were averaged prior to normalization to β-actin, used as a housekeeping gene. The ABI PRISM 7300 Sequence Detection System Software, Version 1.1 (Applied Biosystems) was used to carry out data analysis.
The expression of CYP1A, CYP1B1, and CYP1C1 was examined in NI and AHR1A-mo-injected embryos after exposures to various PAHs and PCB-126. Primers for the three CYPs were published previously (Timme-Laragy et al., 2007). β-actin primers were also published previously (Grimes et al., 2008; Van Tiem and Di Giulio, 2011). The average mRNA fold induction of each target gene was calculated by comparing the Ct (threshold cycle) of the target gene to that of β-actin (Livak and Schmittgen, 2001). β-actin was determined to be a valid housekeeping gene as its expression was not altered by morpholino or treatment.

4.2.8 Statistical Analysis

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

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

The efficacy of the AHR1A morpholino was previously validated by Incardona et al. (2006), and we also confirmed its efficacy in our laboratory (data not shown). The efficacy of the newly designed AHR1B morpholino was determined by comparing amplification of a cDNA fragment in the region targeted by the morpholino, exon 1 – 3, between NI and AHR1B-mo embryos. NI embryos treated with DMSO or 50 μg/L BkF exhibited the same degree of amplification of the AHR1B cDNA fragment (Fig. 1, lane 2 and 3, respectively). AHR1B appeared to have strong constitutive expression and did not appear to be induced by BkF treatment. AHR1B-mo embryos treated with DMSO or 50 μg/L BkF appeared to exhibit weaker amplification of the AHR1B cDNA fragment (Fig. 13, lane 3 and 4, respectively). However, we were not able to detect a smaller band, which would suggest deletion of exon 2, or a larger band, which would suggest insertion of intron 2.

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

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

NI, Co-mo, and AHR1A-mo embryos were exposed to various individual PAHs, PAH mixtures and individual exposures to PCB-126. The pericardial areas of AHR1A-mo embryos exposed to individual doses of AHR ligands (BkF and BaP) and a CYP1 inhibitor (FL) were not different than the areas of NI embryos exposed to the same treatments or NI controls (Fig. 15).

AHR1A knockdown exacerbated the cardiac deformities caused by the three PAH mixtures examined. BaP + FL co-exposure resulted in an average pericardial area of 111±6% in NI embryos, which was not significantly greater than NI controls (Fig. 15A). AHR1A knockdown resulted in embryos with moderate but significant pericardial effusion as compared to NI controls and NI BaP + FL-exposed embryos (132±5%, p < 0.001 vs. NI DMSO and NI BaP + FL). NI embryos exposed to 100 μg/L BkF + 100 μg/L FL had an average pericardial area of 122±6% control levels, which was not significantly greater than controls, and AHR1A knockdown resulted in 177±10% pericardial effusion, which was significantly greater than NI DMSO controls and NI embryos of the same treatment (Fig. 15B; p < 0.0001). AHR1A knockdown also exacerbated the deformities caused by co-exposure to a mixture of different concentrations of BkF + FL. NI embryos exposed to 50 μg/L BkF + 150 μg/L FL had significant pericardial effusion compared to NI controls (149±5%; p < 0.0001), while
AHR1A knockdown resulted in even greater pericardial effusion (Fig. 15C; 214±11%; p < 0.0001 vs. NI DMSO and NI BkF + FL).

AHR1A knockdown also induced deformities in embryos exposed to 1 μg/L PCB-126 (Fig. 16). NI embryos exposed to PCB-126 had an average pericardial area of 110±4%, which was not significantly greater than the pericardial areas of control fish exposed to DMSO. AHR1A knockdown resulted in significant pericardial effusion in embryos exposed to 1 μg/L PCB-126 (140±5%, p < 0.001 vs. NI DMSO and NI PCB-126).

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

The effect of AHR1A knockdown on the expression of CYP1A, CYP1B1, and CYP1C1 in response to PAHs and PCB-126 was examined. Injection of the Co-mo or AHR1A-mo did not affect gene expression in DMSO-treated embryos. As determined by two-way ANOVA, there was an effect of treatment but not morpholino on expression of the three CYPs. FL alone did not upregulate any of the CYPs in any of the morpholino treatments. BkF and BaP alone induced CYP expression but induction was not altered by morpholino. CYP expression in response to the individual PAH treatments is excluded in the figures for graphical clarity. Exposure to 50 μg/L BkF + 150 μg/L FL significantly upregulated expression of all three CYPs in NI, Co-mo, and AHR1A-mo embryos (CYP1A - 87±6-, 84±10-, and 80±4-fold; CYP1B1 - 19±1-, 19±3-, and 18±1-fold; and CYP1C1 - 75±12-, 74±11-, and 67±8-fold, respectively; all p < 0.0005 vs. NI
DMSO) (Fig. 17). Exposure to 100 μg/L BaP + 500 μg/L FL also significantly induced expression of all three CYPs in NI, Co-mo, and AHR1A-mo embryos (CYP1A - 33±8-, 34±5, and 43±6-fold; CYP1B1 - 6±1-, 6±1, and 8±1-fold; and CYP1C1 - 29±4-, 29±4-, and 25±4-fold, respectively; all p < 0.001 vs. NI DMSO) (Fig. 18). These increases in CYP expression in response to PAHs were not statistically different between NI, Co-mo, and AHR1-mo embryos.

CYP1A expression in NI, Co-mo, and AHR1A-mo embryos exposed to 1 μg/L PCB-126 was induced 60±3-, 55±8-, and 47±3-fold, respectively, above NI controls; CYP1B1 was upregulated 27±5-, 24±4-, and 16±3-fold, respectively; and CYP1C1 was induced 104±22-, 118±17-, and 111±13-fold, respectively (all p < 0.002 vs. NI DSMO) (Fig. 19). Once again, CYP induction was not statistically different between the different morpholino treatments.

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

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

Once again, exposure to each PAH individually did not result in pericardial effusion in any embryos. In NI embryos, 100 μg/L BkF + 100 μg/L FL did not cause significant pericardial effusion and thus, there was no gross toxicity from which AHR2-mo embryos could be protected; however, AHR2-mo embryos had a slightly, but not significantly, smaller average pericardial effusion (105±5%) than NI embryos of the same treatment (114±3%) (Fig. 20A). AHR1A knockdown caused significant pericardial effusion in embryos exposed to 100 μg/L BkF + 100 μg/L FL (167±10%, p = 0.004 vs. NI BkF + FL and p < 0.0001 vs. NI DMSO). AHR1A/2 co-knockdown resulted in an average pericardial area of 124±2%, which was significantly less than the AHR1A-mo BkF + FL embryos (p = 0.012) but not different than NI DMSO or NI BkF + FL embryos.

Using a different dosing ratio for BkF and FL, NI embryos exposed to 50 μg/L BkF + 150 μg/L FL had significant pericardial effusion compared to NI DMSO embryos (146±6%, p < 0.001) (Fig. 20B). As expected based on previous studies, AHR2 knockdown prevented this toxicity; the average pericardial area of AHR2-mo embryos exposed to 50 μg/L BkF + 150 μg/L FL was 116±4%, which was not significantly different than NI controls. AHR1A knockdown caused more extreme pericardial effusion (182±8%, p < 0.001) than in NI embryos of the same treatment. AHR1A/2 co-knockdown
did not protect or exacerbate the pericardial effusion induced by BkF + FL (137±6%, p < 0.001 vs. NI DMSO, not different than NI BkF + FL).

To examine if the effect of AHR1A/2 co-knockdown was unique to the combination of the strong AHR agonist BkF in conjunction with FL, fish were also exposed to a combination of FL and another AHR agonist, BaP. Exposure to 100 µg/L BaP + 500 µg/L FL caused significant pericardial effusion in NI embryos compared to NI controls (124±5%, p < 0.0001) (Fig. 20C). AHR2 knockdown completely prevented this effusion; AHR2-mo embryos had an average pericardial area of 105±3% (not different from NI controls). AHR1A knockdown exacerbated BaP + FL-induced toxicity, resulting in embryos with an average pericardial area of 141±5% (p < 0.0001 vs. NI DMSO and NI BaP + FL). The same effect of AHR1A/2 co-knockdown that occurred in 50 µg/L BkF + 150 µg/L FL-exposed embryos was observed in BaP + FL-exposed embryos: AHR1A/2 co-knockdown neither prevented nor exacerbated the deformities caused by BaP + FL compared to NI BaP + FL embryos (125±6%, p < 0.0001 vs. NI DMSO, not different than NI BaP + FL).

Embryos were also exposed to single doses of the strong AHR agonist PCB-126, which is capable of producing deformities without the addition of a CYP1 inhibitor. In these experiments, 1 µg/L PCB-126 did not appear to be as toxic as in previous experiments. However, NI embryos exposed to 2 µg/L PCB-126 had significant pericardial effusion, with an average pericardial area of 140±4% (p < 0.0001) (Fig. 21).
AHR2 knockdown prevented the cardiac deformities caused by this dose of PCB; AHR2-mo embryos had an average pericardial area of 110±2% (not different than NI DMSO).

AHR1A knockdown resulted in embryos with significant pericardial effusion (164±12%, p < 0.0001 vs. NI DMSO and NI PCB-126). Unlike the effect seen with the PAH co-exposures, AHR1A/2 co-knockdown completely prevented the pericardial effusion caused by 2 μg/L PCB-126, just as AHR2 knockdown did. AHR1A/2 embryos had an average pericardial area of 111±3% (p < 0.0001 vs. NI 2 μg/L PCB-126, not different than NI DMSO).

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

For evaluation of the effect of AHR1A knockdown, AHR2 knockdown, and AHR1A/2 co-knockdown on CYP enzyme activity, NI and injected embryos were exposed to the AHR ligands individually without FL. CYP1 activity, as measured via the EROD assay, varied depending on the agonist used, dose of the agonist, and morpholino. CYP1 activity in NI embryos exposed to 100 μg/L BaP was significantly induced to 232±43% of NI control levels (p < 0.001) (Fig. 22A). AHR2 knockdown prevented induction of CYP1 activity (112±20%). In contrast, AHR1A knockdown resulted in increased CYP1 activity (491±20%, p < 0.001 vs. NI DMSO and NI BaP). AHR1A/2 knockdown appeared to prevent CYP1 induction but the induction of 149±14% was not statistically different from NI controls or NI BaP-exposed embryos.
NI and mo-injected embryos were also exposed to 50 and 100 µg/L BkF, which is a stronger AHR agonist than BaP. NI embryos exposed to 50 µg/L BkF had CYP1 activity 732±49% of NI control levels (p < 0.0001) (Fig. 22B). AHR2 knockdown prevented induction of CYP1 activity compared to NI embryos of the same treatment (567±71%, p < 0.001) but the CYP1 activity was still significantly higher than NI controls (p < 0.01). AHR1A knockdown slightly increased CYP1 activity to 989±130%, but this increase was not significantly different than the activity in NI 50 µg/L BkF-exposed embryos. AHR1A/2 knockdown resulted in 723±77% NI control CYP1 activity, which was not statistically different than control or NI 50 µg/L BkF levels. Exposure to 100 µg/L BkF significantly induced CYP1 activity in NI embryos (874±87%, p < 0.001), and AHR2 knockdown significantly reduced CYP1 activity (502±96%, p < 0.001 vs. NI DMSO and NI 100 µg/L BkF). AHR1A knockdown did not significantly increase or decrease CYP1 activity compared to NI 100 µg/L BkF-exposed embryos (831±67%) nor did AHR1A/2 co-knockdown (665±50%).

Exposure to 1 µg/L PCB-126 significantly induced CYP1 activity to 314±14% control levels in NI embryos (Fig. 22C). AHR1A knockdown resulted in increased CYP1 activity (451±68%) compared to NI embryos of the same treatment (p < 0.0001 vs. NI DMSO and NI 1 µg/L PCB-126). AHR2 and AHR1A/2 knockdown both significantly reduced CYP1 activity (205±20% and 255±18%, respectively) compared to NI treated embryos but the activity was still higher than NI controls. NI embryos exposed to 2
μg/L PCB-126 had significantly increased CYP1 activity above control levels (473±24%, p < 0.0001). AHR1A knockdown did not increase CYP activity compared to NI treated embryos (458±34%). AHR2 and AHR1A/2 knockdown both significantly reduced CYP1 activity compared to NI 2 μg/L PCB-126-exposed levels and the activity was not different than NI DMSO controls (168±20% and 248±11%, respectively). Even though AHR1A/2 knockdown did not cause significantly different CYP1 activity compared to NI exposed embryos exposed to 1 μg/L PCB-126, there was a trend toward the co-knockdown resulting in a level of CYP1 activity that was intermediate between NI embryos and AHR2-mo embryos.

4.4 Discussion

Limited information about the AHR1 isoforms exists but the findings that AHR1B knockdown had no impact on PAH- and PCB-126-induced cardiac toxicity while AHR1A knockdown exacerbated this toxicity were quite unexpected. The zebrafish AHR1s and AHR2 arose from a gene duplication event that occurred before the divergence of ray- and lobe-finned fishes and thus, before the teleost gene-duplication event that is responsible for the existence of many other paralogous genes in fish (Hahn, 2002). AHR1B is adjacent to AHR2 on chromosome 22 while AHR1A is located on chromosome 16 (Karchner et al., 2005). AHR1B is expressed in zebrafish embryos at 24 hpf but was not inducible by TCDD at 48 or 72 hpf. However, using
velocity sedimentation analysis, AHR1B was shown to have high-affinity binding for 
[3H]TCDD and was shown to be transcriptionally active in response to TCDD in COS-7 
cells with efficiency similar to AHR2 but sensitivity approximately eight-fold lower than 
AHR2 (Karchner et al., 2005). Based on these results, we hypothesized that AHR1B 
knockdown would have similar effects to AHR2 knockdown, providing a protective role 
against PAH- and PCB-126-induced toxicity but perhaps to a lesser extent than AHR2 
knockdown. However, AHR1B knockdown had no effect on the cardiac deformities 
caused by PAH mixtures and PCB-126 in our studies. This lack of apparent effect could 
be due to the fact that we only examined the gross morphological condition of embryos. 
AHR1B knockdown may result in a more discrete effect that could be elucidated via 
finer examination with histopathology or immunohistochemistry. Alternatively, 
AHR1B could have functional redundancy with AHR2. However, because AHR2 is 
necessary for some PAH and PCB-126 toxicity and appears to have a higher affinity for 
TCDD, it is apparent that AHR2 has a greater role in xenobiotic metabolism and toxicity 
than AHR1B. Additionally, though our PCR results showed lesser amplification of 
AHR1B cDNA in morpholino-injected embryos compared to NI embryos, amplification 
was still evident. Our knockdown of AHR1B may have been insufficient to cause gross 
phenotypic effects in response to PAH and PCB-126 exposure.

Andreasen et al. (2002a) showed that AHR1A mRNA expression was detectable 
at 24 hpf in zebrafish and was slightly induced by TCDD. In adult tissues, AHR1A
mRNA was most highly expressed in the liver and to a lesser extent in the heart and kidney but was not induced by TCDD exposure. In contrast, AHR2 was found to be expressed in all tissues and was significantly induced by TCDD in the heart. In the same study, AHR1A was shown to be able to dimerize with ARNT2b and recognize XREs \textit{in vitro} but with DNA binding activity much lower than the AHR2-ARNT2b heterodimer. AHR1A was also not transcriptionally active in response to TCDD in COS-7 cells and did not exhibit specific binding with $[^{3}H]$TCDD or $[^{3}H]$BNF. These findings led to the hypothesis that AHR1A is a nonfunctional incipient pseudogene (Andreasen et al., 2002a; Karchner et al., 2005). However, in the only other study of which we are aware that examined the effect of AHR1A knockdown \textit{in vivo}, Incardona \textit{et al.} (2006) showed that knockdown delayed onset of abnormal development and pericardial effusion caused by high doses of the four-ringed PAH pyrene, but that embryos eventually succumbed to these deformities. Based on these two previous studies, we hypothesized that AHR1A knockdown would either have no effect on the toxicity induced by high molecular weight PAH mixtures and PCB-126 or would have a slight protective effect. Hence, the exacerbation of toxicity by AHR1A knockdown was quite surprising.

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

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

Various lines of evidence suggest that AHR1A may be acting in a manner similar to AHRR. An example from the mammalian literature shows that AHRR deficient mice lack any overt signs of developmental deformities and exhibit increased CYP1A1 mRNA induction in the skin, stomach and spleen but not in the liver, lung, or heart (Hosoya et al., 2008). While we did not observe increased CYP1 mRNA expression in AHR1A-mo
embryos, we did observe greater CYP1 activity in AHR1A-mo embryos. This difference could be explained by the fact that the EROD assay measures the cumulative activity of the CYP1s until the time of the assay (96 hpf) while QPCR was used to measure expression of each of the three CYPs (1A, 1B1, and 1C1 in this study) individually at one time point (48 hpf). The lack of increased CYP1 expression could be due to species differences or the early developmental stage at which expression was examined. Also, due to the early time point, we were not able to examine tissue-specific differences in gene expression. AHRR lacks a ligand-binding region but is capable of dimerizing with ARNT (Mimura et al., 1999) and downregulates the classic AHR2-mediated pathway by competing with the AHR2/ARNT complex for binding to XRES and also by XRE binding-independent mechanisms (Evans et al., 2008). Two AHRRs, initially termed AHRR1 and 2, now identified as AHRRa and AHRRb, have been identified in zebrafish (Evans et al., 2005; Jenny et al., 2009). Jenny et al. (2009) knocked down both AHRRa and AHRRb individually and simultaneously in zebrafish and found that CYP1 expression in response to TCDD exposure was not affected at 48 hpf but was increased at 72 hpf and that this enhancement of induction was due to AHRRb knockdown. In the current study, CYP1 expression was also unaffected by AHR1A knockdown. We did not examine gene expression at later time points; future experiments would be informative for this. Furthermore, AHRRa knockdown in untreated embryos resulted in a TCDD-like phenotype with embryos displaying pericardial effusion, cardiac deformities, and
craniofacial malformations while AHRRb morphants were normal; however, knockdown of either repressor individually resulted in enhanced toxicity in TCDD-exposed embryos (Jenny et al., 2009). A similar enhancement of PAH- and PCB-126-induced deformities was evident in AHR1A-mo embryos in our study. If AHR1A acts as a repressor of AHR2 in some manner, then AHR1A knockdown would result in increased and inappropriate signaling from AHR2, resulting in increased toxicity in response to AHR ligand exposure.

In conclusion, the results presented herein indicate that AHR1A and AHR1B have distinct, though still not fully elucidated, roles in zebrafish development as well as response to xenobiotics. AHR1B knockdown via morpholino injection did not affect PAH- or PCB-126-induced cardiac toxicity. On the other hand, AHR1A knockdown unexpectedly exacerbated PAH- and PCB-126-induced toxicity in developing zebrafish embryos. AHR1A knockdown did not affect CYP1 mRNA expression but did enhance CYP1 activity. Concurrent knockdown of AHR1A and AHR2 did not exacerbate or prevent PAH-induced deformities was but was protective with respect to PCB-126-induced deformities. Further experiments examining the interaction of the AHR1 isoforms with other members of the classical AHR2 pathway as well as examination of gene expression at different time points and further characterization of the effects of the AHR1 isoforms on cardiac structure and function will help to elucidate the developmental and/or toxicological role of AHR1 isoforms in embryonic zebrafish.
Figure 13: Knockdown of AHR1B via splice junction morpholino. Embryos were dosed with DMSO or 50 μg/L BkF at 24 hpf. Representative image is from RT-PCR amplification of 10 pooled embryos per each treatment. Ld = 100 bp DNA ladder. NI embryos exposed to BkF show strong amplification of the AHR1B exon 1-3 cDNA fragment. AHR1B-mo embryos exposed to BkF show weaker amplification of the fragment.
Figure 14: Effect of AHR1A and AHR1B knockdown on PAH- and PCB-126-induced deformities.
Non-injected (NI; white bars), control morpholino (Co-mo; grey bars), AHR1A-mo (black bars), and AHR1B-mo (patterned bars) embryos were dosed with PAHs (A) and PCB-126 (B) at 24 hpf and scored at 96 hpf. Values are expressed as percent NI DMSO pericardial area ± SEM (n = 9 per treatment; each n represents the average of five embryos). Groups not sharing a common letter are significantly different (p ≤ 0.05; ANOVA, Tukey adjusted LSMeans).
Figure 15: Effect of AHR1A knockdown on PAH-induced deformities.
Non-injected (NI; white bars), control morpholino (Co-mo; grey bars), and AHR1A-mo (black bars) embryos were dosed with (A) 100 μg/L BkF and 100 μg/L FL; (B) 50 μg/L BkF and 150 μg/L FL; or (C) 100 μg/L BaP and 500 μg/L FL at 24 hpf and scored at 96 hpf. Values are expressed as percent NI DMSO pericardial area ± SEM (n = 12 per treatment; each n represents the average of five embryos). Groups not sharing a common letter are significantly different (p ≤ 0.05; ANOVA, Tukey adjusted LSMeans).
Figure 16: Effect of AHR1A knockdown on PCB-126-induced deformities. Non-injected (NI; white bars), control morpholino (Co-mo; grey bars), and AHR1A-mo (black bars) embryos were dosed with 1 µg/L PCB-126 at 24 hpf and scored at 96 hpf. Values are expressed as percent NI DMSO pericardial area ± SEM (n = 12 per treatment; each n represents the average of five embryos). Asterisk (*) denotes treatment that is significantly different from NI controls ($p \leq 0.05$; ANOVA, Tukey adjusted LSMeans).
Figure 17: Effect of AHR1A knockdown on BaP + FL-induced CYP1 expression. CYP1 mRNA expression in non-injected (NI; white bars), control morpholino (Co-mo; grey bars), and AHR1A-mo (black bars) embryos exposed to 100 µg/L BaP + 500 µg/L FL at 24 hpf. (A) CYP1A; (B) CYP1B1; (C) CYP1C1. Expression was measured at 48 hpf. Expression is shown as fold induction ± SEM compared to NI controls (n = 6 per treatment; each n represents the average of five embryos). An asterisk (*) represents a significant difference from NI control (p ≤ 0.05; ANOVA, Tukey adjusted LSMeans).
Figure 18: Effect of AHR1A knockdown on BkF+FL-induced CYP1 expression. CYP1 mRNA expression in non-injected (NI; white bars), control morpholino (Co-mo; grey bars), and AHR1A-mo (black bars) embryos exposed to 50 µg/L BkF + 150 µg/L FL at 24 hpf. (A) CYP1A; (B) CYP1B1; (C) CYP1C1. Expression was measured at 48 hpf. Expression is shown as fold induction ± SEM compared to NI controls (n = 6 per treatment; each n represents the average of five embryos). An asterisk (*) represents a significant difference from NI control (p ≤ 0.05; ANOVA, Tukey adjusted LSMeans).
Figure 19: Effect of AHR1A knockdown on PCB-126-induced CYP1 expression.

CYP1 expression in non-injected (NI; white bars), control morpholino (Co-mo; grey bars), and AHR1A-mo (black bars) embryos exposed to 1 μg/L PCB-126 at 24 hpf. (A) CYP1A; (B) CYP1B1; (C) CYP1C1. Expression was measured at 48 hpf. Expression is shown as fold induction ± SEM compared to NI controls (n = 6 per treatment; each n represents the average of five embryos). An asterisk (*) represents a significant difference from NI control (p ≤ 0.05; ANOVA, Tukey adjusted LSMeans).
Figure 20: Comparison of AHR1A, AHR2, and AHR1A/2 knockdown on PAH-induced deformities.

Non-injected (NI; white bars), AHR2-mo (diagonal bars), AHR1A-mo (black bars), and AHR1A/2-mo (dark grey bars) embryos were dosed with (A) 100 μg/L BkF and 100 μg/L FL; (B) 50 μg/L BkF and 150 μg/L FL; and (C) 100 μg/L BaP and 500 μg/L FL at 24 hpf and scored at 96 hpf. Values are expressed as percent NI DMSO pericardial area ± SEM (n = 9 per treatment; each n represents the average of five embryos). Groups not sharing a common letter are significantly different (p ≤ 0.05; ANOVA, Tukey adjusted LSMeans).
Figure 21: Comparison of AHR1A, AHR2, AHR1A/2 knockdown on PCB-126-induced deformities.
Non-injected (NI; white bars), AHR2-mo (diagonal bars), AHR1A-mo (black bars), and AHR1A/2-mo (horizontal bars) embryos were dosed with 1 μg/L and 2 μg/L PCB-126 at 24 hpf and scored at 96 hpf. Values are expressed as percent NI DMSO pericardial area ± SEM (n = 9 per treatment; each n represents the average of five embryos). Groups not sharing a common letter are significantly different (p ≤ 0.05; ANOVA, Tukey adjusted LSMeans).
Figure 22: Effect of AHR1A, AHR2, and AHR1A/2 knockdown on CYP1 activity.
EROD induction in non-injected (NI; black line), AHR2-mo (grey line), AHR1A-mo (dotted line), and AHR1A/2-mo (hashed line) embryos treated with the following: A) 100 μg/L BaP; (B) 50 and 100 μg/L BkF; (C) 1 and 2 μg/L PCB-126. Embryos were dosed at 24 hpf and scored at 96 hpf. Values are expressed as percent NI control EROD ± SEM (n = 9 per treatment; each n represents average of five embryos). Groups not sharing a common letter are significantly different (p ≤ 0.05; ANOVA, Tukey adjusted LSMeans).
5. Heart-specific microarray identification of AHR-dependent and AHR-independent genes involved in the synergistic developmental toxicity of PAHs

Parts of this chapter will be submitted for publication with Lindsey A. Van Tiem, Joel N. Meyer, Kyle N. Erwin, Huai-Jen Tsai, Margaret L. Kirby, and Richard T. Di Giulio as authors.

5.1 Introduction

The aryl hydrocarbon receptor (AHR) is a ligand-activated transcription factor that regulates the transcription of numerous phase I and II metabolic genes. Agonists for the AHR include certain dioxins (most notably 2,3,7,8-tetrachlorodibenzo-\(p\)-dioxin (TCDD)), coplanar polychlorinated biphenyls (PCBs), and many polycyclic aromatic hydrocarbons (PAHs). Upon ligand-binding by these agonists, the AHR undergoes a conformational change, translocates from its constitutive position in the cytoplasm to the nucleus, dissociates from chaperone proteins, and forms a heterodimer with the aryl hydrocarbon nuclear translocator (ARNT). The heterodimer then binds to xenobiotic response elements (XREs, also known as dioxin response elements or aryl hydrocarbon response elements) and activates expression of target genes. In mammals, the best characterized components of the AHR gene battery are cytochrome p450 1A (CYP1A), CYP1B1, aldehyde dehydrogenase 3 (ALDH3), glutathione s-transferases (GSTs),
NAD(P)H:quinone oxidoreductase 1 (NQO1), and UDP-glucuronosyltransferase 6 (UDPGT6) (Nebert et al., 2000). CYP1A, 1B1, 1C1, and 1C2, AHRR (aryl hydrocarbon receptor repressor) as well as certain GSTs are also induced by AHR ligand binding in fish (Jonsson et al., 2007; Timme-Laragy et al., 2007; 2009). These genes are involved in the detoxification of numerous lipophilic compounds, including some AHR ligands. In mammals, birds, and fishes, numerous PAHs, as well as TCDD and coplanar PCBs, are known to produce a wide range of toxic effects including carcinogenesis, mutagenesis, disruption of immune and endocrine function, and developmental defects. The heart is a target organ of this developmental toxicity. Exposures of fish early life stages to three- and four-ringed PAHs and weathered crude oil containing PAHs have been shown to cause impaired heart looping, atrioventricular conduction block, and cardiac arrhythmia in zebrafish (*Danio rerio*) (Incardona et al., 2005). The PAHs benzo[k]fluoranthene (BkF) and the model PAH β-naphthoflavone (BNF) cause pericardial effusion and an elongated heart in killifish (*Fundulus heteroclitus*) embryos (Clark et al., 2010). It was originally hypothesized that inhibition of CYP1A would result in protection from the toxicity induced by a PAH that is an AHR agonist due to evidence of this occurrence from TCDD studies (Cantrell et al., 1996; Smith et al., 2001; Uno et al., 2004). However, our laboratory has shown that exposure to a simple mixture consisting of a PAH that is an AHR agonist with one that is a CYP1 inhibitor results in pericardial effusion and an elongated heart (the “stringy heart” phenotype) (Billiard et al., 2006; Van
Tiem and Di Giulio, 2011; Wassenberg and Di Giulio, 2004). Furthermore, knockdown of CYP1A using morpholino antisense oligonucleotides exacerbates toxicity of BNF in zebrafish and killifish embryos (Billiard et al., 2006; Matson et al., 2008a). On the other hand, knockdown of AHR2 (zebrafish have three identified AHRs (Andreasen et al., 2002a; Karchner et al., 2005; Tanguay et al., 1999)) prevents the toxicity of these AHR agonist + CYP1 inhibitor mixtures (Billiard et al., 2006; Van Tiem and Di Giulio, 2011). AHR2 knockdown also prevents PAH- and PCB-126-induced cardiac toxicity in killifish (Clark et al., 2010). In addition to mediating PAH toxicity, AHR2 mediates TCDD toxicity in zebrafish (Dong et al., 2004; Prasch et al., 2003). Furthermore, AHR null mice are protected from the toxicity caused by TCDD as well as the carcinogenicity of BaP (Fernandez-Salguero et al., 1996; Shimizu et al., 2000). While it is well-established that the AHR, specifically AHR2 in zebrafish, mediates the toxicity of many PAHs, there is some evidence that some PAH toxicity may occur through an AHR2-independent mechanism. For example, Incardona et al. (2005) showed that AHR2 knockdown did not prevent the cardiac arrhythmia caused by the tricyclic PAHs dibenzothiophene and phenanthrene, which are very weak AHR agonists.

Despite a plethora of data characterizing the phenotypic effects of PAH toxicity and mediation of this toxicity via the AHR, the exact mechanisms by which PAHs cause cardiac developmental toxicity remain unknown. In this study, we sought to identify novel genes involved in the cardiac toxicity caused by a simple PAH mixture and to
determine if these genes are controlled in some part by the AHR. We injected zebrafish embryos with an AHR2 morpholino or control morpholino, exposed them to the AHR agonist BaP and the CYP1 inhibitor fluoranthene (FL), and extracted the hearts. We then used microarrays to identify heart-specific gene expression changes in PAH-exposed vs. control embryos across various time points and to determine which PAH-induced genes are controlled by AHR2. The most abundant gene expression changes occurred at the last time point after dosing (18 h), and all of the gene expression changes appeared to be regulated by AHR2. Most notably, many of the genes upregulated by PAH exposure are not currently associated with AHR regulation.

5.2 Materials and Methods

5.2.1 Fish care

AB(cmlc2::GFP) (a kind gift from Debbie Yelon (Skirball Institute, New York) with permission from Dr. Huai-JenTsai (National Taiwan University, Taiwan) zebrafish (Danio rerio) were maintained according to Nusslein-Volhard (2002). All adult handling and reproductive techniques were approved by the Duke University Institutional Animal Care & Use Committee (A047-04-02).

Embryos were obtained from the natural spawning of adult zebrafish and were collected in 60 mg/L salt water embryo medium. Embryos were maintained at 28°C on a 14 h: 10 h light:dark cycle.
5.2.2 Morpholino injection

Morpholino antisense oligonucleotides were designed and produced by Gene Tools, LLC (Philomath, OR, USA). A morpholino previously shown to block translation of AHR2 (AHR2-mo: 5’-TGTACCGATACCCGCCGACATGGTT-3’) was used to knock down AHR2 (Teraoka et al., 2003). Gene Tools’ standard control morpholino (Co-mo, 5’-CCTCTACCTCAGTTACAATTATA-3’) was used as a control for the process of injection. Both morpholinos were fluorescein-tagged at the 3’ end to allow for determination of injection success under fluorescence microscopy. Morpholinos were diluted to 100 μM working stocks in 30% Danieau (Nasevicius and Ekker, 2000).

Approximately 3 nL of morpholino was injected into the yolk of embryos at the 1-4 cell stage (0-1 hour post fertilization (hpf)) using a FemtoJet Microinjector (Eppendorf, NY, USA). Embryos exhibiting normal development and strong, uniform fluorescence indicating successful incorporation of the morpholinos were used for experiments.

5.2.3 Chemicals and dosing

Benzo[a]pyrene (BaP) and fluoranthene (FL) were purchased from Absolute Standards, Inc. (Hamden, CT, USA), and dimethyl sulfoxide (DMSO) and tricaine methanesulfonate (MS-222) were purchased from Sigma-Aldrich (St. Louis, MO, USA).
BaP and FL stocks were dissolved in DMSO and kept protected from light at -20°C until use.

5.2.3.1 Dosing for deformity assessment

At 24 hpf, non-injected (NI), Co-mo-injected, and AHR2-mo-injected embryos were dosed with DMSO, 100 µg/L BaP, 500 µg/L FL, and 100 µg/L BaP + 500 µg/L FL. Embryos were dosed in 7.5 mL 30% Danieau in 20-mL glass scintillation vials with five embryos per vial and three vials per treatment. Final DMSO concentration was < 0.03% in each vial.

5.2.3.2 Dosing for microarray experiment

The normal dosing paradigm of our laboratory includes dosing embryos at 24 hpf. However, attempts to extract hearts after dosing at 24 hpf resulted in heart fragmentation into pieces too small to collect. In order to successfully extract whole hearts from zebrafish embryos at time points shortly after dosing, a later dosing time point was necessary. Thus, Co-mo- and AHR2-mo-injected embryos were dosed at 36 hpf with the same treatments as were used for deformity screening. To ensure that a sufficient number of whole hearts could be extracted and collected per treatment, two vials per morpholino injection per treatment per time point containing five embryos each were dosed (n = 10 embryos per experimental replicate). The experiment was replicated four times within a three-week period and a fifth time approximately two
months later due to low RNA yield from previous samples for a final n = 5 pools of 5 hearts/morpholino/dose/time point.

5.2.4 Deformity Assessment

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

5.2.5 Statistical analysis for deformity experiments

The deformity data were analyzed via two-way analysis of variance (ANOVA) to determine an overall effect of the morpholino injection and dose followed by least square means (LSMeans) procedures using JMP 8.1.1 (SAS Institute Inc., Cary, NC, USA). Tukey’s post-hoc test was used to determine differences between groups. After initial range-finding experiments, the dosing with the BaP and FL concentrations
described in section 2.3.1 were replicated four times with three samples per treatment per experiment. No differences between experimental replicates were observed for any test. Data are represented as mean ± standard error of the mean (SEM). Values were considered significantly different at p ≤ 0.05.

5.2.6 Heart extraction

Four heart extraction time points were chosen to capture early and late gene expression changes. Hearts were extracted at 2, 6, 12, and 18 hours post dosing (hpd), corresponding to 38, 42, 48, and 54 hpf, respectively. Hearts were extracted using a method modified from Burns and MacRae (2006). Briefly, zebrafish were placed in 1.5-mL Eppendorf tubes and anesthetized on ice. Embryos were then washed three times with approximately 1 mL embryo disruption media (EDM) consisting of Leibovitz’s L-15 Medium containing 10% FBS (Invitrogen, Carlsbad, CA) and then resuspended in 1.25 mL EDM. For the 2 and 6 hpd time points, approximately 1 mL EDM and the embryos were drawn into a 6-mL syringe with a 21-gauge needle and expelled back into the Eppendorf tube, and this was repeated a total of 5 times. For the 12 and 18 hpd time points, embryos and the same volume of EDM were drawn into a 6-mL syringe with a 19-gauge needle and then expelled a total of 8 times. Upon the last aspiration, embryos were expelled into a 35 x 10 mm sterile suspension dish (Nalge Nunc International, Rochester, NY, USA). A new dish was used for each replicate. Zebrafish hearts were
collected with a p10 pipet under fluorescent microscopy, placed into unused, sterile 1.5-mL Eppendorf tubes, immediately flash frozen, and stored at -80°C until RNA extraction.

5.2.7 RNA extraction and microarray

RNA extraction and microarrays were carried out by Beckman Coulter Genomics (formerly Cogenics; Morrisville, NC, USA). The quantity of each of the 128 RNA samples was determined using a NanoDrop ND-100 (NanoDrop Technologies, Wilmington, DE, USA), and the quality and size distribution of RNA was assessed using an Agilent Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA). This study used a universal reference design meaning that all experimental samples were compared to pooled RNA extracted from a zebrafish embryonic fibroblast cell line, Zf4 cells derived from one-day old zebrafish embryos, kindly supplied by Dr. Elwood Linney, Duke University Medical Center (Driever and Rangini, 1993). Aliquots of the isolated RNA were stored at -80°C.

Microarray hybridizations were performed on Agilent Zebrafish 4x44K Custom Microarrays that included all standard probes plus 439 custom probes encompassing additional neural and retinoic acid genes from Dr. Elwood Linney. Fifty nanograms of total RNA was converted into labeled cRNA with nucleotides coupled to a fluorescent dye (either Cy3 or Cy5) using the Low Input Quick Amp Kit (Agilent Technologies)
following the manufacturer’s protocol. The amount and quality of the labeled cRNA were determined using a NanoDrop ND-1000 and an Agilent Bioanalyzer, respectively. Four replicates from each treatment were analyzed. Equal amounts of Cy3- and Cy5-labeled cRNA from the reference and experimental samples were hybridized onto the arrays. Due to an insufficient amount of the Zf4 reference sample, 675 ng of each sample was hybridized, which is less than the 825 ng recommended by Agilent. This may have resulted in loss of detectability of very low abundance transcripts. Agilent’s Feature Extraction Software (Agilent Technologies) was used to extract data from scanned images of each array.

5.2.8 Microarray data analysis

Raw data from the Feature Extraction were analyzed with GeneSpring GX 10 software (Agilent Technologies). Due to the high degree of variability in the data and potentially developmental stage-specific gene changes, the time points were analyzed individually. Identification of differentially expressed genes between treatments was completed using the GeneSpring Guided Workflow with the defaults to threshold all values to 1 and perform a baseline transformation using the median of all samples. The Guided Workflow also includes quality control parameters whereby the quality of the hybridizations was examined and samples outside of the QC range were excluded. Probesets were then filtered so that any gene missing in every sample and the genes
with expression in the lowest 15% percentile and below were excluded. A two-way ANOVA followed by the Benjamini-Hochberg post-hoc test was used to determine significance. Genes were considered to be significantly differentially expressed when they had a fold-change ≥ 1.3 and a \( p \)-value ≤ 0.05.

### 5.3 Results

#### 5.3.1 Deformity assessment

Exposure to 100 \( \mu \text{g}/\text{L} \) BaP and 500 \( \mu \text{g}/\text{L} \) FL individually did not cause pericardial effusion in NI, Co-mo, or AHR2-mo embryos (Fig. 23). Exposure to the BaP + FL mixture resulted in significant pericardial effusion in NI and Co-mo embryos. NI embryos had an average pericardial area of 241±16% and Co-mo embryos had an average pericardial area of 233±3% (both \( p \) < 0.0001 vs. NI DMSO, but not different from one another). AHR2 knockdown prevented BaP +FL-induced pericardial effusion; AHR2-mo embryos had an average pericardial area of 108±6%, which was not different than NI controls.

#### 5.3.1 Microarray data

Due to the vastness and complexity of the data from 128 arrays, each time point (2, 6, 12, or 18 hpd) was examined individually. Because AHR2 knockdown prevents
BaP + FL-induced cardiac toxicity, focus was placed on differential gene expression between Co-mo hearts exposed to BaP + FL and AHR2-mo hearts exposed to BaP + FL.

5.3.1.1 Two hours post dosing time point

At 2 hpd, only six genes were found to be differentially expressed between the Co-mo and AHR2-mo hearts exposed to BaP + FL. All six genes were upregulated in Co-mo BaP + FL-exposed hearts compared to AHR2-mo BaP + FL-exposed hearts, i.e., AHR2 knockdown prevented the upregulation of genes that occurred in Co-mo BaP + FL hearts. Of the six upregulated genes, five have been at least partially annotated (Table 4). The one unannotated gene (Agilent Probe ID A_15_P117633) was upregulated 18- and 21-fold in AHR2-mo BaP + FL embryos and Co-mo BaP + FL embryos, respectively, compared to Co-mo DMSO. Due to the rather large induction in both Co-mo and AHR2-mo hearts, it would be interesting to know the identity of this gene.

5.3.1.2 Six and twelve hours post dosing time point

No genes were found to be differentially expressed between Co-mo BaP + FL embryos and AHR2-mo BaP + FL embryos at the 6 hpd time point. At 12 hpd, one gene, zgc:162816, was upregulated 1.3-fold (p < 0.0001) in Co-mo BaP + FL hearts compared to AHR2-mo BaP + FL hearts. This gene is an ortholog of the yeast (Saccharomyces cerevisiae) gene DSD1, D-serine dehydratase, which converts D-serine to pyruvate and ammonia (Ito et al., 2008).
5.3.1.3 Eighteen hours post dosing time point

The greatest abundance of genes differentially expressed was identified at the 18 hpd time point. Comparing Co-mo BaP + FL and AHR2-mo BaP + FL hearts, 189 genes were differentially expressed. Of these genes, only 6 were downregulated, 2 of which have been annotated. Tlr2 was downregulated 2.2-fold and Gabbr1 was downregulated 2.6-fold in the Co-Mo BaP + FL treatment compared to the AHR2-mo BaP + FL treatment. Tlr2 is a membrane-bound receptor that is involved in the innate immunity of cells, and the TLRs are highly conserved across vertebrates (Aderem and Ulevitch, 2000). Gabbr1 encodes half of the GABA_B receptor heterodimer, which is also composed of Gabbr2. The GABA_B receptor mediates the metabotropic actions of the inhibitory neurotransmitter GABA through activation of G proteins to modulate the action of ion channels and second messengers (Mody et al., 1994).

Of the 183 genes upregulated in Co-mo BaP + FL compared to AHR2-mo BaP + FL, 84 have been at least partially annotated. Many of these genes are involved in various processes including cell adhesion (e.g., cadherin 23), oxidation-reduction processes or monooxygenase activity (e.g., various CYPs and NADH dehydrogenase (ubiquinone) flavoprotein 1), regulation of apoptosis and cell proliferation (e.g., rho/rac guanine nucleotide exchange factor (GEF) 18), zinc ion binding (zinc finger, matrin type 5), and transforming growth factor beta (TGF-β) receptor binding (TGF-β itself). Many
are also components of the cellular membrane, Golgi apparatus membrane (phosphotidylinositol transfer protein, beta), ribosome (mitochondrial ribosomal protein L48), or endoplasmic reticulum (translocating chain-associating membrane protein 1) membrane and involved in transfer of compounds in and out of these membranes (refer to Appendix Table 1 for a complete list of 84 annotated genes).

Comparing gene expression in Co-mo BaP + FL and AHR2-mo BaP + FL, there were 10 upregulated genes known or hypothesized to be involved in heart morphology and function (Table 3). The same genes that were upregulated in Co-mo BaP + FL compared to AHR2-mo BaP + FL were also upregulated when comparing to Co-mo BaP + FL to Co-mo DMSO. However, there were no significant differences between AHR2-mo BaP + FL vs. Co-mo DMSO.

5.4 Discussion

The data obtained from the microarray analysis described herein are highly variable. Part of this variability was likely due to gene changes across developmental time points, which lead use to examine gene expression at each time point independently. Using this approach, we were able to identify particular genes that were upregulated in the Co-mo BaP + FL treatment compared to the AHR2-mo BaP + FL treatment at the 2 hpd time point and 18 hpd time point. At the 2 hpd, the upregulation of Sox9b in the Co-mo BaP + FL treatment is noteworthy. Sox9b is an early neural crest
marker (Li et al., 2002) and is essential for chondrocyte differentiation and cartilage formation (Bi et al., 2001). Sox9b has been shown to be downregulated in the deformed jaws of zebrafish exposed to TCDD (Xiong et al., 2008). Expecting the PAH mixture to have a similar effect to TCDD, it is surprising that the mixture upregulated Sox9b expression. However, tissue responses to TCDD appear to be very tissue-specific, and Xiong et al. (2008) saw very little expression overlap when they compared TCDD-induced gene expression in the jaw and heart. Another gene of interest at 2 hpd is smoothened ( smo). Smo is a critical part of the hedgehog signaling pathway, a key regulator of embryogenesis and body segmentation that is highly conserved amongst invertebrates and vertebrates. Smo appears to be inhibited by Ptch (Ptch2 is upregulated at the 18 hpd time point in Co-mo BaP + FL), but binding of hedgehog to Ptch causes relief of this inhibition, allowing Smo to signal to downstream components of the pathway (Ingham et al., 2000). Upregulation of Smo may result in increased hedgehog signaling and improper development.

Most of the identified gene expression changes between Co-mo BaP + FL and AHR2-mo BaP + FL occurred at 18 hpd. The gene expression differences were due to upregulation in the Co-mo BaP + FL that was different from both AHR2-mo BaP + FL and Co-mo DMSO. There were no significant differences between AHR2-mo BaP + FL and Co-mo DMSO. Thus, none of the differentially expressed genes seem to be regulated in an AHR-independent manner. This finding was somewhat unexpected as
other studies using TCDD have shown that it upregulates certain genes in an AHR-dependent as well as AHR-independent manner. Comparing gene expression changes caused by TCDD exposure in vivo in AHR null and wild-type AHR mice adult liver, Tijet et al. (2006) found that TCDD altered 456 probesets in wild-type AHR mice while only 32 probesets were altered in AHR null mice. In cultured aortic smooth muscle cells (SMCs) from AHR null mice, TCDD was shown to increase expression of TGF-β2, CYP1B1, and other TGF-β-related genes (Guo et al., 2004). So while it appears that TCDD induces most gene expression changes through the AHR, it does influence gene expression in organisms and cultured cells lacking the AHR.

Much of the information known about AHR signaling in zebrafish has come from TCDD studies. Though there are many similarities in the effects and mechanisms of PAHs and TCDD, there are stark differences between the two. Many PAHs (such as BaP) are oxidized via CYP1 enzymes, make good substrates for phase II metabolism, and are readily excreted. On the other hand, TCDD is not readily oxidized by CYPs nor is it a good phase II substrate due to its high degree of halogenation, and it has a half-life of 7-9 years in humans (Geyer et al., 1993). Thus, the slow rate of detoxification and elimination of TCDD is thought to contribute to its toxicity, while the metabolism of PAHs into reactive electrophiles capable of interacting with macromolecules or redox cycling is considered an important mechanism of their toxicity. Comparing the gene expression changes caused by TCDD and BaP in mice aortic SMCs, Karyala et al. (2004)
determined that the two compounds caused vastly different gene expression profiles aside from certain genes, such as CYP1B1, known to be part of the AHR pathway. Additionally, as mentioned above, TGF-β2 has been shown to be upregulated in response to TCDD in AHR null mice but TGF-β1 and ‘similar to TGF-β2 receptor’ were both upregulated approximately two-fold in Co-mo BaP + FL in our experiment.

The upregulated genes in Co-mo BaP + FL also encompassed numerous genes known or suggested to be involved in heart and vascular function and morphology. Ddr1 (discoidin domain receptor family, member 1) is involved in collagen formation, chemotaxis, proliferation and MMP production in smooth muscle cells, and it was upregulated approximately 43-fold in Co-mo BaP + FL compared to AHR2-mo BaP + FL. It was also found to be part of a network of genes upregulated in peripheral blood mononuclear cells from a dioxin-exposed human population in Italy (McHale et al., 2007). And as another indication that PAHs may interfere with normal TGF-β signaling, VSN (vasorin), which is expressed in rat vascular smooth muscle cells and has been shown to bind to TGF-β1 and attenuate its signaling in vitro, was also induced in Co-mo BaP + FL. VSN was found to be downregulated during vessel repair after arterial injury in rat VSMCs and rescue of VSN attenuated injury, indicating that it is involved in vascular injury response and repair (Ikeda et al., 2004b). The contradicting expression patterns may be due to species differences, in vivo vs. in vitro study differences, or timing. Genes involved in TGF-β were also differentially expressed in the hearts of
zebrafish embryos exposed to TCDD (Carney et al., 2006). Upregulation of Npr1a (natriuretic peptide receptor 1a), the receptor for ANP (atrial natriuretic peptide - a vasodilator) occurred in Co-mo BaP + FL; this result is in accordance with TCDD results. Mice gestationally exposed to TCDD exhibited a three-fold upregulation of ANP compared to expression in those whose mothers were exposed to corn oil. This increased level of ANP expression persisted into adulthood, which was concomitant with increased ventricular size compared to controls (Aragon et al., 2008). It is notable that these genes discussed in detail, as well as many of the other 84 annotated genes that were upregulated by BaP + FL treatment in Co-mo embryos, do not currently have a direct association with AHR. Identification of XREs or antioxidant response elements (AREs) in these genes would provide a clear link between AHR and gene upregulation. Alternatively, a lack of XREs or AREs does not rule out interaction with AHR. The metabolism of PAHs into more reactive electrophiles could cause intermediate cell signaling events that in turn could upregulate some of the genes induced in this study.

The lack of any robust gene changes at the 6 and 12 hpd time point was unexpected. In an array study examining gene expression changes in response to TCDD in zebrafish jaws, 64, 53, 65, and 85 transcripts were changed at 1, 2, 4, and 12 hours post exposure, respectively, showing a trend in increased gene expression changes over time (Xiong et al., 2008). It is possible that the effects of the BaP + FL treatment were not apparent at the early time points because more time was necessary for the PAHs to be
metabolized and elicit their effects after dosing, unlike TCDD, which causes toxicity without being readily metabolized. When zebrafish embryos were dosed at 34 hpf with a combination of BNF and the CYP1 inhibitor α-naphthoflavone (ANF) and then washed and placed in untreated water at 60 hpf, they did not develop cardiac deformities, which become apparent by 72 hpf (Timme-Laragy, 2007). Our time points of heart extraction were 38, 42, 48, and 54 hpf. However, we would expect gene expression changes to precede the gross cardiac deformities caused by PAHs and for PAH metabolism to occur within the given timeframe of our study. In a study examining heart-specific gene expression changes caused by TCDD, Carney et al. (2006) identified expression changes 1 hour after exposure to TCDD, though embryos were dosed at a much later stage (72 hpf) than the present study. Our data set has little overlap with the heart-specific TCDD-induced gene changes reported by Carney et al. (2006). This difference could be due to time of dosing, time points of heart extraction, number of hearts used per treatment, and most notably, the compounds used. Also, Carney et al. (2006) found a largely different set of genes to be affected by TCDD treatment than another study examining the effects of TCDD on murine heart gene expression, showing that even when using the same compound, though a different species in this case, microarray results can be different (Thackaberry et al., 2005).

The fact that almost all of the gene expression differences identified were a result of upregulation in the Co-mo BaP + FL treatment suggests that the deformities caused by
this PAH mixture may be a result of inappropriate cell signaling events at the wrong times during development. Before more definitive conclusions can be drawn from this microarray experiment, further data analysis is necessary. Due to the large amount of data obtained from 128 microarrays completed across different morpholino treatments, PAH exposures, and time points, there are various avenues for further analysis. First of all, perhaps analysis with another software program or with different parameters would help to identify differential genes in the 6 and 12 hpd time points and more in the 2 or 18 hpd time points. Secondly, comparisons between the other treatments (e.g., Co-mo DMSO vs. Co-mo BaP or Co-mo BaP vs. AHR2-mo BaP) would be helpful in identifying the contribution of BaP and FL to the toxicity caused by co-exposure of the two. Thirdly, comparing gene expression across time points in Co-mo DMSO vs. AHR2-mo DMSO would elucidate genes regulated by the AHR during zebrafish development. Lastly, verification of the gene expression changes described herein by QPCR is necessary to validate the microarray results.
Figure 23: Effect of PAHs on pericardial effusion in zebrafish embryos. Non-injected (NI), control-morpholino-injected (Co-mo), and AHR2-morpholino-injected (AHR2-mo) cmlc2::GFP embryos were dosed with DMSO, 100 μg/L benzo[a]pyrene (BaP), 500 μg/L fluoranthene (FL), or 100 μg/L BaP + 500 μg/L FL at 24 hpf and scored at 96 hpf. (n = 12 per treatment; each n represents the average of five embryos). An asterisk (*) represents a significant difference from control (p ≤ 0.01; ANOVA, Tukey adjusted LSMeans).
Table 2: Co-mo BaP + FL vs. AHR2-mo BaP + FL gene expression changes in zebrafish hearts extracted 2 hours post dosing

<table>
<thead>
<tr>
<th>Gene Symbol</th>
<th>Gene Name</th>
<th>Fold change</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Slc43a3-like</td>
<td>Solute carrier family 43 member 3-like</td>
<td>3.9</td>
<td>0.01</td>
</tr>
<tr>
<td>Similar to CHRNA7</td>
<td>Novel protein similar to vertebrate cholinergic receptor, nicotinic, alpha polypeptide 7 (CHRNA7)</td>
<td>3.7</td>
<td>0.08</td>
</tr>
<tr>
<td>Smo</td>
<td>Smoothened</td>
<td>7.5</td>
<td>0.01</td>
</tr>
<tr>
<td>PFKL</td>
<td>phosphofructokinase</td>
<td>2.2</td>
<td>0.02</td>
</tr>
<tr>
<td>Sox9b</td>
<td>SRY (sex determining region Y)-box 9</td>
<td>2.6</td>
<td>0.05</td>
</tr>
</tbody>
</table>
Table 3: Differentially expressed genes between Co-mo BaP + FL and AHR2-mo BaP + FL: Genes known or hypothesized to be involved in heart or vascular structure and function

Genes differentially expressed at 18 hpd time point. All $p \leq 0.05$.

<table>
<thead>
<tr>
<th>Gene Symbol</th>
<th>Gene Name</th>
<th>Fold change</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ptch2</td>
<td>patched 2</td>
<td>558</td>
</tr>
<tr>
<td>ddr1</td>
<td>discoidin domain receptor family, member 1</td>
<td>43</td>
</tr>
<tr>
<td>npr1a</td>
<td>natriuretic peptide receptor 1a</td>
<td>11.8</td>
</tr>
<tr>
<td>aggf1</td>
<td>angiogenic factor with G patch and FHA domains 1</td>
<td>8.7</td>
</tr>
<tr>
<td>pkp2</td>
<td>plakophilin 2</td>
<td>6.0</td>
</tr>
<tr>
<td>socs3b</td>
<td>suppressor of cytokine signaling 3b</td>
<td>5.6</td>
</tr>
<tr>
<td>vasn</td>
<td>vasorin</td>
<td>5.0</td>
</tr>
<tr>
<td>efnb2a</td>
<td>ephrin B2a</td>
<td>4.4</td>
</tr>
<tr>
<td>apooa</td>
<td>apolipoprotein O, a</td>
<td>3.8</td>
</tr>
<tr>
<td>tbx3b</td>
<td>T-box 3b</td>
<td>2.4</td>
</tr>
</tbody>
</table>
6. Conclusion

6.1 Summary

In this dissertation I sought to expand our understanding of the mechanisms by which PAHs elicit developmental cardiac toxicity. One hypothesis of this work was that AHR2 mediates this toxicity by regulating or interacting with genes that are not currently part of the typical ‘AHR gene battery’. The second hypothesis was that the toxicity of PAHs is mediated by mechanisms independent of AHR2.

Previous work in our laboratory laid the groundwork for this research using the model PAHs BNF, an AHR agonist, and ANF, a CYP1 inhibitor. The data presented in Chapter 2 show that a combination of the AHR agonist BkF and the CYP1 inhibitor FL, two environmentally PAHs, causes cardiac deformities in developing zebrafish along with induction of CYP1 and redox-responsive gene expression. AHR2 knockdown prevented the cardiac deformities caused by the PAH mixture as well as CYP1 and potentially ARE-mediated gene expression. These data provide evidence that AHR2 play an important role in the toxicity of these PAHs and appears to be involved in the oxidative stress response of zebrafish to PAHs.

In Chapter 3, I examined the role of GSTp2 in mediating PAH- and PCB-126-induced toxicity. This work stemmed from findings of Chapter 2 and past studies in our lab showing that GSTp2 is induced after PAH exposure (Timme-Laragy et al., 2009). In addition to BkF and FL, the AHR agonists BaP and PCB-126 were used. GSTp2
exacerbated PAH toxicity but had no effect on PCB-126 toxicity, indicating that GSTp2 plays a protective role against PAH toxicity but not PCB-126 toxicity.

In Chapter 4, the role of AHR1 isoforms in mediating PAH- and PCB-126-induced toxicity was examined and compared to the role of AHR2. We were surprised to find that knockdown of AHR1A exacerbated toxicity of PAH mixtures and PCB-126 while AHR1B knockdown did not appear to affect the toxicity of any mixtures or PCB-126. Knockdown of AHR1A also increased CYP1 enzyme activity in response to PAHs and PCB-126 but did not affect CYP1 mRNA expression. These effects are in contrast to those of AHR2, the knockdown of which causes reduced CYP1 activity and mRNA expression, as shown in this chapter and Chapter 2. Co-knockdown of AHR1A and AHR2 did not exacerbate nor ameliorate PAH toxicity but did prevent PCB-126 toxicity. These data suggest that AHR1A and AHR2 may have opposite, or at the very least different, roles in mediating PAH and PCB-126 toxicity.

In Chapter 5, I used microarrays to identify novel AHR2-dependent and AHR2-independent genes in zebrafish hearts involved in PAH mixture toxicity at multiple time points after exposure. Various genes involved in TGF-β signaling, cell adhesion, and oxidation-reduction processes, as well as genes involved in heart and vascular function and morphology, were upregulated by PAHs. Furthermore, these data show that differential gene expression in response to PAHs is regulated by AHR2. However, many of the upregulated genes are not currently associated with AHR2 regulation,
perhaps suggesting intermediate or indirect signaling between the AHR and these genes.

6.2 Implications

6.2.1 Mechanisms of PAH toxicity

The results in this dissertation have implications for our understanding of the mechanisms by which PAHs exert toxicity in fish as well as in higher vertebrates. A great deal of the knowledge about downstream targets of the AHR, its role in mediating toxicity of certain xenobiotics, its potential role in normal developmental processes, and its cross-talk with other signaling pathways has come from extensive research using TCDD and DLCs, particularly coplanar PCBs (Fernandez-Salguero et al., 1996; Prasch et al., 2003; Walker et al., 1991; Yeager et al., 2009). Previous studies have shown that PAH and DLC mechanisms of toxicity vary. For example, CYP1A knockdown has been shown to exacerbate PAH toxicity (Billiard et al., 2006; Matson et al., 2008a), but it does not affect TCDD toxicity (Carney et al., 2004). Some of the results from this dissertation further highlight the similarities and differences in the mechanisms of PAH toxicity and DLC toxicity. For example, while PAH mixtures and PCB-126 both induced GSTp2 mRNA expression, GSTp2 knockdown exacerbated PAH but not PCB-126 toxicity (Chapter 3). It appears that this facet of phase II metabolism is protective against PAH but not PCB-126 toxicity. Knockdown of AHR1A exacerbated PAH mixture and PCB-
126 toxicity; however, co-knockdown of AHR1A with AHR2 did not affect PAH toxicity yet it prevented PCB-126 toxicity to the same extent as AHR2 knockdown (Chapter 4). While studies using DLCs can help inform those who carry out PAH toxicity research, it is important to recognize that the similar phenotypic effects caused by PAHs and DLCs in developing fish may be caused by different molecular mechanisms.

The fact that zebrafish often have two or more isoforms of genes of which mammals have only one offers both a challenge and opportunity for using zebrafish as model organisms and extrapolating these findings to higher vertebrates, including humans. For instance, zebrafish have three identified AHRs and mammals have one. Untreated fish injected with AHR1-mo did not appear to have any gross defects in our studies whereas AHR null mice exhibit multiple abnormalities including cardiomyopathy and gastric hyperplasia. The ability to study each zebrafish AHR isoform individually in apparently normal individuals may lead to new insights into how the singular AHR functions in mammals. The challenge comes in identifying which zebrafish gene(s) perform(s) functions similar to mammalian genes and examining if and how fish and mammals respond differently to PAH toxicity.

Additionally, microarray results have identified genes and pathways that may play important roles in mediating or ameliorating PAH-induced developmental toxicity; these genes are not part of the classic ‘AHR gene battery.’ These findings not only hold
opportunities for future research but also further suggest the complexity with which PAHs induce developmental toxicity, even in a tissue-specific manner.

6.2.2 Risk assessment

Current risk assessment models for PAH mixtures assume additivity and estimate risk based on each individual PAH’s AHR agonism strength in relation to TCDD; this number is known as a toxic equivalency factor (TEF) (Barron et al., 2004). This method of risk assessment is relatively accurate at estimating risk for DLCs such as PCBs. However, using this current method of risk assessment, a combination of BkF and FL or BaP and FL would not be predicted to be more toxic than BkF or BaP alone, respectively, because FL is not an AHR agonist. In addition to past work in our laboratory (Billiard et al., 2006; Wassenberg and Di Giulio, 2004), the experiments in this dissertation show that this assumption is incorrect and that this assessment method greatly underestimates the risk of PAH mixtures in the context of causing developmental toxicity in fish. Recognizing the disparity between current models and evidence of synergism between PAHs is particularly important because PAHs are present as complex mixtures in the environment.
6.3 Future directions

For every question answered in science, several new ones arise. To begin, previous work in our laboratory (Timme-Laragy et al., 2009) has indicated that PAH mixtures are capable of inducing redox-responsive gene expression and data from this dissertation (Chapter 2) show that this upregulation occurs in an AHR2-dependent manner. It is important to identify if these redox-responsive genes in fish contain XREs, AREs, or both to determine if AHR2 can directly bind to these genes or if AHR2 affects their expression through cross-talk with the Nrf2 pathway or some other pathway. Furthermore, we only examined gene expression at one time point (24 hours after dosing, 48 hpf). Examination of the expression of these genes at earlier and later time points would help to elucidate the transcriptional response to PAHs of various genes over time.

It would also be interesting to explore if the AHR1 isoforms have any capacity to regulate redox-responsive or phase II metabolic genes. Though AHR1A knockdown did not affect the expression of the CYP1 genes analyzed, it could interact with other genes. It is also possible that CYP1 expression might be affected by AHR1A at different time points. Our results showing that AHR1A knockdown exacerbated PAH- and PCB-126-induced toxicity were quite surprising; determining the effect of overexpression of AHR1A in embryos exposed to AHR agonists would help to further elucidate the role of AHR1A under normal and PAH-exposed conditions. To further explore the role of
AHR1B, use of another morpholino would be helpful in validating the results of our preliminary experiments in which AHR1B knockdown had no effect on PAH- or PCB-126-induced toxicity. Injection of another morpholino individually or even co-injection of our morpholino with another targeting AHR1B could result in a more complete knockdown, allowing identification of the role of AHR1B in PAH toxicity. Also, co-knockdown of AHR1B with AHR2 or with AHR1A could give further insight into the role of AHR1B. Moreover, simultaneous knockdown of all three zebrafish AHRs could potentially lead to deformities in untreated embryos, mimicking effects seen in AHR null mice and indicating that perhaps all three have some role in normal zebrafish development.

As mentioned in Chapter 5, the data from our microarray study afford virtually endless avenues of data analysis and future experiments. Comparisons across time points and at each time point for each treatment and each morpholino still need to be completed. Examining expression across time points would give insight into which genes respond earliest to PAHs and potentially cause downstream genes to turn on or off at later time points. From other reports of PAHs and DLCs affecting TGF-β signaling (Guo et al., 2004; Karyala et al., 2004) as well as the results of our study, further exploration into the interaction between the AHR pathway and TGF-β could elucidate novel mechanisms of PAH toxicity.
Lastly, the studies described in this dissertation rely heavily on pericardial effusion as a representative measurement for cardiac toxicity. An elongated heart resulting in the ‘stringy heart’ or ‘tube heart’ phenotype and bradycardia occurred along with the pericardial effusion but we have not quantified either in our laboratory. Additionally, examining the histopathology of whole zebrafish embryos could help to elucidate how an AHR agonist and a CYP1 inhibitor interact with one another to cause gross morphological deformities. At the histopathological level, small perturbations in normal development could potentially be seen in embryos exposed to individual PAHs, identifying if one PAH is exacerbating small effects caused by the other. Similarly, we measure CYP activity in ovo, and aside from the microarray experiment, we measured gene expression in whole embryos. Many other studies have shown that there are tissue-specific effects of AHR activation and CYP expression in response to low molecular weight PAHs and TCDD (Andreasen et al., 2002b; Incardona et al., 2006). Measuring heart size and length, cardiac output, and stroke volume, counting cardiomyocyte number, examining tissue-specific gene and protein expression, and examining the histopathology of exposed fish would give a more complete picture of the mechanisms by which PAHs induce cardiac toxicity in developing zebrafish. Extending these experiments into juvenile and adult zebrafish would also increase our knowledge regarding the implications of embryonic PAH exposure for later life stages.
Appendix– Annotated genes significantly differentially expressed at 18 h – Co-mo BaP + FL vs. AHR2-mo BaP + FL

Table 4: Annotated genes differentially expressed between Co-mo BaP + FL and AHR2-mo BaP + FL treatment

List of annotated genes differentially expressed in zebrafish hearts at 18 hpd (54 hpf). GO category and gene name given when available. Fold changes have been rounded off to nearest whole number, all p ≤ 0.05. All genes are upregulated in Co-mo BaP + FL treatment vs. AHR2-mo BaP + FL treatment except two highlighted in grey, gabbr1 and tlr1, which are downregulated.

<table>
<thead>
<tr>
<th>GO category and gene name given when available</th>
<th>Gene Symbol /ID</th>
<th>Gene Name</th>
<th>Fold change - Co-Combo vs. AHR2-mo-Combo</th>
</tr>
</thead>
<tbody>
<tr>
<td>GO:0007155 : cell adhesion hapln2</td>
<td>hyaluronan and proteoglycan link protein 2</td>
<td>1736</td>
<td></td>
</tr>
<tr>
<td>GO:0010002 : cardioblast differentiation Ptch2</td>
<td>patched 2</td>
<td>558</td>
<td></td>
</tr>
<tr>
<td>GO:0006811 : ion transport gb</td>
<td>BC092684</td>
<td>ATPase, H+ transporting, lysosomal V1 subunit B2</td>
<td>290</td>
</tr>
<tr>
<td>GO:0016337 cell-cell adhesion GO:0005509 : calcium ion binding cdh23</td>
<td>cadherin-23</td>
<td>163</td>
<td></td>
</tr>
<tr>
<td>GO:0005509 : calcium ion binding ldlr</td>
<td>low density lipoprotein receptor</td>
<td>71</td>
<td></td>
</tr>
<tr>
<td>GO:0008285 : negative regulation of cell proliferation ddr1</td>
<td>discoidin domain receptor family, member 1</td>
<td>43</td>
<td></td>
</tr>
<tr>
<td>GO:0004674 : protein ref</td>
<td>NC_007117.3</td>
<td>Lkb1 ortholog</td>
<td>39</td>
</tr>
<tr>
<td>GO:0035023 : regulation of Rho protein signal transduction</td>
<td>arhgef18</td>
<td>rho/rac guanine nucleotide exchange factor (GEF) 18 - 35</td>
<td></td>
</tr>
<tr>
<td>-----------------------------------------------</td>
<td>-------</td>
<td>--------------------------------------------------</td>
<td></td>
</tr>
<tr>
<td>GO:0008654 : phospholipid biosynthetic process</td>
<td>si:dkey-30h14.2</td>
<td>acyl-CoA:lysophosphatidyl glycerol acyltransferase 1 24</td>
<td></td>
</tr>
<tr>
<td>GO:0030097 : hemopoiesis</td>
<td>runx3</td>
<td>runt-related transcription factor 3 23</td>
<td></td>
</tr>
<tr>
<td>GO:0002009 : morphogenesis of an epithelium</td>
<td>ref</td>
<td>XM_689890.3</td>
<td>similar to carbonic anhydrase 9 21</td>
</tr>
<tr>
<td>GO:0004497 : monooxygenase activity</td>
<td>cyp2j30</td>
<td>cytochrome P450, family 2, subfamily J, polypeptide 27 18</td>
<td></td>
</tr>
<tr>
<td>GO:0007275 : multicellular organismal development</td>
<td>ref</td>
<td>NC_007127.3</td>
<td>POU5F1 ortholog 17</td>
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<tr>
<td>GO:0016491 : oxidoreductase activity</td>
<td>tyr</td>
<td>tyrosinase 16</td>
<td></td>
</tr>
<tr>
<td>GO:0006355 : regulation of transcription, DNA-dependent</td>
<td>klhl31</td>
<td>kelch-like 31 15</td>
<td></td>
</tr>
<tr>
<td>GO:0055114 : oxidation-reduction process</td>
<td>ndufv1</td>
<td>NADH dehydrogenase (ubiquinone) flavoprotein 1</td>
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</tr>
<tr>
<td>GO:0005575 : cellular_component</td>
<td>trpc4apb</td>
<td>transient receptor potential cation channel, subfamily C, member 4 associated protein b 13</td>
<td></td>
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<tr>
<td>GO:0043547 : positive regulation of GTPase activity</td>
<td>LOC100004299</td>
<td>chimerin (chimaerin) 1-like 13</td>
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</tr>
<tr>
<td>GO:0006355 : regulation of</td>
<td>roraa</td>
<td>RAR-related orphan 13</td>
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</tr>
<tr>
<td>ID</td>
<td>Description</td>
<td>Gene/Protein</td>
<td>Description</td>
</tr>
<tr>
<td>----</td>
<td>-----------------------------------------------------------------------------</td>
<td>----------------------------------------------</td>
<td>-----------------------------------------------------------------------------</td>
</tr>
<tr>
<td>GO:0004222</td>
<td>transcription, DNA-dependent receptor A, paralog a - ROR-α</td>
<td>pmpca</td>
<td>peptidase (mitochondrial processing) alpha</td>
</tr>
<tr>
<td>GO:0006182</td>
<td>cGMP biosynthetic process</td>
<td>si:dkey-27c15.1</td>
<td>npr1a natriuretic peptide receptor 1a</td>
</tr>
<tr>
<td>GO:0072019</td>
<td>proximal convoluted tubule development</td>
<td>ift88</td>
<td>intraflagellar transport 88 homolog</td>
</tr>
<tr>
<td>GO:0042074</td>
<td>cell migration involved in gastrulation</td>
<td>lbr</td>
<td>lamin B receptor</td>
</tr>
<tr>
<td>GO:0042612</td>
<td>MHC class I protein complex</td>
<td>mhc1uxa2</td>
<td>major histocompatibility complex class I UXA2 gene</td>
</tr>
<tr>
<td>GO:0016787</td>
<td>hydrolase activity</td>
<td>ppmh1</td>
<td>protein phosphatase, Mg2+/Mn2+ dependent, 1H</td>
</tr>
<tr>
<td>GO:0032588</td>
<td>trans-Golgi network membrane</td>
<td>scamp2</td>
<td>secretory carrier membrane protein 2</td>
</tr>
<tr>
<td>GO:0003723</td>
<td>RNA binding</td>
<td>rbm19</td>
<td>RNA binding motif protein 19</td>
</tr>
<tr>
<td>LOC559539</td>
<td>similar to very large inducible GTPase 1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>GO:0045766</td>
<td>positive regulation of angiogenesis</td>
<td>aggf1</td>
<td>angiogenic factor with G patch and FHA domains 1</td>
</tr>
<tr>
<td>GO:0005575</td>
<td>cellular_component</td>
<td>zgc:112091</td>
<td>grap2a - GRB2-related adaptor protein 2a</td>
</tr>
<tr>
<td>GO:0016021</td>
<td>integral to membrane</td>
<td>CFTR</td>
<td>Cystic Fibrosis transmembrane conductance regulator</td>
</tr>
<tr>
<td>GO:0004053</td>
<td>arginase activity</td>
<td>arg2</td>
<td>arginase, type II</td>
</tr>
<tr>
<td>GO:0000139</td>
<td>Golgi membrane</td>
<td>slc35b4</td>
<td>solute carrier family 35, member B4</td>
</tr>
<tr>
<td>GO:0004674</td>
<td>protein serine/threonine kinase activity</td>
<td>snrk</td>
<td>SNF related kinase 1</td>
</tr>
<tr>
<td>GO:0007507 : heart development GO:0016337 : cell-cell adhesion</td>
<td>pkp2</td>
<td>plakophilin 2</td>
<td>6</td>
</tr>
<tr>
<td>-------------------</td>
<td>------</td>
<td>--------------</td>
<td>---</td>
</tr>
<tr>
<td>GO:0016055 : Wnt receptor signaling pathway</td>
<td>aes</td>
<td>amino-terminal enhancer of split</td>
<td>6</td>
</tr>
<tr>
<td>GO:0035556 : intracellular signal transduction</td>
<td>socs3b</td>
<td>suppressor of cytokine signaling 3b</td>
<td>6</td>
</tr>
<tr>
<td>GO:0004497 : monoxygenase activity GO:0055114 : oxidation-reduction process</td>
<td>cyp26c1</td>
<td>cytochrome P450, family 26, subfamily C, polypeptide 1</td>
<td>5</td>
</tr>
<tr>
<td>GO:0043434 : response to peptide hormone stimulus</td>
<td>smtlb</td>
<td>somatolactin beta</td>
<td>5</td>
</tr>
<tr>
<td>GO:0005515 : protein binding</td>
<td>vasn</td>
<td>vasorin</td>
<td>5</td>
</tr>
<tr>
<td>GO:0043065 : positive regulation of apoptosis</td>
<td>bida</td>
<td>BH3 interacting domain death agonist</td>
<td>5</td>
</tr>
<tr>
<td>GO:0007155 : cell adhesion</td>
<td>ncam2</td>
<td>neural cell adhesion molecule 2</td>
<td>5</td>
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<tr>
<td>GO:0008484 : sulfuric ester hydrolase activity</td>
<td>arsa</td>
<td>arylsulfatase A</td>
<td>5</td>
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<tr>
<td>GO:0005789 : endoplasmic reticulum membrane</td>
<td>tram1</td>
<td>translocating chain-associating membrane protein 1</td>
<td>5</td>
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<td>GO:0035475 : angioblast cell migration involved in selective angioblast sprouting</td>
<td>efnb2a</td>
<td>ephrin B2a</td>
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</tr>
<tr>
<td>GO:0003735 : structural constituent of ribosome</td>
<td>mrpl48</td>
<td>mitochondrial ribosomal protein L48</td>
<td>4</td>
</tr>
<tr>
<td>GO:0016831 : carboxy-lyase activity</td>
<td>amd1</td>
<td>adenosylmethionine decarboxylase 1</td>
<td>4</td>
</tr>
<tr>
<td>GO:0016021 : integral to membrane</td>
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References


Robertson, I.G.C., Guthenberg, C., Mannervik, B., Jernström, B., 1986. Differences in Stereoselectivity and Catalytic Efficiency of Three Human Glutathione Transferases in the Conjugation of Glutathione with 7β,8α-Dihydroxy-9α,10α-oxy-7,8,9,10-tetrahydrobenzo(a)pyrene. Cancer Research 46, 2220-2224.


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Biography

Lindsey Anne Van Tiem was born in Royal Oak, MI, USA on November 23, 1983 to Paul and Cynthia Van Tiem. She received a Bachelor of Science degree, magna cum laude, in Biology with a minor in Chemistry from Aquinas College in May 2005.

Publications:


Honors and Awards:

James B. Duke Fellowship, Duke University, 2005-present

Jerome C. Byrne Full Tuition and Room Scholarship, Aquinas College, 2001-2005

Outstanding Senior Biology Student, Aquinas College, 2005

Outstanding Freshman Biology Student, Aquinas College, 2001