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AhR-related Activities in a Creosote-Adapted Population of Adult Atlantic Killifish, Fundulus heteroclitus, Two Decades Post-EPA Superfund Status

Josephine Wojdylo
Clemson University, jwojdyl@clemson.edu

AHR-RELATED ACTIVITIES IN A CREOSOTE-ADAPTED POPULATION OF ADULT ATLANTIC KILLIFISH, *FUNDULUS HETEROCLITUS*, TWO DECADES POST-EPA SUPERFUND STATUS

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by
Josephine Veronique Wojdylo
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Accepted by:
Charles D. Rice, PhD, Committee Chair
Lisa J. Bain, PhD
Peter Van den Hurk, PhD
Thomas E. Schwedler, PhD

ABSTRACT

Atlantic killifish, Fundulus heteroclitus, have adapted to, or at least developed a level of resistance to creosote-based polycyclic aromatic hydrocarbons (PAHs) found at the Atlantic Wood (AW) superfund site in the southern branch of the Elizabeth River, VA USA. Historically, 90+% of the mummichogs at this site have hepatic lesions of varying severity, and 30+% of these fish have tumors of various origin. Many xenobiotics, including select PAHs found at the Atlantic Wood site, are ligands for the aryl hydrocarbon receptor (AhR), which is a promiscuous ligand-dependent transcription factor involved in developmental regulation, environmental sensing, and induction of a suite of phase I, II, and III drug metabolizing enzymes. Subsequent to the discovery of the AW killifish population, investigators demonstrated that these fish were recalcitrant to AhR activation and CYP1A induction following exposure to model PAHs. Moreover, killifish embryos collected from the AW site are still resistant to the cardiac and craniofacial deformities typically associated with exposure to PAHs and sediments collected from the AW site. In this study, the issue of CYP1A inducibility in AW fish collected in situ was re-examined nearly 2 decades after first discovery, and shown that the gene expression of this sentinel biomarker of AhR activation was significantly higher in intestines in comparison to a reference population. The livers of AW mummichogs were examined by IHC to discover that CYP1A and AhR2 protein patterns reflect tissue damage from lesions, and/or intrinsic cellular physiology of tumors, rather than recalcitrant induction of CYP1A. Gene expression profiles also indicated that liver

COX-2 is elevated in livers of these fish. A monoclonal antibody was then generated against COX-2 (mAb CX53-1) and used to observe the possible role of COX-2 and inflammation in Atlantic Wood fish liver lesions. COX-2 protein expression was very high in macrophage aggregates and surrounding tumors, suggesting chronic inflammation. Further characterization of the gut innate immune system focusing on eosinophilic cell infiltration and lysozyme levels demonstrated higher activity. Overall, these findings suggest that AW fish are not necessarily recalcitrant to CYP1A induction, and thus seem to have active functional AhR2 protein(s) in non-damaged tissues. These fish are also under chronic inflammatory stress.

DEDICATION

I dedicate this work to my family and all my loved ones that are no longer here with me, including, but not limited to, Jozefa and Bronislaw Wozniak, Micheline Cizove, Julianna Kolodynska, Jurek Wojdylo, Vixey, Skrat, Bear, Ash, Cinnabun, Wildling, and Constantine. I could not have done any of this without their love and support.

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REVIEW OF LITERATURE

Introduction to *Fundulus heteroclitus*

As human populations grow and urban sprawl and pollution continues, surrounding natural environments are being exposed to xenobiotics of anthropogenic origin such as PCBs (polycyclic biphenyls), PAHs (polycyclic or polynuclear aromatic hydrocarbons), metals and other pollutants. PAHs can result from incomplete combustion of organic material that can be found naturally in the environment such as volcanoes and forest fires or from anthropogenic sources such as burning of fossil fuels and other industrial sources. Oftentimes, the majority of them are of anthropogenic origin which is creating major concerns for neighboring biotic populations such as harbor estuaries (Menzie, et al. 1992).

One sentinel species occupying these harbor estuaries as well as saltwater marshes and tidal creeks along the east coast of North America is a teleost fish known as *Fundulus heteroclitus,* more commonly referred to as the mummichog or Atlantic killifish (Kneib 1986, Teo and Able 2003). Mummichogs are quite ubiquitous and can be found from Newfoundland, Canada to as far south as northern Florida (Bigelow and Schroeder 1953, Hardy 1978). These euryhaline fish are also very hardy and can occupy

bodies of water that have ranges in salinity from freshwater, 0 parts per thousand (ppt), to salinities of upwards to 120.3ppt (Abraham 1985, Griffth 1974). These fish have a high home range fidelity throughout their entire life cycle and can be used as a sentinel species to monitor the health of an ecosystem (Lotrich 1975). Due to their limited migration (Duvernell, et al. 2008) along with the mummichog's high reproduction rate and gene plasticity, these fish have adapted to their surroundings and are abundant in both pristine as well as polluted environments (Kelly, et al. 2012, Nacci, et al. 2010, Nacci, et al. 1999).

Fundulus heteroclitus as research animals

Mummichogs can spawn readily in the wild or in captivity by lunar cues during high tide during each new and full moon from spring till fall. Since these fish are sexually dimorphic, males can easily be distinguished by their horizontal stripes and colorful spots compared to females, which lack them in comparison (Hardy 1978). Female mummichogs are oviparous egg layers capable of laying several hundred eggs. These eggs are transparent and well documented throughout embryonic development (Bigelow and Schroeder 1953, Overstreet, et al. 2000), which are key features for developmental studies. While the zebrafish is a very popular model for development, it is not native to polluted environments and does not have the key ecological position of the killifish. In addition, zebrafish are a freshwater species and do not drink water. Killifish are marine fish, and thus drink water and serve as a potential model for intestinal contaminants from the water column. The killifish model has been used extensively in biomedical research and toxicological research and a large literature base is thus available (Armknecht, et al.

1998b, Frederick, et al. 2007, Munns, et al. 1997, Nacci, et al. 2010, Nacci, et al. 1999, van den Hurk, et al. 1998, Vogelbein, et al. 1990, Vogelbein, et al. 1999, Wassenberg, et al. 2002, Wassenberg and Di Giulio 2004, Weis 2002). Furthermore, the genome of *Fundulus heteroclitus* is currently being sequenced, where 100x sequence coverage is complete, contig assembly is also complete, and the scaffold assembly is currently in progress (Andrew Whitehead, personal communication).

Fundulus heteroclitus inhabiting superfund sites

There have been several documented subpopulations of mummichogs that have adapted or developed a resistance to environments with PAHs Atlantic Wood on the Elizabeth River, VA (Frederick, et al. 2007), PCBs in New Bedford Harbor (NBH), MA (Arzuaga, et al. 2004, Bello, et al. 2001), polychlorinated dibenzodioxins (PCDDs) such as 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) in Newark Bay, New Jersey (Prince and Cooper 1995), halogenated aromatic hydrocarbons (HAHs) and PAHs (Arzuaga and Elskus 2002) as well as a multitude other pollutants. A common factor that has been discovered in these subpopulations is a recalcitrant CYP1A induction phenotype (Arzuaga and Elskus 2002, Arzuaga, et al. 2004, Bello, et al. 2001, Frederick, et al. 2007).

Atlantic wood is a superfund site located in the southern branch of the Elizabeth river in Portsmouth, Virginia. Having reported concentrations of PAHs that reached upwards of 2200mg/kg of dry weight of sediment (Bieri, et al. 1986), Atlantic wood is one of the most heavily polluted superfund sites in the world (Walker, et al. 2004). From

1926 to 1992 it was a wood treatment facility, producing creosote, a toxic distillate of coal tar that contains up to 85% PAHs that include immunotoxic and carcinogenic PAHs, such as benzo[a]pyrene (BaP), chrysene, and dibenzo[a,h]anthracene (Jung, et al. 2011). Despite these conditions, there is a population of mummichogs that appear to be thriving. However, upon closer examination in 1990 by Volgelbein et al., it has been discovered that 93% of the mummichogs in this site have hepatic lesions and 33% of these fish had hepatocellular carcinomas. In 1995, Van Veld and Westbrook published a paper that has shown that adult killifish from this site are resistant to PAH mediated induction of Cyp1a. Other observations in this population were the increased expression of P-glycoproteins, manganese superoxide dismutase (MnSOD) and glutathione concentrations (Armknecht, et al. 1998a, Cooper 1999, COOPER 1999, Meyer, et al. 2003).

One theory that developed from observing recalcitrant CYP1A phenotypes in a heavily polluted site is that it offers a level of protection against generating potential metabolites that could be more toxic and detrimental than the existing parent compounds. Mummichog embryos from Atlantic Wood do not express toxic and teratogenic effects such as pericardial edema and heart deformations. However, a study in 2004 demonstrated that CYP1A inhibition in a reference embryo population (Kings Creek, VA) exposed to sediment extracts from Atlantic wood was not sufficient to suppress all toxicities (Wassenberg and Di Giulio 2004), suggesting that the mechanism behind this population phenotype is far more complex and is not limited to phase I enzymes (Wassenberg and Di Giulio 2004, Wills, et al. 2009).

Introduction to the aryl hydrocarbon receptor (AhR)

Many xenobiotics, including some found in polluted environments, such as PAHs, can act as ligands for the aryl hydrocarbon receptor, AhR, which is a promiscuous ligand-dependent transcription factor that is highly conserved in vertebrates and invertebrates (Hahn, et al. 2006a). It belongs to a multigene family of transcription factors involved in developmental regulation and environmental sensing. AhR belongs to a family of proteins that have a signature PAS domain; the domain is named after the letter of the first three founding proteins in the family, Per (period), ARNT(aryl hydrocarbon receptor nuclear transporter), and Sim (single-minded), respectively. The PAS domain is responsible for allowing AhR to form either a homotypic interaction between another PAS protein or a heterotypic interaction that involves the interaction with a chaperone protein or ligand. AhR also contains a second domain that consists of basic helix-loop-helix (bHLH) motifs that immediately follow the N-terminal of the PAS domain. Within the helix-loop-helix domain, there is homotypic interaction between a pair of bHLH motifs that forms a basic dimerization region which allows proteins to bind to the regulatory elements within the DNA (reviewed by (Gu, et al. 2000, Kewley, et al. 2004)).

In its inactivated or latent state, AhR is located in the cytoplasm where it is stabilized by chaperone proteins that involve two 90 kDa molecular chaperone heat shock proteins (Hsp90), one p23 protein and one hepatitis B virus X-associated protein (also known as XAP2/AIP/Ara9) (reviewed by (Denison, et al. 2011, Gu, et al. 2000, Kewley, et al. 2004).

Once AhR binds to its ligand, a conformational change occurs for AhR that allows a NLS, nuclear localization signal, to be exposed which allows it to translocate into the nucleus and dissociate from its chaperone proteins. Once AhR is located in the nucleus, it heterodimerizes with a ubiquitous class II bHLH/PAS protein, known as the aryl hydrocarbon receptor nuclear translocator, ARNT. The ligand-heterodimer complex then binds to a specific sequence of nuclear DNA motifs termed xenobiotic response elements (XREs) or dioxin-like response elements (DREs), which are located upstream of ligand-responsive genes (Kewley, et al. 2004). The consensus sequence for these elements is 5'- (T/G)NGCGTG-3' (Whitlock Jr 1999, ZeRuth and Pollenz 2007).

The binding of these XREs promotes the transcription of a multitude of genes, many of which are involved in phase I and II xenobiotic metabolism such as cytochrome P450 enzymes like CYP1A, CYP1B, CYP1C, as well as phase II enzymes like glutathione-S-transferase (GST), glucuronosyl transferase which are involved in detoxification of xenobiotics. Other genes include phase III transporters such as multidrug resistance protein (MDR or P-glycoprotein), as well as many others that may be involved in cell growth, metabolism and cellular differentiation (DeGroot, et al. 2011, Hahn, et al. 2006a). AhR activation can also cause transcription for the aryl hydrocarbon receptor repressor, AhRR, which acts as a negative regulatory loop that down regulates the induction of AhR by sequestering ARNT and blocking XRE sequences (Denison and Nagy 2003, Denison, et al. 2011, Kewley, et al. 2004, Mimura, et al. 1999, Mimura and Fujii-Kuriyama 2003, Vondracek, et al. 2011, Xu, et al. 2005).

While the purpose of xenobiotic metabolism is to make the toxicant more soluble in water to promote its excretion, it can also lead to the production of metabolites that can be more toxic than the parent compound (Nebert, et al. 2004). For instance, the monooxygenase enzyme Cyp1a, which is considered to be the hallmark of AhR activation (Aluru, et al. 2011), has the potential to aid in the detoxification of xenobiotics or generating more toxic metabolites which can damage the cell and lead to further toxicity. Exposure to certain ligands of the AhR such as PAHs, PCBs, and dioxins can cause detrimental effects such as cardiac edema, cardiac deformities (looping, malformed heart valve), immunosuppression (Bizzarri, et al. 2011, Clark, et al. 2010, Frederick, et al. 2007), wasting syndrome, neurotoxicity, and carcinogenesis (Fan, et al. 2010, Poland and Knutson 1982, Williamson, et al. 2005).

Ligands of AhR

AhR has a large and promiscuous range of ligands that can bind to its ligand binding pocket. How these ligands interact with this domain will dictate differences in AhR-dependent gene expression. There are two main categories of AhR ligands, classical AhR ligands and non-classical AhR ligands. Classical AhR ligands are characterized as hydrophobic compounds that are planar and aromatic properties. A classic example of such ligand is TCDD (2, 3, 7, 8 tetrachlorodibenzo-p-dioxin, or dioxin) which has a high affinity towards AhR (Brown, et al. 2002, DeGroot, et al. 2011, Denison, et al. 2011).

More recently, potential endogenous AhR ligands have been discovered such as indole-containing compounds, sterols, tryptophan photoproducts, flavonoids, carotenoids, to name a few. These ligands are referred to as non-classical AhR ligands due to structural and physicochemical characteristics that differ from classic dioxin related compounds. These compounds have a relatively low affinity towards AhR and are metabolically labile. Many are moderate inducers of AhR and are thought to play normal physiological roles for AhR (DeGroot, et al. 2011, Denison and Nagy 2003, Denison, et al. 2011, Mitchell and Elferink 2009) such as regulating hematopoiesis (Lindsey and Papoutsakis 2013), liver and vascular development, as well as immune function (Stevens, et al. 2009).

The most common and highest affinity ligands of the AhR are exogenous compounds, which are either produced anthropogenically or non-biologically in the environment by means such as the combustion of fossil fuels or petroleum products (Shur-Hueih Cherng, et al. 1996). Some of the classical AhR ligands include halogenated aromatic hydrocarbons, HAHs, such as dioxin, polyaromatic hydrocarbons, PAHs, such as benzo(a)pyrene and 3-methylcholanthrene, and polychlorinated biphenyls such as PCB-126 ((DeGroot, et al. 2011) reviewed by (Hahn 1998)).

Different types of ligands will have different binding affinities for AhR. Classical AhR ligands typically have a high affinity for AhR compared to non-classical AhR ligands. Halogenated aromatic hydrocarbons have relativity high binding affinities in the range of picomolar to nanomolar and generally produce toxic effects, such as

immunotoxicity, hepatotoxicity, cardiotoxicity, reproductive toxicity, dermal toxicity, teratogenesis, endocrine disruption, lethality, wasting syndrome, carcinogenesis, diabetes, and porphyria ((DeGroot, et al. 2011) reviewed by (Denison, et al. 2011)). On the other hand, PAHs have affinities for AhR that range from nanomolar to micromolar and while they are able to have biological effects such as altered gene expression, they do not typically express the same magnitude in toxicity as higher affinity, dioxin-like compounds. It is thought that these differences in toxicity are related in part to the metabolic stability of the compound. Ligands that are metabolically stable and are not readily broken down, such as HAHs, persistently activate or repress the expression of key responsive genes of the AhR pathway which can in turn produce highly toxic effects. Comparatively, ligands that are metabolically labile and are exposed to the AhR pathway transiently can have a shorter response time in induction that often leads to less toxic effects (Denison, et al. 2011).

AhR1 and AhR2 in fish

While there is only one mammalian AhR, which mediates toxic cellular responses to environmental contaminants, studies on teleostean fishes have found two aryl hydrocarbon receptors: AhR1 and AhR2 (Abnet, et al. 1999, Hahn, et al. 1997, Merson, et al. 2006, Roy and Wirgin 1997), with up to six AhR paralogs discovered the Atlantic salmon (*Salmo salar*) (Merson, et al. 2006). In *Fundulus heteroclitus*, there have been two highly divergent AhRs identified, fhAhR1 and fhAhR2 (Hahn, et al. 2006b, Karchner, et al. 1999a, Merson, et al. 2006). It has been speculated that gene duplication

events in vertebrate evolution lead to multiple AhR genes. Both AhR1 and AhR2 in *Fundulus* share similar mechanistic properties of mammalian AhR in that they share co-factors (e.g., ARNT, AhR Repressor) in the AhR induction and regulation pathways (Hahn 2002a).

It has been shown in mice that having even slight differences in amino acids in mouse AhR strains can impact their affinity to different ligands and affect toxicity (Hahn 2002b). The most significant AhR polymorphisms in terms of its functionality have been observed at the 375 position that is in the ligand binding domain (reviewed by(Okey, et al. 2005). Studies using C57BL/6 mice, which have polymorphism that encodes for alanine at the AhR codon 375, showed that they had a 15-fold higher affinity for β-naphthoflavone (BNF) (Maier, et al. 1998) and a 10-fold higher affinity for TCDD (Okey, et al. 1989) compared to DBA/2 AHR mice which have a polymorphism that encodes for valine at the 375 position of AhR (Okey, et al. 2005).

Not only can the level of expression of AhR vary in different organs, tissues, and cell types but it can also vary depending upon the developmental stages of animals (review by (Hahn 1998)). In mammals, such as rats, AhR is predominately found and expressed in the lungs, liver, thymus, kidney, placenta and to a lesser extent can be present in the spleen, heart, brain, muscle, pancreas, and gonads (Kewley, et al. 2004). This pattern of ubiquitous expression is thought to be expressed in all mammals (reviewed by (Denison and Nagy 2003, Hahn 1998, Kewley, et al. 2004). In fish, the level of expression and organ location varies between AhR1 and AhR2. AhR2 is

predominant and is the most widely and highly expressed aryl hydrocarbon receptor in teleost fish including *Fundulus*. In gene knock-down study, using morpholino-modified oligonucleotides (MO) that have been used in AhR2 MO zebrafish, it was determined that AhR2 helps mediate several toxic effects of TCDD (Prasch, et al. 2003).

AhR2 is expressed in most tissues in Fundulus, whereas, AhR1 is most often expressed in the heart, brain, and gonads (Karchner, et al. 1999b, Merson, et al. 2006). Analyzing the varying degrees of AhR expression can be vital in determining the effects of different ligands and possibly predicting which organs may be more sensitive or less sensitive to certain ligands (reviewed by (Hahn 1998)). Studies performed on killifish from a PCB and dioxin contaminated superfund site known as New Bedford Harbor, Massachusetts, have shown that while there are no differences promoter methylation of AhR1 and AhR2 between mummichog populations, there were differences in methylation between the receptors in the livers suggests that tissue specific expression of AhR1 and AhR2 may be controlled by methylation of AhR promoter regions. AhR1 had hypermethylation in its promoter region which correlated with low AhR1 transcripts in the livers of both populations. AhR2, on the other hand, was hypomethylated in its promoter region and correlated with a high AhR2 mRNA. (Aluru, et al. 2011).

Cross-talk in the AhR pathway

It is thought that by having a recalcitrant cyp1a phenotype in a heavily polluted site, it offers a level of protection against generating potential metabolites that could be more toxic and detrimental than the existing parent compounds. Mummichog embryos

from Atlantic Wood do not express teratogenic effects such as pericardial edema and heart deformations, and it is thought that being recalcitrant to cyp1a induction offers this protection from potential teratogenic effects. However, a 2004 study performed by Di Giulio and Wassenberg demonstrated that cyp1a inhibition in a reference embryo population (Kings Creek, VA) exposed to sediment extracts from Atlantic wood was not sufficient to suppress all toxicities, suggesting that the mechanism behind this population phenotype is far more complex and is not limited to phase I enzymes and may also be due to the complex cross-talk that the AhR pathways has with other pathways (Wassenberg and Giulio 2004, Wills, et al. 2009).

AhR can crosstalk with a transcription factor known as nuclear factor erythroid 2-related factor (Nrf2) that is a member of the Cap'n' collar family of transcription factors involved in antioxidant and phase II enzyme gene regulation in response to oxidative stress (Denison and Nagy 2003). Firstly, if AhR is activated, it can bind to a XRE of one of its many target genes that includes Nrf2. Secondly, under non-stress conditions Nrf2 is found in the cytoplasm bound by a repressor protein called kelch-like ECH-associated protein 1 (Keap1) where it can be targeted for degradation (Harbeitner, et al. 2013). In the presence of reactive oxygen species or other stressors such as electrophiles (some of these stressors can be due to AhR agonists that are poorly metabolizing by P450 enzymes), Nrf2 dissociates from its repressor protein and translocates into the nucleus where it can bind with a protein called Maf. The Nrf2-Maf complex can then bind to antioxidant response elements (AREs) that are located in the promoter region of target genes such as phase II enzymes (such as glutathione S-transferase (GST) and

NADPH:quinone oxidoreductase (NQO1)) as well as AhR (Harbeitner, et al. 2013, Muller, et al. 2010). Another thing worth noting is that some of the target genes of AhR are also target genes of Nrf2 as they have XREs and AREs at their promoter regions (some examples of genes are glutathione-S-transferase alpha, superoxide dismutase 1 (Sod1), NAD(P)H dehydrogenase, quinone 1 (Nqo1) to name a few) (Nguyen, et al. 2003).

Other AhR crosstalk can involve other pathways such hormone receptor pathways. Among those best understood is the estrogen receptor (ER) pathway. One way the two pathways can crosstalk is during AhR activation, where the CYP1A1/1B1 enzymes can use estrogen as their substrate and catabolize it, which in turn down regulates estrogen levels and estrogen response. Another way AhR can interfere with the ER pathway is the ARNT:AHR complex can bind to its XRE, xenobiotic response elements, and block the ability of that gene to be activated by ER. Another method of interference is by ARNT, which can bind to ER and enhance its transcriptional activity (or decrease it if AhR is activated and directly competes for ARNT) (Denison, et al. 2011).

Besides ER and the numerous ways that AhR can crosstalk with that pathway, another well-known interaction of AhR can be seen with the hypoxia inducible factor (HIF-1α) pathway. HIF-1α is a transcription factor that regulates a cassette of genes that are involved in adaption to low oxygen availability. HIF-1α uses ARNT (also known as

HIF-1β) as its dimerization partner and during hypoxic conditions, and competes directly with AhR this way, affecting its expression, including CYP1A (Denison, et al. 2011).

Inflammation and carcinogenesis in Atlantic Wood killifish

A previous study by Frederick and Rice in 2007 has shown that mummichogs from Atlantic wood seem to have a dampened humoral immune system while the innate immune system is primed. These fish seem to express chronic inflammation through elevated levels of lysozyme and cox-2.

One common pathway that is associated with inflammation is the NF-κB pathway. This transcription factor plays a major role in development, apoptosis, proliferation, immunity, and inflammation. In the mammalian system, NF-κB consists of five members (NF-κB1 (p50), NF-κB2 (p52), RelA (p65), RelB, and c-Rel) that exist as either homo- or heterodimers that are bound in the cytoplasm by inhibitory factor (IKβ). When stimulated by a pro-inflammatory signals such as cytokine tumor necrosis factor α (TNFα), IL-1β, binding of lipopolysaccharide (LPS) to toll-like receptors (TLR4), carcinogens (cigarette smoke, for example), and tumor promoters, IKβ is phosphorylated and ultimately degraded, allowing NF-κB to translocate into the nucleus and promote the transcription of a multitude of genes such as inflammatory enzymes cyclooxygenase 2 (cox-2), type II nitric oxide synthase (iNOS), TNFα, vascular endothelial growth factor (VEGF). NF-κB has been shown to be constitutively active in most tumors and is

thought to be the link between cancer and inflammation (Aggarwal, et al. 2006a, Hayden and Ghosh 2004, Tian 2009).

In the last few decades, studies are showing that there is a relationship between disease and the immune system. Inflammation is a localized response either to tissue damage, irritation, or invading pathogen that is characterized by redness, swelling, pain, fever, or loss of function. Acute inflammation is a defense response to protect the host and involves wound healing and/or clearance of the invading pathogen. If this process goes awry, which may happen if the macrophages become chronically stimulated or cannot find and clear the pathogen, chronic inflammation may occur which has been shown to contribute to the pathophysiology and development of many diseases such as heart disease, irritable bowel diseases, diabetes, many cancers, arthritis, among many others (Aggarwal, et al. 2006b, Mantovani, et al. 2008). The Atlantic wood population has significantly elevated levels of COX-2 (cyclooxygenase 2) which is indicative of an inflammatory state (Frederick, et al. 2007). Over 50% of Atlantic Wood mummichogs have hepatic lesions, but to date these tissues have not been examined for indices of inflammation.

There are two isoforms of the cyclooxygenase enzyme, Cox-1 and Cox-2. Cox-2 is the inducible form of prostaglandin G/H synthase, while Cox-1 is constitutively active. Cox-2 can be induced by cytokines, mitogens, tumor necrosis factor (TNF-α), lipopolysaccharide (LPS), interleukin (IL)-1β and tumor promoters. Cox-2 has a dual activity that involves a cyclooxygenase activity that converts arachidonic acid to

prostaglandin (PG)G2 and a peroxidase activity that will convert(PG)G2 to PGH2, which is a prostanoid precursor. Cox-2 is expressed in macrophages and has shown some ability to metabolize some PAHs like to diol-epoxide intermediates through bystander xenobiotic metabolism where there is a xenobiotic nearby with the right configuration for Cox-2 cyclooxygenase activity to insert an oxygen (Amano, et al. 2003, Frederick, et al. 2007, Parkinson 2001). Cox-2 has also been shown to increase the expression of p-glycoprotein (COOPER 1999) which has been shown in AW mummichogs which have recently been found to express high levels of Cox-2 (Frederick, et al. 2007).

COX-2 has been shown to be over expressed in many types of cancers such as pancreatic (Yoshida, et al. 2005), colorectal (Gupta and DuBois 2001), liver (Bae, et al. 2001), lung (Khuri, et al. 2001, Sandler and Dubinett 2004), breast (Zhao, et al. 2008) and ovarian. Its expression is highly associated with the prognosis of the cancer; high expression usually corresponds to poor prognosis (Aggarwal, et al. 2006a, Khuri, et al. 2001, Nasi and Castiglione 2002). COX-2 inhibitors, such as non-steroidal anti-inflammatory drugs (NSAIDS) have been shown to inhibit the progression of many cancers, possibly suggesting that COX-2 may have a role in tumor formation and progression (Mantovani, et al. 2008). COX-2 has a promoter region that can be recognized by the AhR (Degner, et al. 2009) and inhibitors of AhR activation such have also been shown to inhibit Cox-2 expression.

The relationship between NF-kB and AhR pathways is complicated. There have been findings that there is cross talk between them. Both share and potentially compete

for the same coactivators, SRC-1 (steroid receptor coactivator) and p300/CPB. NF-κB (RelA) and AhR interaction has demonstrated findings that were contradictory, some studies showing inhibition and others activation. It has been demonstrated that inflammatory cytokines, LPS, and TNFα can suppress the expression of CYP1A1 which is mediated through the interaction of AhR and NF-κB, suggesting an inhibitory interaction between these pathways (Ke, et al. 2001, Tian, et al. 1999, Tian, et al. 2002, Tian 2009). In other studies, there have been suggestions of cooperative role between the two pathways through a novel finding in which AhR/RelA-containing NFκB element binding complex was identified (Kim, et al. 2000).

Do fish have an inflammatory response?

Polarized T-helper cells, such as Th1 involved in inflammation and Th2 involved in immunosuppression, have been described in mammals but they have not been characterized in fish. Immune cells such as B cells, that have only been found to secrete IgM, T cells, neutrophils, macrophages, and eosinophilic granular cells (EGCs, thought to be the mast cell equivalents) have been described in fish. EGCs morphologically resemble mast cells with the basophilic components (which show metachromasia, characteristic of mast cells) easily being washed out leaving behind the acidophilic red granules, hence the name. EGCs in teleosts produce chemical effector agents that are involved in the inflammatory reaction. Studies in salmonids have shown the EGCs recruit toward the site where persistent inflammatory reactions are occurring (Reite and Evensen 2006). In higher teleosts, melano-macrophage centers containing aggregates of

lymphocytes and macrophages, thought to be analogous to germinal centers of lymph nodes, have also been discovered. These aggregates are found primarily in haemopoietic tissues such as the head kidney and spleen. They have also been found in the liver and can develop elsewhere in the body in lesions due to chronic inflammation. The size and amount of these aggregates can vary within a species of fish due to age, stress, pathogenic and inflammatory conditions and thus have been suggested a biomarker for environmental stress (Agius and Roberts 2003).

Overview and Specific Aims of My Dissertation Research

Atlantic killifish, Fundulus heteroclitus, have adapted to, or at least are resistant to creosote-based polycyclic aromatic hydrocarbons (PAHs) found at the Atlantic Wood (AW) superfund site in the southern branch of the Elizabeth River, VA USA. As far back as the early 1990s, it was documented that 93% of the mummichogs at this site hade hepatic lesions of varying severity, and 33% of these fish had hepatocellular carcinomas. Many xenobiotics, including select PAHs found at the Atlantic Wood site, are ligands for the aryl hydrocarbon receptor (AhR), which is a promiscuous ligand-dependent transcription factor involved in developmental regulation, environmental sensing, and induction of a suite of phase I, II, and III drug metabolizing enzymes. Subsequent to the discovery of the AW killifish population, investigators demonstrated that these fish were refractory to CYP1A induction following exposure to model PAHs. Moreover, killifish embryos collected from the AW site are still resistant to the cardiac and craniofacial

deformities typically associated with exposure to PAHs and sediments collected from the AW site.

The purpose of this dissertation is to first develop and characterize new monoclonal antibodies against Ahr-1, AhR-R, and COX-2 to then use in a larger field study. In the field study, I will re-examine the issue of CYP1A expression in AW fish exposed and collected in situ, to characterize AhR expression profiles in these fish, and to explore the possible role of COX-2 and inflammation in Atlantic Wood fish liver lesions. An additional purpose of this research is to further expand an in-hand tool box of antibodies for examining protein expression in AW fish compared to reference fish. The major hypothesis to be tested is that adult killifish from the AW site are not refractory to CYP1A induction by PAHs in situ, and that the intestines are the major anatomical location for CYP1A expression. A secondary hypothesis to be tested is that liver lesions make most of the liver unfit for normal AHR and CYP1A expression and function.

Specific Aims:

A. **Characterize gene expression profiles of killifish livers, intestines, and lymphoid organs from Atlantic Wood (AW) Superfund Site, VA and King's Creek (KC), VA reference site.** Livers, intestines, and lymphoid organs from Atlantic Wood Superfund Site and a King's Creek reference site will be assessed for gene expression of phase I, II and III xenobiotic drug metabolizing enzymes, the transcription factors Ahr-2 and Ahr-1 along with their repressor (AhR-R), as well as COX-2.

B. **Develop, characterize, and optimize technical applications for *Fundulus heteroclitus* AHR-1, AhR-R, and COX-2- specific monoclonal antibodies.** Monoclonal antibodies against *Fundulus* recombinant AHR-1 and AhR-R, and a designed COX-2 peptide will be developed and characterized as part of a larger panel of in-hand antibodies for quantifying and localizing protein expression, and validating qPCR results.

C. **Examine the histology and immunohistochemistry of killifish livers and intestines, from Atlantic Wood Superfund Site, VA and the Kings Creek, VA reference site.** Livers and intestines from Atlantic Wood Superfund Site and King's Creek reference site will be assessed for protein expression of the transcription factor AHR-2, CYP1A as well as COX-2. Inherent in this objective is to examine the prevalence and types of hepatic lesions found in killifish at the AW site.

D. **Determine if conditions at the AW site are associated with markers of inflammation in intestines.** Intestines will be examined for the presence of major basic protein (MBP) as in indicator of EGC degranulation, a marker of pro-inflammatory status.

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CHAPTER TWO

DEVELOPMENT OF MONOCLONAL ANTIBODIES AGAINST *FUNDULUS HETEROCLITUS* AHR-1, AHR-R, and COX-2.

INTRODUCTION

Many environmental contaminants, such as planar polycyclic aromatic hydrocarbons (PAHs) and halogenated aromatic hydrocarbon (HAHs), exert their toxicities by first binding to and activating the aryl hydrocarbon receptor (AhR). The AhR is a ligand activated transcription factor belonging to a large family of bHLH-PAS transcription factors involved in numerous physiological activities such as circadian rhythms, cell cycle regulation, growth and development, and oxidative stress, to name a few (Denison *et al.*, 2002; Knockaert *et al.*, 2004; Nguyen and Bradfield, 2008; Shimba and Watabe, 2009; Zhao *et al.*, 2013) . One of the most characterized functions of the AhR is in xenobiotic sensing and detoxication of ligands through the activation of phase I, II, and II xenobiotic metabolizing genes (Nebert *et al.*, 2000; Denison *et al.*, 2002; Hu *et al.*, 2007).

Gene duplication events in early lower vertebrates, followed by deletions during vertebrate evolution yield a wide variety of AhRs among vertebrate taxa, with two Ahr lineages in vertebrates; AhR1 and AhR2 (Hahn *et al.*, 1997; Karchner *et al.*, 1999).

More primitive teleostean fishes, like Atlantic salmon (*Salmo salar*), have two AhR1 isoforms (α, β) and four AhR2 isoforms (α, β, γ, δ), each expressed at different levels and

tissues (Hansson and Hahn, 2008). In more modern perciform teleosteans, like *F. heteroclitus*, two isoforms of Ahr1 (α, β) and two isoforms of AhR2 (α, β) have been found (Hahn *et al.*, 2004a), though nothing other sequence information about the beta forms of each are currently known. The same is true in zebrafish, *Danio rerio*, a very common model for biomedical research (Andreasen *et al.*, 2002a). A third class of AhR, known as the repressor (AhR-R) has two isoforms (α,β) in zebrafish, but only the alpha form has been described in killifish. AhR-R is expressed during AhR activation, and has a negative regulatory effect on expression of the other two AhR (Karchner, 2001; Hahn *et al.*, 2009; Jenny *et al.*, 2009). AhR-R seems to have a key role in early embryonic development, and probably related to the dynamics of cell proliferation and differentiation (Jenny *et al.*, 2009). As with CYP1A expression, AhR-R expression is considered a marker of AhR activation (Mimura *et al.*, 1999; Nguyen and Bradfield, 2008; Nguyen *et al.*, 2013). Throughout the evolution of vertebrates, leading to mammals, AhR2 has been deleted in mammals, with AhR1 being more homologous to the single AhR found in mammals. In fish, AhR2 is the dominant form associated with contaminant ligand binding and activation leading to toxicity of planar HAHs and PAHs (Hahn *et al.*, 1997; Karchner *et al.*, 1999; Karchner *et al.*, 2005; Clark *et al.*, 2010).

Specific roles of multiple AhR forms have not been elucidated, but several studies suggest that AhR1 in killifish and zebra fish may have functions in early development (Powell *et al.*, 2000; Karchner *et al.*, 2002; Jonsson *et al.*, 2007), while AhR2 in both fish seem to have a stronger role in the toxicity of select PAHs and HAHs (Carney *et al.*, 2006; Clark *et al.*, 2010; Chang *et al.*, 2013). The affinity for very toxic compounds

such as 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD), for ahr1 is lower than for AhR2 (Andreasen *et al.*, 2002a). Moreover, genetic suppression using morpholinos against AhR2, but not AhR1 greatly reduce the developmental toxicities of planar PAHs, PCBs, and TCDD (Clark *et al.*, 2010; Van Tiem and Di Giulio, 2011). Additional studies using zebrafish show that expression of AhR1 peaks during early embryonic development, while AhR2 is high throughout development and after hatching and larval development (Andreasen *et al.*, 2002b; Franks *et al.*, 2005).

Exactly why there is differential expression of AhR1 vs AhR2 is not known at this point, but these above observations came from studies examining gene expression, and we know nothing about the differential expression of protein (and functions) for these two gene products in developing fish embryos and on into adult stages. One study in particular, generated polyclonal antibodies against AhR1α, AhR2α, and AhR-R in killifish using recombinant proteins expressed in *E. coli* cells (Merson *et al.*, 2006), but whether or not these antibodies recognize their respective protein in fixed tissues is not known. To that end, the study described herein used those same recombinant proteins to generate very specific (epitope-specific) monoclonal antibodies selected for non-cross-reactivity, and then determined tissue-specific expression of AhR1, AhR2, and AhR-R using immunohistochemistry as well as whole tissue lysates. This will be important for determining tissue-specific and xenobiotic-specific effects on expression, and mostly because these antibodies will allow researcher to follow translocation of the particular AhR to the nucleus during ligand binding and activation, and to determine cellular

location of the Ahr in fixed tissues. Antibodies for this endpoint are available for mammals (Knockaert *et al.*, 2004), but not yet for fish.

One of the hallmark toxicological effects of AhR ligands is immunotoxicity and immunomodulation, and depending on the specific ligand, AhR activation may lead to either pro-inflammatory or anti-inflammatory outcomes (Vogel *et al.*, 2007a; Quintana *et al.*, 2008; Veldhoen *et al.*, 2008; Quintana *et al.*, 2010; Nguyen *et al.*, 2013), as well as cancer (Hawk *et al.*, 2002). Cyclo-oxygenase-2 (COX-2), or prostaglandin-H-endoperoxiase-2, is known to be expressed in cells and tissues following exposure to a strong AhR ligand like TCDD (Jönsson *et al.*, 2012), and blocking COX-2 activity may alleviate developmental toxicity, at least in the cardiovascular system of zebrafish (Dong *et al.*, 2010; Teraoka *et al.*, 2014). Moreover, COX-2 expression correlates with several types of cancers (Wölfle *et al.*, 2000; Hawk *et al.*, 2002; Ladetto *et al.*, 2005; Martín-Sanz, 2010), and being able to visualize COX-2 expression in fish tumors would be an asset to those investigating chemical carcinogenesis. At the moment there is a lack of concordance between phenotypic effects of TCDD on COX-2 gene expression, reduction of developmental toxicity by COX-2 inhibition, and actual protein detection to visualize levels of COX-2 protein. In most fishes, there are multiple isoforms of COX-2 due to gene duplication events described for the AhR(s), but killifish have only one COX-2 gene (Havird *et al.*, 2008). Previous work in our lab developed an antibody (mAb CX5-3) for detection of killfish COX-2 (Frederick *et al.*, 2007), but that particular clone was not effective at detecting protein in fixed tissues. As described herein, a killifish-specific monoclonal antibody was developed to localize COX-2 in various tissues by

immunohistochemistry, and to at least appreciate the circumstances surrounding expression of this pro-inflammatory protein, and especially in relation to AhR/CYP1A expression patterns.

MATERIALS AND METHODS

I. Generation and characterization of a mAb against AhR-1 in *F. heteroclitus*:

A C-terminus portion of Fundulus heteroclitus AhR-1 cDNA was cloned into a pQE80/82 6-HIS expression plasmid (Qiagen) and used to transfect the BL21-CodonPlus (RP) strain of *E. coli* for protein expression (Merson *et al.*, 2006) and provided as a gift from Dr. Mark E. Hahn's lab, WHOI. The expression plasmid was isolated using a GeneJet Plasmid Miniprep Kit (Thermofisher), and used to transform DE3 *E. coli* (Stratagene) harboring cold-adapted chaperone proteins for the option of improved expression at low temperatures over extended periods of time (Arctic Express system, Agilent). The transformed cells were incubated overnight at 37^0 C on LB agar plates containing 100 ug/ml ampicillin and 50 ug/ml chloramphenicol antibiotic concentrations. After the appropriate bacterial colony was selected following the Arctic Express DE3 protocol (Agilent Technologies), it was incubated in a shaker (250 rpm) overnight at 37^0 C in 5 ml of LB broth containing 100ug/ml ampicillin and 50ug/ml chloramphenicol antibiotic concentrations. All 5 ml were then transferred to a sterile bottle containing 500 ml LB broth with no antibiotics. The culture was incubated at 30^0 C at 250rpm for 3 hrs followed by induction with 2 mM of IPTG and a final incubation at 37^0 C at 250 rpm for 3 additional hours. The culture was then centrifuged at 4,000g for 20 minutes at 4^0C.

Following the directions provided by a Ni-NTA Fast Start Kit (Qiagen), the pellet was then frozen at -20^0 C and suspended in 20 ml of lysis buffer for denaturing conditions under constant shaking overnight at 4^0C. Next, the mixture was centrifuged at

14,000g for 30 minutes and the supernatant was collected. Ni-agarose columns and buffers were provided in the kit. Per instructions from the kit, the recombinant AhR1 protein was isolated over Ni-agarose columns. The purity of recombinant protein throughout washing and elution steps was determined visually by SDS-PAGE on 4-20% Criterion™ gels (Biorad) stained with Coomassie blue stain, followed by de-staining to visualize separated proteins. The presence of HIS-tag on recombinant proteins was verified by repeating the above SDS-PAGE using washing and elution fractions and transferring proteins to Immulon PVDF membranes (Fisher) and probing with Ni-HRP as part of commercially available kit (SuperSignal, Pierce). HRP activity was visualized using 4-chloro-1-napthol as a substrate.

Immunization and generation of mAb against AhR1

Only the most visually pure elution was used to immunize 6 – 8 week old balb/c mice at the Godley-Snell facilities at Clemson University, and under ALAC approved conditions. Following one primary and three boosting immunizations, spleen cells were isolated and fused with SPO/14 myeloma cells and subsequent hybridoma supernatants screened for reactivity by ELISA against rAhR1 using techniques previously described (Rice *et al.*, 1998). Supernatants from positive primary hybridomas were further evaluated by SDS-PAGE/immunoblotting steps as follows. Adult mummichogs were collected using baited minnow traps from the Belle Baruch Marine Lab, University of SC, euthanized in Tricaine (MS-222) and livers quickly removed, homogenized and centrifuged to obtain tissue S9 fraction protein. Thirty ug of liver protein, 10 µg each of

COS (African green monkey kidney fibroblast cells) expressing either full length AhR1 or AhR-2 (both were gifts from Dr. Mark E. Hahn, WHOI), and rAhR-1 were subjected to SDS-PAGE/immunoblotting on 10% gels probed with supernatants from clones that were positive in ELISA screenings. This would show whether the antibody was specific against AhR-1 and not both AhR-1 and AhR-2 which share a 40% overall amino acid sequence (Merson *et al.*, 2006).

Following a 5 min wash with 0.1 M phosphate buffered saline containing with 0.05% Tween-20 (PBS-Tw) the blot was covered with blocking buffer (10% FBS in PBS-Tw) and gently rocked for 2 hr at room temperature (RT). Following three 5 min washes with PBS-Tw, the blot was probed for 1 hr with supernatants from clones under consideration. Blots were washed x 3 with PBS-Tw and further incubated with alkaline phosphatase-conjugated goat-anti-mouse IgG (1:2000) for 1 hr at RT. After four washings with PBS-TW, alkaline phosphatase activity was visualized using the chromagen NBT/BCIP (Fisher Scientific) in alkaline phosphatase buffer.

II. mAb specific to *F. heteroclitus* AhR2

A mAb against F. heteroclitus rAhR2 was generated in a previous study (Josephine V. Wojdylo, M.S. Thesis, Clemson University, 2009).

III. Generation and characterization of a mAb against AhR-R in *F. heteroclitus*:

Generation and purification of AhR-R recombinant protein

A C-terminus portion of Fundulus heteroclitus AhR-R cDNA that was cloned into a pQE80/82 6-HIS expression plasmid (Qiagen) and used to transfect the BL21-CodonPlus (RP) strain of *E. coli* was a generous gift from Dr. Mark E. Hahn, WHOI (Merson *et al.*, 2006). The expression plasmid was purified and used to transform DE3 cells as described above, as were further steps for recombinant AhR-R protein expression. Purity of rAhR-R proteins throughout washing and elution steps was determined visually by SDS-PAGE on 4-20% Criterion™ gels (Biorad) stained with Coomassie blue stain, followed by de-staining to visualize separated proteins. The presence of HIS-tag on recombinant proteins was verified by repeating the above SDS-PAGE using washing and elution fractions and transferring proteins to Immulon membranes (Fisher) and probing with Ni-HRP as part of commercially available kit (SuperSignal, Pierce). HRP activity was visualized using 4-chloro-1-napthol as a substrate.

Immunization and generation mAb AhR-R from hybridomas in balb/c mice

Only the most visually pure elutions of rAhR-R were used to immunize mice as described above. Subsequent ELISA and immunoblotting steps were the same as described above.

IV. Screening for specificity of mAbs against AhR1, AhR2, and AhR-R.

One hundred ul of transformed bacterial cultures containing rAhR-1, rAhR-R, or rAhR-2 expression plasmids were collected after 3 hr of induction with IPTG (as described above) were subjected to SDS-PAGE/immunoblotting on 10% gels probed with supernatants from hybridoma clones isolated during previously screening steps. From screening assays, it was determined that mAb 5B6 (anti-AhR2), mAb 7B8 (anti-AhR1), and mAb 9R1 (anti-AhR-R) were best suited for their respective protein, and were used to determine if they cross-reacted with each other's intended protein. The above bacterial cultures were first boiled with sample buffer and then loaded onto the same gel, but in separate lanes, to have membranes for probing with each of the mAbs. The gels ran for 45min at 200V and were then transferred to a methanol treated 0.45µM Immunlon (PVDF) membrane (Fisher Scientific) at 4^0C overnight at 35 V.

Following a 5 min wash with PBS-Tw, the blot was covered with blocking buffer (10% FBS in PBS-Tw) and gently rocked for 2 hr at room temperature (RT). Following a 5 min wash with PBS-Tw, each of the three blots was incubated 1 hr with either mAb 5B6, mAb 7B8, or mAb 9R1. Blots were washed x 3 with PBS-Tw and further incubated with alkaline phosphatase-conjugated goat-anti-mouse IgG (1:2000) for 1 hr at RT. After four washings with PBS-TW, alkaline phosphatase activity was visualized using the chromagen NBT/BCIP (Fisher Scientific) in alkaline phosphatase buffer.

V. Generation and characterization of a mAb against COX-2 in *F. heteroclitus*:

A 10 residue synthetic peptide was designed from the complete cDNA sequence of rainbow trout (accession #CAB46017), and targeted the c-terminus end. This particular sequence is highly conserved in teleostean fishes, with only one amino acid difference between *F. heteroclitus* (accession #CAB46017) (Choe, 2006), and two different residues in croaker, *M. undulates* (accession #BAF52620). The peptide was synthesized and conjugated to KLH by Genosys-Sigma, and used as a hapten-carrier system to immunize mice as described previously (Rice *et al.*, 1998; Frederick *et al.*, 2007). Spleen cells were isolated and fused with FOX myeloma cells (ATCC, VA) and subsequent hybridomas screened for reactivity by ELISA against BSA-conjugated peptide. Supernatants from positive primary hybridomas were further evaluated by SDS-PAGE/immunoblotting steps as described (Frederick *et al.*, 2007). Positive clones were then screened by immunohistochemistry in various tissues to determine which is more suitable for antigen retrieval steps under low and high pH, and microwave vs. steaming conditions as described below.

VI. Optimizing mAb against AhR-1, AhR-R and COX-2 for Immunohistochemistry

Using mummichog liver cross sections, the monoclonal antibodies against AhR1, AhR2, AhR-R, and Cox-2 were optimized for use in immunohistochemistry. The slides were deparaffinized with xylene followed by decreasing ethanol concentration washes with the final hydration step in deionized water. Antigen retrieval was determined by

testing out the following conditions of either: having no antigen retrieval, microwaving in sodium citrate buffer (10mM sodium citrate, .05% tween 20, pH 6.0), microwaving in tris-EDTA buffer (10mM tris base, 1mM EDTA solution, .05% tween 20, pH 9.0), steaming in sodium citrate buffer (10mM sodium citrate, .05% tween 20, pH 6.0), and steaming in tris-EDTA buffer (10mM tris base, 1mM EDTA solution. This was followed by a thirty minute quenching step in .3% H_2O_2. The Mouse IgG Vectastain® Elite ABC Kit (Vector Laboratories) was using for blocking the slides using 10% horse serum. This was followed by blocking using an avidin/biotin blocking kit (Vector Laboratories). The primary antibodies against either AhR-1, AhR2, AhR-R, or COX-2 were applied as supernatants and incubated overnight at 4°C. Using the Vectastain® kit, the secondary biotinylated antibody against mouse IgG was incubated for thirty minutes at room temperature, followed by applying the Vectastain ABC Reagent, staining with ImmPACT ™ NovaRED™ (Vector Laboratories) and finally counterstaining with Hematoxylin QS (Vector Laboratories).

RESULTS AND DISCUSSION

The overall purpose for generating novel antibodies for Ahr1, 2, and Repressor is to further expand an in-hand tool box of antibodies for examining protein expression in multiple populations of killifish, and under various environmental conditions. Having these antibodies will also provide researchers the ability to validate gene expression data, either from microarrays or from qPCR studies, as well as IHC. Using expression plasmids provided by collaborators at WHOI, recombinant his-tagged AhR-1 and AhR-R were expressed in DE3 cells and purified using standard Ni-agarose procedures, and were found to be the predictable size as originally expressed (Merson *et al.*, 2006) (Figure 1). Killifish AhR-2 was previously expressed as a recombinant protein also provided as an expression plasmid by collaborators at WHOI (Merson *et al.*, 2006), and after expression in DE3 cells and purification steps, it too is of the expected size (Figure 1).

Attempts to purify rAhr1 were successful in that subsequent elution steps were reasonably pure and were indeed his-tagged proteins (Figure 2). Using rAhR-1, immunizations were carried out and after screening by ELISA and immunoblotting, mAb 7B8 was then selected and further characterized as an IgG2a *K1* immunoglobulin, then tested for non-cross reactivity with COS-expressed full length AhR2, and shown to be specific for AhR1 only (Figure 3). Multiple tissues and at several stages of development from killifish were examined by IHC, and for the most part AhR1 is not expressed. However, in developing cartilage and early ossification of the lower jaw, AhR1 is

expressed (Figure 4). This expression of AhR1 in early connective tissues could possibly indicate that AhR1 plays a role in early ossification. A study that looked at development tissue expression of AhR1 mRNA in a population of mummichogs from a superfund site in New Bedford Harbor, MA concluded that adult reference site fish expressed AhR-1 in the brain, heart, and gonads, while fish from the superfund site included expression in gill, gut, kidney, liver, and spleen organs (Powell *et al.*, 2000), but the study examined mRNA expression, not the protein.

Purification of rAhR-R over Ni-agarose columns resulted in relatively pure elution steps, and these proteins were shown to be his-tagged (Figure 5). Using rAhR-R for immunizations and subsequent screenings of hybridomas, mAb 9R1 (IgG1 *K1* isotype) emerged as the single antibody recognizing rAhR-R, but not rAhR1 and rAhR2 (Figure 6), and thus a very specific mAb for each of the three proteins is now available. At the time of this dissertation publication, mAb 9R1 does not recognize protein using IHC approaches, and this antibody may require additional, untested novel antigen retrieval methods - not all antibodies are suited for IHC. To my knowledge, the ability to correlate expression of AhR-R protein with gene expression in fish collected directly from the field has not been demonstrated. While AhR-R expression is a result of AhR activation in a manner similar to other gene products (e.g., CYP1A, CYP1B, phase II & III metabolism genes) (Nguyen *et al.*, 2013), it is unknown if fish AhR-R expression follows the same temporal or magnitude of other AhR-related products. At this point, the developing toolbox of antibodies for killifish has mAb 5B6 against AhR2 and mAb 9R1 against AhR-R, and together with mAb C10-7 against CYP1A (Rice *et al.*, 1998)

researchers are able to explore the expression of two key indicators of AhR activation. Based on my findings here with mAb 7B8 against AhR-1, this antibody is unlikely to be of benefit for future studies other than perhaps using this antibody as a negative control (irrelevant antibody) in immunoassays.

The next monoclonal antibody created was against COX-2. Using mAb 53-1, COX-2 is detected in mummichog macrophages throughout tissues, and especially within melanomacrophage aggregates surrounding a parasite in the liver (Figure 7). Macrophage aggregations (MA), sometimes referred to as melano-macrophage centers, or pigmented macrophage aggregations, contain activated macrophages and are part of the inflammatory response of fish (Blazer *et al.*, 1987; Camp *et al.*, 2000; Harper and Wolf, 2009). MA increase in both size and number in fish as they age, and due to exposure to environmental contaminants (Hinck *et al.*, 2007), therefore having an antibody that readily stains these macrophages allows us to localize and quantify sites of inflammation. The significance of this antibody to my dissertation research is that I may be able to correlate the localization of activated macrophages with different types of lesions in fish from the Atlantic Wood superfund site on the southern branch of the Elizabeth River near Portsmouth, VA. Over 90% of the killifish from this site have hepatic lesions, and with over 30% harboring malignant cancers of varying types (Vogelbein *et al.*, 1999; Vogelbein and Unger, 2006; Vogelbein *et al.*, 2008), and to date – no studies examining COX-2 protein expression within liver lesions of AW fish have been published.

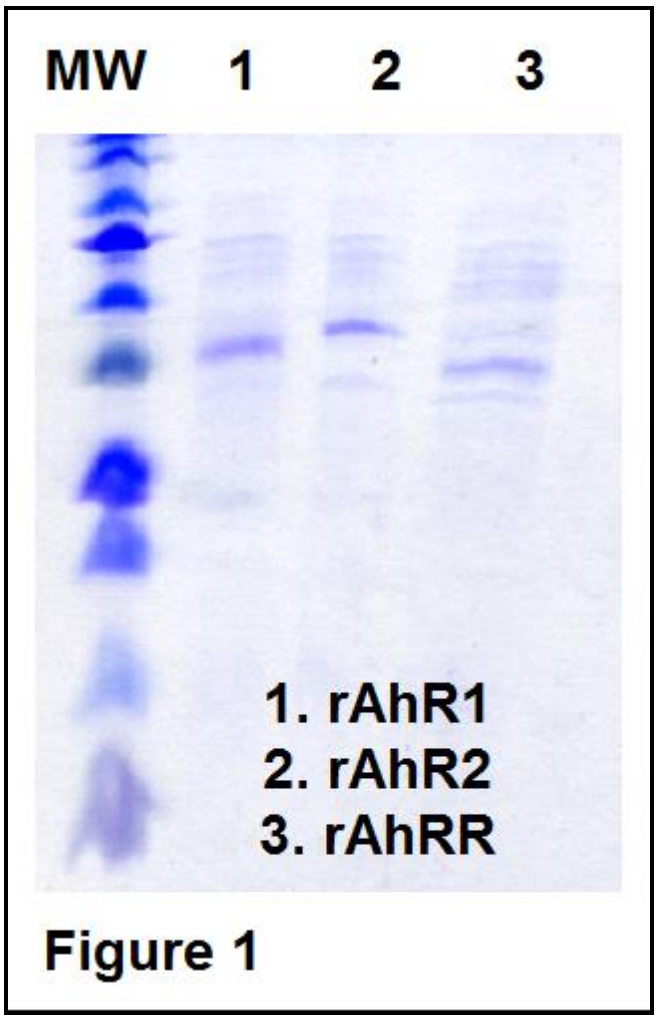

Figure 1. rAhR1, rAhR2, and rAhR-R lysates. Lysates from DE3 cells transfected with expression plasmids and subjected to SDS-PAGE and subsequent Coomasie Blue staining . Recombinant proteins among bacterial debris are visualized at the expected molecular weight.

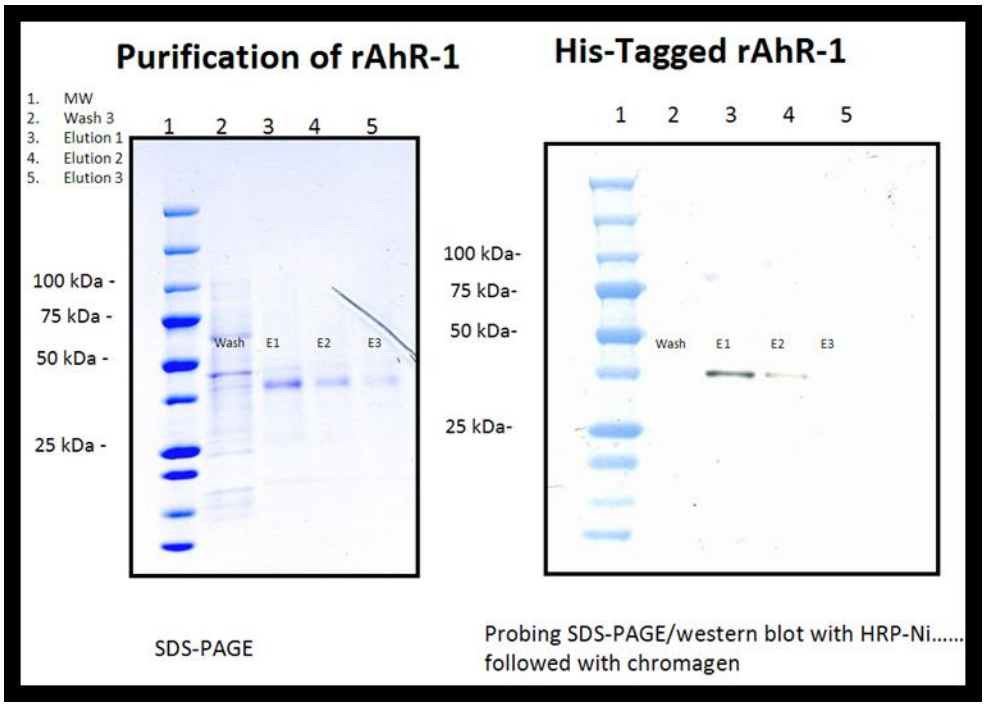

Figure 2. Visualization of purified recombinant AhR-1 protein through the use of Coomassie blue staining and Ni-HRP probing techniques. The gel to the left was loaded with wash 3 and elutions 1-3 and determined visually by SDS-PAGE on 4-20% Criterion™ gels (Biorad) stained with Coomassie blue stain, followed by de-staining to visualize separated proteins. The presence of HIS-tag on recombinant proteins was verified by repeating the above SDS-PAGE using washing and elution fractions and transferring proteins to Immulon membranes followed by probing with Ni-HRP, nickel horse radish peroxidase.

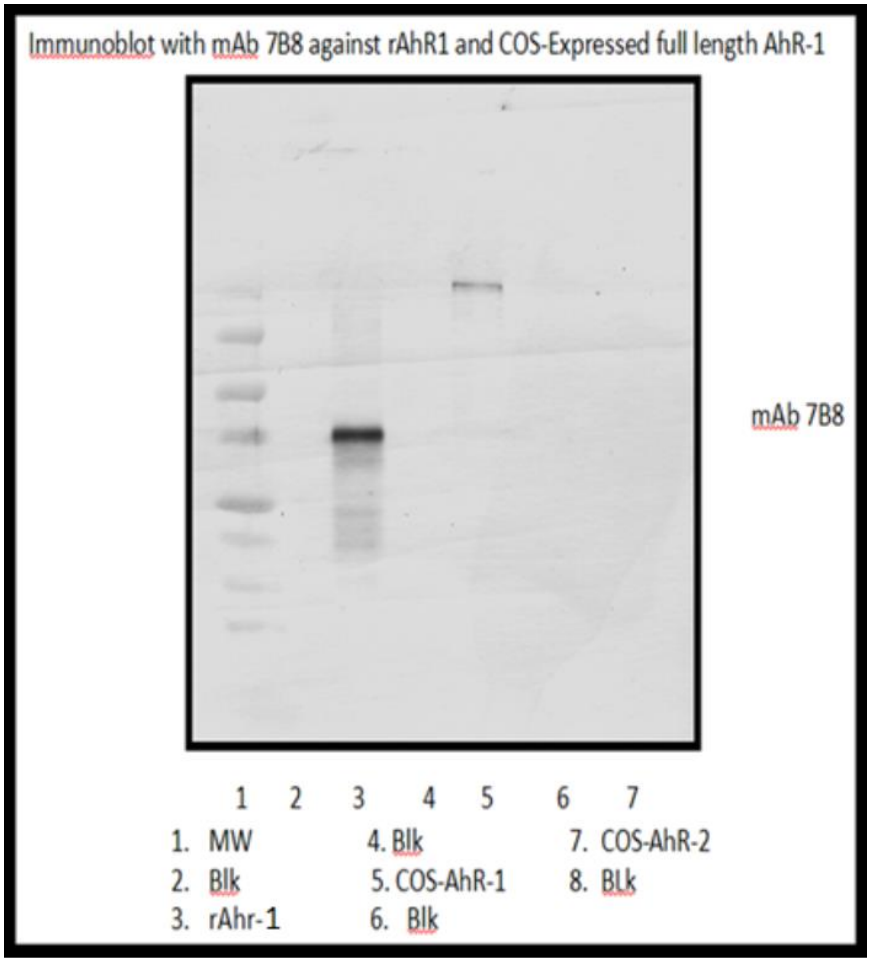

Figure 3. Immunoblot probed against AhR-1 using mAb 7B8. Thirty µg of rAhR-1 protein, 10 µg each of COS-expressed AhR1 and AhR-2, and rAhR-1 were subjected to SDS-PAGE/immunoblotting on 10% gels probed with mAb 7B8.

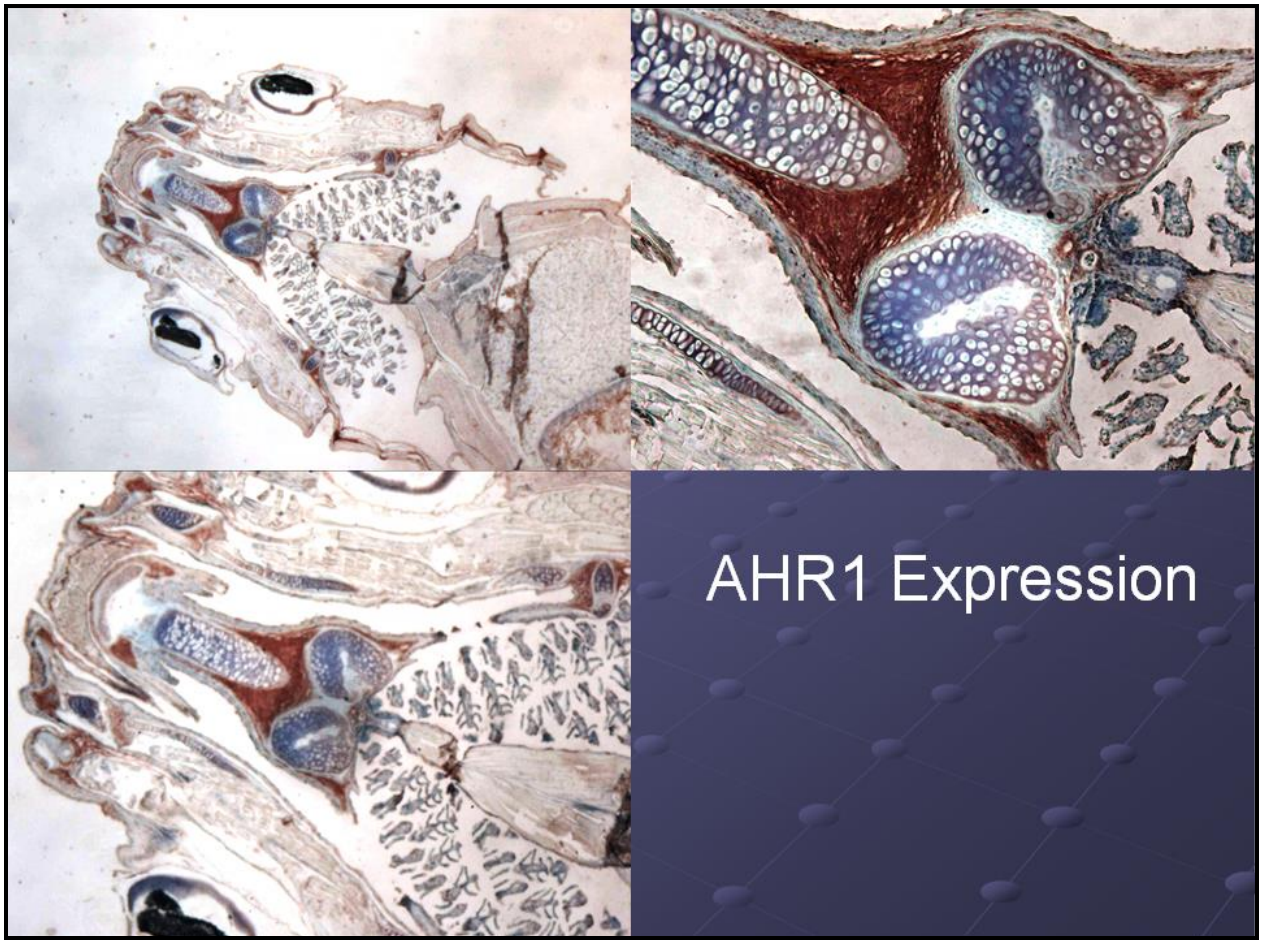

Figure 4. Immunohistochemisty using mAb 7B8 to localize the expression of AhR-1 in *F. heteroclitus* fry. NovaRed was used as the primary stain and Hematoxylin QS was used as a counterstain. Fry shown is two weeks post hatching.

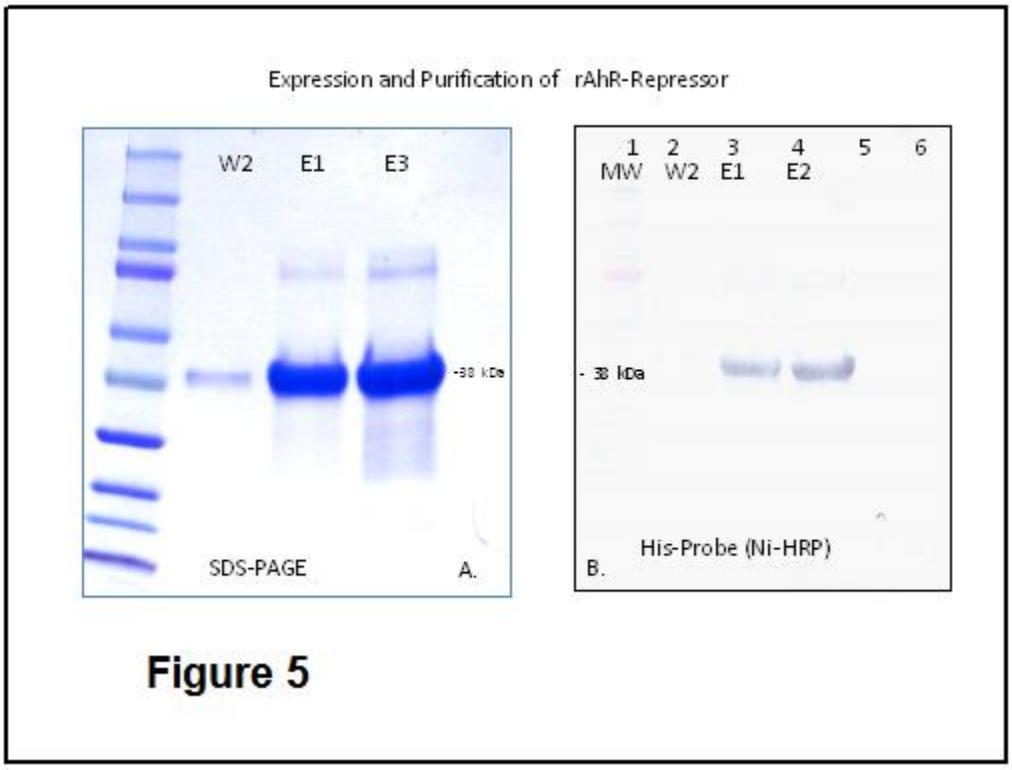

Figure 5. Purification and elutions of rAhR-R from Ni-agarose columns showing that E1 and E2 were relatively pure, and that these proteins were his-tagged.

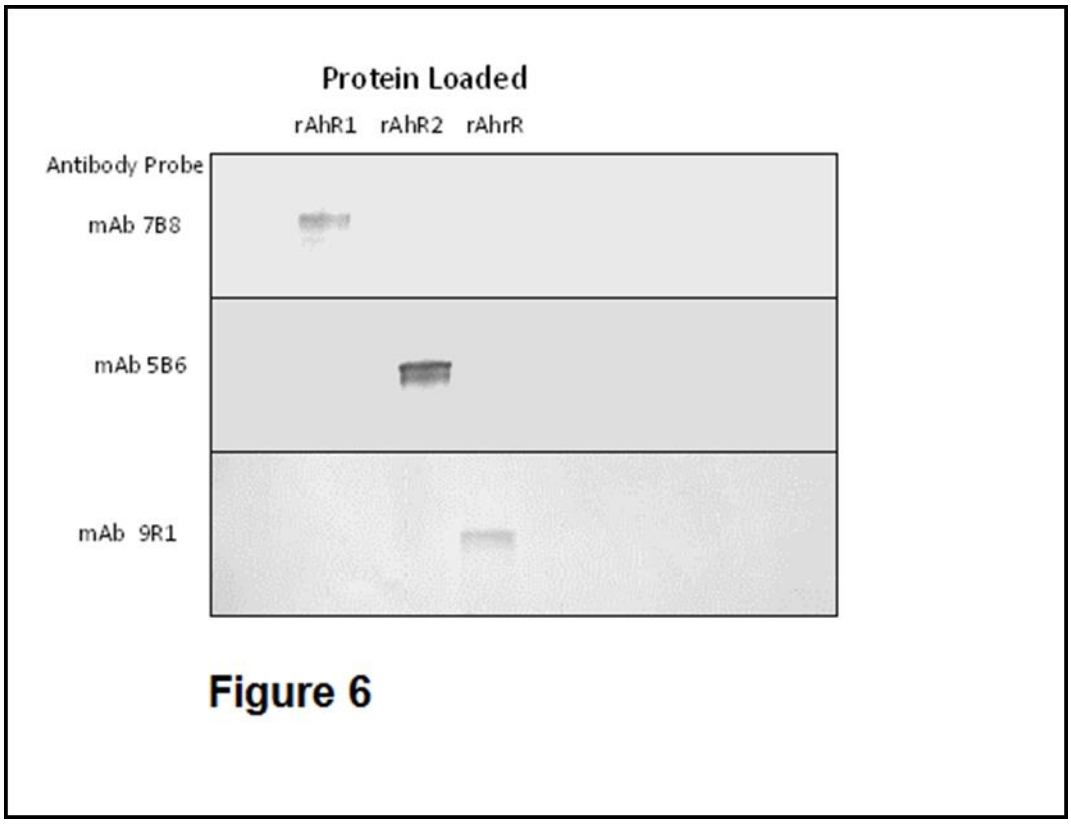

Figure 6. Test for cross-reactivity of mAbs against rAhr1, rAhr2, and rAhr-R. Purified recombinant proteins were subjected to SDS-PAGE and immunoblotted with all three antibodies. As shown, each each antibody is specific and does not cross-react with other two proteins.

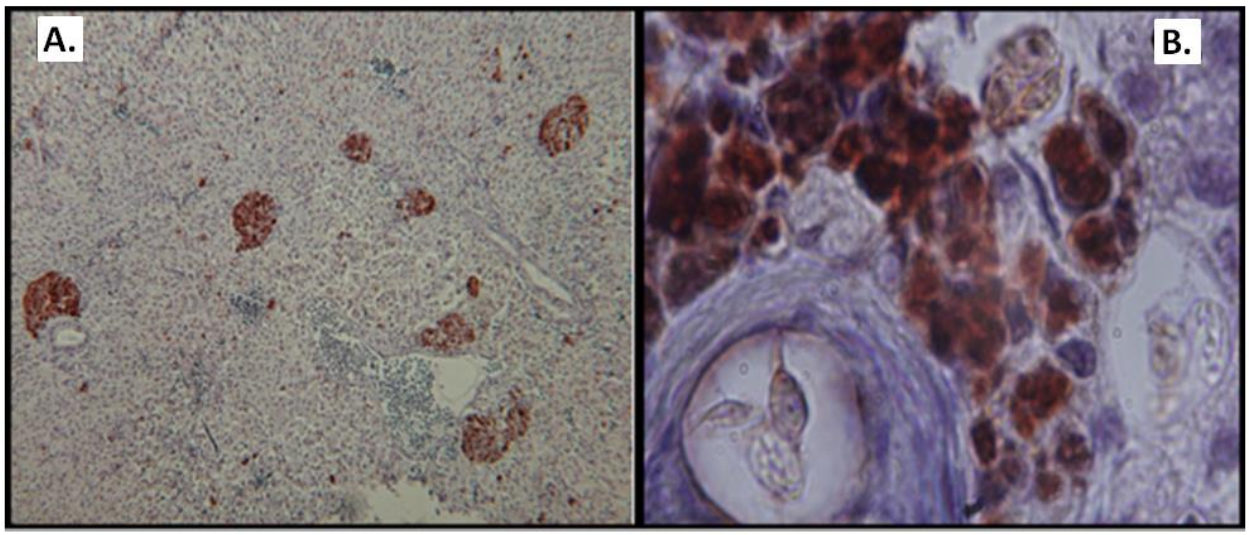

Figure 7. IHC of *F. heteroclitus* liver probed against COX-2 using mAb CX53-1. Image labeled A shows IHC of an AW liver probed for COX-2, using Nova Red as the primary stain and Hematoxylin QS as the counterstain. Image B is a close up, emphasizing a group of macrophage aggregations surrounding a parasite. Note the postiive staining of individual and clumps of fewer cells outside of the macrophage aggregate.

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CHAPTER THREE

AhR-RELATED ACTIVITIES IN A CREOSOTE-ADAPTED POPULATION OF ADULT ATLANTIC KILLIFISH, *FUNDULUS HETEROCLITUS,* TWO DECADES POST-EPA SUPERFUND STATUS

INTRODUCTION

Between 1926 and 1992, Atlantic Wood Industries operated a wood-treating facility on the Southern Branch of the Elizabeth River. In 1990, the Atlantic Wood (AW) site was added to the National Priorities List (NPL) of most hazardous waste sites due to the high volumes of creosote and pentachlorophenol (PCP) that were discharged, the storage of treated wood, and the disposal of wastes. At one time, the Navy leased part of the site for waste disposal, including abrasive sand-blasting materials. From such intense commercial use and released pollution over the years, the site contains polynuclear aromatic hydrocarbons (PAHs), PCP, dioxins, and several metals, including arsenic, chromium, copper lead, and zinc. In 1995, the US EPA issued a Record of Decision describing how the Agency would address the contamination of soil and sediments at the AW site, including studies to determine the environmental impact of local contaminants. This above information is gathered from the US EPA Mid-Atlantic Superfund page (http://www.epa.gov/reg3hwmd/npl/VAD990710410.htm).

Several key studies were initiated in the early 1990s to determine environmental impacts of local contaminated sediments at the AW site, along with studies to examine the impact on the Elizabeth River at sites both proximal and distal to the areas of highest contamination. One of the earliest series of studies demonstrated higher glutathione-s-transferase (GST) activity in intestines and livers, and altered natural cytotoxic cell activity associated with basic immune functions (Faisal *et al.*, 1991; Van Veld *et al.*, 1991), as well as intense incidence of hepatic lesions in Atlantic killifish, *Fundulus heteroclitus,* at the AW site (Vogelbein *et al.*, 1990; Van Veld *et al.*, 1991). A high incidence of hepatic lesions (90%) and neoplasia (30%) were evident (Vogelbein *et al.*, 1990; Fournie and Vogelbein, 1994; Vogelbein *et al.*, 1999), that ranged from altered hepatocyte foci, and bile duct and cholangiocellular cell proliferations, to exocrine pancreatic neoplasms and hepatoblastomas, but no lesions were found in less contaminated sites.

Concomitant with studies showing several pathologies in adult killifish, a key study emerged suggesting that inducibility of the AhR-related induction of hepatic CYP1A was compromised in adult AW killifish (Van Veld and Westbrook, 1995). Killifish from AW and KC received an i.p. injection of benzo-*a*-pyrene, and hepatic CYP1A protein and enzymatic activity (EROD assay) were measured 24 and 48 hr later; activity in AW fish was depressed compared to KC fish. This was one of the earliest studies in AW killifish to suggest a "recalcitrant" CYP1A phenotype. However, it was also demonstrated for the first time in AW fish that livers with lesions expressed CYP1A, but in a mottled fashion, and never uniform from one lesion type to the other (Van Veld

et al., 1992). In another study, embryos of AW killifish were tolerant of high concentration of sediments found at the site, while embryos from a reference site were highly susceptible (Ownby *et al.*, 2002). This phenomenon was heritable up through F2 generations.

Other studies were demonstrating possible mechanisms of toxicity, carcinogenesis, and what appeared to be tolerance, or at least adaptation, to contaminants at the site. For example, both lymphoid and hepatic DNA-adduct formation was high in adult fish at the site (Rose *et al.*, 2000; Rose *et al.*, 2001). In our own lab, we showed that antibody production was compromised in adult AW killifish in comparison to a reference site, and that indicators of innate and pro-inflammatory immune functions were elevated even up until 2002 and 2003 (Frederick *et al.*, 2007). Other studies were demonstrating that the toxicity of PAHs and typical cardiovascular deformities in embryos resulting from exposure to prototypical AhR ligands in AW killifish occurred at only high doses of compounds or high concentrations of sediment extracts (Meyer *et al.*, 2002; Wassenberg *et al.*, 2002; Wassenberg and Di Giulio, 2004; Wassenberg and Giulio, 2004; Wills *et al.*, 2009; Wills *et al.*, 2010; Clark *et al.*, 2013b) Because several PAHs, and especially those found at the AW site, are metabolized to teratogenic, carcinogenic, and immunotoxic intermediates through AhR-related CYP1A/CYP1B pathways, the general consensus at the time was that altered CYP1A induction (lowered CYP1A activity), was the key to understanding tolerance in AW killifish.

Tolerance to local heavily contaminated environments by Atlantic killifish is not confined to the AW site in VA, but has been documented in several populations, including New Bedford Harbor, MA, a site contaminated with PCBs (Nacci *et al.*, 1999; Powell *et al.*, 2000; Bello *et al.*, 2001; Bard *et al.*, 2002). Killifish from a dioxin-contaminated site in NJ also demonstrate a resistance to toxicity of local contaminants (Prince and Cooper, 1995a; Prince and Cooper, 1995b; Arzuaga and Elskus, 2002). Previous studies demonstrated that pre-exposure to mercury led to larval tolerance in killifish (Weis and Weis, 1982a), as was the case with metal mixtures in the environment (Weis and Weis, 1982a) resulting from several industries, including Landfills, a plating company, solvent companies, and mercury refining plants (http://www.epa.gov/superfund/programs/recycle/live/region2_ny.html). It was these early studies by the Weis's that laid the foundation for later New Bedford Harbor work (Weis and Weis, 1982b). At least one other early study demonstrated tolerance to the insecticide Kepone in killifish (Rice and Mills, 1987).

With the initial cloning and future expression studies of the AhR in fish, it became clear that early vertebrates exhibited multiple forms of this ligand-activated transcription factor (HAHs) (Hahn *et al.*, 1997). As presented in Chapter 2 of this dissertation, gene duplications in early vertebrates yield AhR-1, AhR-2, and AhR-repressor in modern fishes, and there are subforms (e.g., α, β, γ, δ etc) of each (Karchner *et al.*, 1999; Andreasen *et al.*, 2002a; Hahn *et al.*, 2004b; Hansson and Hahn, 2008; Hahn *et al.*, 2009; Jenny *et al.*, 2009). At least in the zebrafish and Atlantic killifish models, AhR2α (hereafter, referred to as AhR2) is involved in typical teratogenic responses of

embryos to contaminants, such as planar PCBs, dioxins, and PAHs, as can be demonstrated with anti-AhR2 morpholinos (Jonsson *et al.*, 2007; Clark *et al.*, 2010; Jönsson *et al.*, 2012). Population-wide studies examining a wide-array of genes that differ among adapted vs. sensitive killifish show that AhR2 and AhR2-related pathways were the most expressed and differed between sites (Nacci *et al.*, 2002; Roark *et al.*, 2005; Whitehead *et al.*, 2012; Proestou *et al.*, 2014; Reitzel *et al.*, 2014). Of particular note, non-synonymous single nuclear polymorphisms (nsSNP) in AhR2 within the ligand binding domain are present in both tomcod adapted to PCBs in the Hudson River, and in killifish adapted to PCBs are New Bedford Harbor (Wirgin *et al.*, 2011; Reitzel *et al.*, 2014), suggesting evolutionary pressure and selection for reduced AhR2-related activities (e.g., CYP1A expression, developmental toxicity). Mechanistically, these nsSNP-associated polymorphisms in AhR2 ligand binding domain bind typical AhR ligands (e.g., BAP, PCB-126, TCDD) with much less affinity, or lack thereof. There are also nsSNPs in AhR1 genes of killifish at the New Bedford Harbor site, but not in the ligand binding domain (Reitzel *et al.*, 2014).

Whether or not similar nsSNPs occur in the ligand binding domain of AhR2 in AW-adapted killifish is currently unknown. The previously described recalcitrance of CYP1A induction and developmental toxicity following exposure to typical AhR2 ligands in AW embryos (Clark *et al.*, 2013b) suggest altered AhR2 structure and function, but very few studies of AhR2-related functions have been examined in adult AW fish. Most of the studies with adult AW killifish have been limited to either lymphoid organs and plasma (Frederick *et al.*, 2007), or livers as the primary source of

tissue as an indicator organ of toxicity (Vogelbein *et al.*, 1990; Vogelbein *et al.*, 1999; Vogelbein and Unger, 2006). Yet, other organs, and especially intestines, are in direct contact with water overlying the contaminated AW sediments since these fish consume both epi-fauna and interstitial prey items, and they "drink" water. To date, the intestines of AW fish have been examined for GST activity only, but not for CYP1A and other AhR2-mediated functions.

In addition to the paucity of information on non-liver tissues of AW-adapted killifish, there have been significant changes to the Elizabeth River in general. In the years since the initial discovery of the AW population of killifish there have been three major hurricanes and thousands of tidal cycles through the Elizabeth River system, and sediments and fish populations may have been flushed from out of this superfund site. These possible physical and geological changes at the AW site, along with killifish's high reproduction rate and gene plasticity may have altered its resistance phenotype. This scenario is unlikely since very recent studies strongly suggest continued resistance/tolerance, at least with embryos collected at the AW site (Clark and Di Giulio, 2011; Clark *et al.*, 2013b).

The primary focus of this dissertation is to re-examine the issue of CYP1A expression in AW fish collected in situ 20+ years since the first description of the site and indigenous killifish, to characterize AhR expression profiles in these fish, and to explore the possible role of COX-2 and inflammation in Atlantic Wood fish liver lesions and different tumor types. Based on earlier studies in our lab (Frederick *et al.*, 2007), AW

killifish exhibited a "proinflammatory" state, including elevated COX-2 protein expression. COX-2 is an inducible enzyme, and activated primarily through the NF-β (Rel-A) pathway, as are iNOS, activation of inflammasomes, and other inducible systems (Lawrence, 2009). The downstream products of COX-2 induction, namely prostaglandins, thromboxanes, and leukotrienes, collectively are involved both acute and chronic inflammation, and therefore expression of this enzyme serves as a hallmark biomarker of overall inflammatory events. In addition to the role of COX-2 in inflammation, we know that COX-2 is also linked to AhR activation (Vogel *et al.*, 2007a; Degner *et al.*, 2008), and that both may have a role in carcinogenesis. Therefore, by localizing and quantifying expression of both in AW killifish we may be able to at least infer a possible connection in feral fish exposed to AW sediments in situ. Also, already in our antibody toolbox for the killifish model are mAb 2C11 and mAb M24-2, specific for the eosinophilic granular cell (EGC) granule protein major basic protein (MBP) and lysozyme, respectively. Our former work with AW fish demonstrated that circulating and leukocyte lysozyme levels and activity were higher in AW killifish than in KC reference fish (Frederick *et al.*, 2007), but MBP levels in the intestines as an indicator of local inflammatory conditions have not been examined. The intestine is the major site of eosinophilic cells, so it is logical to focus on these tissues as an indicator of inflammation-like conditions. Moreover, MBP induces intestinal epithelial cell apoptosis, followed by over-turn of the intestine lining (Powell *et al.*, 2010), also referred to as "sloughing". Using the molecular and antibody tool box now in hand, the major hypothesis to be tested is that adult killifish from the AW site are not refractory to

CYP1A induction by PAHs in situ, and that the intestines are the major anatomical location for CYP1A expression. Secondary to the primary hypothesis is a revisit earlier studies from two decades ago showing that CYP1A is expressed differentially in severe liver lesions of AW fish (Van Veld *et al.*, 1992), and to compare CYP1A expression to expression of AhR2 and AhR-Repressor. This study will also provide an opportunity to examine more closely the relationship between exposure to the harsh sediments at the AW site and state of intestinal inflammation-like conditions, and if COX-2 expression is elevated in livers harboring lesions and cancers.

MATERIALS AND METHODS

Examining the expression of genes representative of basic AhR-associated toxicology of NPL PAHs

In order to generate the qPCR primers for *Fundulus heteroclitus* (NCBI taxon id: 8078), the gene accession numbers of interest were obtained from the EMBL-EBI database and used to generate qRT-PCR primers from Integrated DNA Technologies (https://www.idtdna.com) as part of an ongoing attempt to develop a molecular and antibody toolbox for the Atlantic killifish model.

AhR1 (alpha form)

AhR2 (alpha form)

AhR-R (repressor)

CYP1A

CYP1B

UGT (UDPGT2)

GST-alpha

MT (metallothionein)

COX-2

RAG-1

P-glycoprotein (MDR-1)

18S

Primer set optimization and validation

Lab raised mummichogs collected from the Belle Baruch Marine Lab, near Georgetown SC, using baited minnow traps, were euthanized with MS-222. Head kidneys, intestines, and livers were collected and added to RNAzol®. Following the manufacturer's directions, individual samples of each organ were separately homogenized. The DNA, proteins and polysaccharides were precipitated and discarded. RNA was subsequently precipitated, washed and solubilized. The RNA purity was measured and used to make cDNA using the RT² Easy Strand Kit (Qiagen) according the to manufacturer's directions.

Platinum® PCR SuperMix (Invitrogen) was used to create PCR products from the mummichog cDNA that was previously generated, along with the mummichog primers. In a PCR thermal cycler, 40 cycles of PCR amplification were performed as followed: denature at 94°C for 15s, anneal (temperature will be dependent on the primer used) for 15s, and extension at72°C for 1min per kb.

PCR products were loaded along with a DNA ladder onto a 2.5% agarose gel with ethidium bromide and electrophoresis was performed at 80 volts. Next, the gel was viewed under an ultraviolet light and using a Biorad gel-documentation chamber to determine if there any dimers formed, which would have indicated that the annealing temperature was too low, or if there was no band formation, that possibly would have indicated that the annealing temperature was too high, or that the primer set was inadequate.

Once the PCR products from their corresponding primers were generated in the expected bp size and a single band shown, the next step was to perform a melt curve analysis using the Bio-Rad iCycler iQ5 PCR Thermal Cycler. Primer sets and conditions are provided in Table 1.

Table 1: qRT-PCR primer sets

Gene Name	Accession #	Primer Sequence (5′ → 3′)	Tm °C	Product size (bp)
AhR1	AF024591.3	F: AGC AAC CCT GGA TTC CCT TAC CTT R: TTC CAG AGC TCC AAA CAG CTC ACT	58	75
AhR2	U29679.3	F: ATC GAC AGC AGT ATG CCA CCT CTT R: TTA GCA GGG AAG GAA GCG TTG ACT	58	100
AhRR	AF443441.1	F: TTG TCT CGA AGC TGT ATG GCT CGT R: ATC TTA ATG GGC GGC ATT TCA GGC	57	124
CYP1A	AF 026800.1	F: AAG CAA GAG GGA GAG AAG GTC CTT R: TGT GCT TCA TCG TGA GGC CAT ACT	57	150
CYP1B	AF235140.1	F: CCA AAG AAT ACA CAG AGG CAA CGG R: ATG AAG GCA TCC AGG TAA GGC AT	58	175
UGT	AY725222.1	F: TTA CCG TAA CAA CAT CCA GCG CCT R: CAG CTC CTT TGT TCC TGA TGT CGT	59	100
MT	AB426465.1	F: AAG ACT GGA AAG TGT AGC TGC GGA R: ATC CTC ACT GAC AGC AGC TCT T	60	175
COX-2	AY532639.2	F: TAC CCG CCA CTG GTT AAG GAT GTT R: TTG TGT TCA CGG AGC CAA ATG GTG	57	146
RAG-1	DQ250438.1	F: TGT TCA GGC GGT TCA GGA AGA TGA R: AGG TGT AGA GCC AGT GGT GTT TCAA	57	100
GST	AY725219.1	F: TCT GAC AGA AAG CAC TGC GAT CCT R: TGA GCA GGA AGA CCT TTG AGC AGT	57	150
18S	M91180.1	F: TTC GTA TTG TGC CGC TAG AGG TGA R: TTC GAA CCT CCG ACT TTC GCT CTT	57	125

qRT-PCR of mummichog livers, intestines, lymphoid organs from AW vs KC

Adult killifish from Atlantic Wood and Kings Creek were collected using baited minnow traps and euthanized with MS-222. Livers, intestines, and lymphoid organs were quickly removed and placed in RNAZol. For gene expression analysis, three pools of three organs for males, and another three pools of three organs for females were processed for RNA following the manufacturer's instructions. After collecting RNA from each tube, genomic DNA contamination was removed using elimination mixture

supplied by the manufacturer, and first strand cDNA synthesis was carried out using the RT^2 Easy First Strand Kit (SABioscience Corporation) as described by the manufacturer. Gene expression was analyzed by quantitative real-time PCR with a BioRad iC5 detection system, RT^2 SYBR green/ fluorescein master mix, and primer sets designed using Integrated DNA Technology (IDT) software, and validated prior to use (Table 1). The quantity of these mRNAs was expressed as fold-changes in gene expression compared to 18S expression. Fold-increase or decrease in gene expression was subjected to appropriate parametric statistics if the data were normally distributed and met the criteria for parametric approaches (Pfaffl method). For data analysis, the Pfaffl method was used to determine fold increase or decrease in expression. Expression data were compared between treatment groups using ANOVA, followed by a Bonferroni's post-test using GraphPad5 statistical package. Also, when data revealed a difference between males and female at each site, these data were graphically represented as a separate data set.

Liver and intestine CYP1A and AhR-R protein expression

To follow up on intestine CYP1A and liver AhR-R gene expression data, killifish were collected again at the AW site and King's Creek, near White Marsh, VA, on the Mobjack Bay, using baited minnow traps. Although any protein expression profiles from the two populations at this sampling would not be from the same fish as used for gene expression profiles, the larger sample size and use of individual animals should be representative of fish previously sampled. Livers and intestines from 8 males and 8 females from each

site were quickly removed and flash frozen in liquid nitrogen, then frozen at -80^0 C at the Virginia Institute of Marine Science until shipping overnight on dry ice to Clemson University, and again stored at -80^{0}C until further use. Livers and intestines were placed in cold homogenization buffer containing 2X concentration of HALT protease inhibitor cocktail (Pierce), and sonicated on ice. Homogenates were centrifuged for 10 min at 1000 x g to settle cellular organelles and tissue debris. Supernatants overlying debris were then centrifuged at 12,000 x g for 20 min to obtain S9 fractions. Protein concentrations of S9 preparations were determined and diluted to 2 mg/ml in lysis buffer and mixed with 5X sample buffer prior to boiling for 5 min. Thirty µg of sample were subjected to SDS-PAGE on 4-20% gels. The gels ran for 45 min at 200 V and were then transferred to 0.45µM Immunlon (PVDF) membrane (Fisher Scientific) at 4^{0}C overnight at 35 V.

Following a 5 min wash with 0.1 M phosphate buffered saline containing with 0.05% Tween-20 (PBS-Tw) the blot was covered with blocking buffer (10% FBS in PBS-Tw) and gently rocked for 2 hr at room temperature (RT). Following a 5 min wash with PBS-Tw, the blot of intestine proteins was incubated with mAb C10-7 against fish CYP1A (Rice et al., 1998), and liver proteins were probed with our newly developed mAb R91 against AhR-Repressor (Chapter 2). Blots were washed x 3 with PBS-Tw and further incubated with alkaline phosphatase-conjugated goat-anti-mouse Ig (h+l) (1:2000) for 1 hr at RT. After four washings with PBS-Tw, alkaline phosphatase activity was visualized using the chromagen NBT/BCIP (Fisher Scientific) in AP buffer.

Collecting organs from AW and KC killifish for histopathology and IHC

Based on studies to examine CYP1A protein expression in livers and intestines and qPCR results, livers and intestines from AW and KC adult killifish were collected and fixed in Z-fix buffered formalin for 72 hr, then placed in 70% histological grade ETOH until ready for processing. The organs were later paraffin-embedded and processed for cutting and mounting by the Clemson University Histology facility. The livers from each population were examined and diagnosed by Dr. Wolfgang Volgelbein, Virginia Institute of Marine Science, College of William and Mary.

Select livers, based on diagnosis and variety of tumor types, were probed with mAb against AhR-2 (5B6), CYP1A (C10-7), and/or COX-2 (CX53-1) following antigen retrieval steps as follows. Slides were heated in Tris-EDTA buffer, pH 9 by microwave on 100% power for 5 minutes followed by having the solution cool for 5 min, followed by a final 5 minutes 100% power cook, and a final 20 min rest in the container. Tissues on slides were encircled with a Liquid Blocker Super mini pen to separate tissue slices on each slide, then the appropriate antibody as diluted hybridoma supernatants was added and incubated overnight at 4^0 C. Each slide with tissue slices contained one slice receiving secondary antibody only. The next day each slide was then washed endogenous peroxidase was quenched with hydrogen peroxide. Tissues were further processed using a goat-anti-mouse IgG Vectastain ABC-Ultra kit. Antibody labeling was detected with Nova Red staining, and hematoxylin counter stain.

Staining for COX-2 expression and EGCs in mummichog livers and intestines, respectively.

To visualize COX-2 and contents of EGC granules (presumably major basic protein), peroxidase-based IHC staining was performed on livers for COX-2 expression and on intestines for major basic protein expression. The slides were deparaffinized with xylene followed by decreasing ethanol concentration washes with the final hydration step in deionized water. Antigen retrieval tris-EDTA buffer (10mM tris base, 1mM EDTA solution, .05% tween 20, pH 9.0) was used following by 5 minute cooking in the microwave on 100% power, followed by 5 minute cooling, followed by another 5 minute cooking in the microwave for 5 minute, and lastly following by a 20 minute period of cooling. The Mouse IgG Vectastain® Elite ABC Kit (Vector Laboratories) was using for blocking the slides using 10% horse serum. The primary antibody, mAb CX53-1 (Chapter 2) or mAb 2C11 (Marsh, 2007) was applied neat and incubated overnight at 4°C. Following five two minute washes in PBS-tw20, the slides were then further processed using Vectastain ABC Ultra kit for peroxidase-based IHC following the manufacturer's instructions.

Immunoblot expression of MBP in Intestines:

To demonstrate that mAb 2C11 recognizes its predicted protein target of 12 kDa in intestines, thirty µg of intestine lysate from 8 mummichogs from AW and 8 mummichogs for KC were subjected to SDS-PAGE/immunoblotting on 4-20% gels. Samples were first boiled with sample buffer and then loaded onto the gel. The gels ran

for 45 min at 200 V and were then transferred to a methanol-treated 0.45µM Immulon (PVDF) membrane (Fisher Scientific) at 4^0C overnight at 35V.

Following a 5 min wash with 0.1 M phosphate buffered saline containing with 0.05% Tween-20 (PBS-Tw) the blot was covered with blocking buffer (10% FBS in PBS-Tw) and gently rocked for 2 hr at room temperature (RT). Following a 5 min wash with PBS-Tw, the blot was incubated with mAb 2C11. Blots were washed x 3 with PBS-Tw and further incubated with alkaline phosphatase-conjugated goat-anti-mouse Ig (h+l) (1:2000) for 1 hr at RT. After four washings with PBS-Tw, alkaline phosphatase activity was visualized using the chromagen NBT/BCIP (Fisher Scientific) in AP buffer.

ELISA for quantifying lysozyme and major basic protein in Atlantic Wood vs. King's Creek Intestines

Killifish intestines were placed in cold homogenization buffer containing 2X concentration of HALT protease inhibitor cocktail (Pierce), and lysed using a Bead-Beader shaker with 1 mM glass beads following the manufacturer's instructions. Lysate protein concentrations were determined and diluted to 2 mg/ml in lysis buffer. Twenty ug of protein were added to poly-L-lysine coated ELISA plates in duplicate and incubated overnight at 4^0 C. Contents of each well were removed by flicking the plate, and the plates were washed x 3 with PBS-Tw. One hundred ul of blocking buffer (10% FBS in PBS) were added to all wells and allowed to incubate at RT for 2 hr, at which time the contents were removed by flicking. ELISA plates were washed x 3 and then received 100 ul of undiluted hybridoma supernatants: 2C11 for EGCs (Marsh, 2007),

M24-2 for lysozyme (Marsh and Rice, 2010), then incubated at RT for 2 hr. Plates were emptied by flicking and washed x 3 with PBS-Tw, then received 100 ul of goat anti-mouse IgG-AP (1:2000; Southern Biotechnology) and were incubated at RT for another 1 hr period. As the final step, plates were washed x 4 with PBS-tw, then incubated with 100 ul per well of a 1 mg/ml para-nitrophenyl phosphate in alkaline phosphatase buffer (AP), incubated for 30 minutes, and the optical density at 405 nM recorded. The O.D. values for duplicate wells for each sample were averaged.

RESULTS

Gene expression of CYP1A and CYP1B, two gene products under the control of AhR-related activity, were different between populations of killifish, but only for intestine CYP1A and lymphoid CYP1B (Figure 1A, 1B). CYP1A gene expression was highest in intestines, with lymphoid and liver expression being comparable. An analysis of AhR2 gene expression showed no differences between populations, or between organs (Figure 1C). Considering that AhR-R is often considered a marker of AhR activation, expression was evaluated as well. Liver and lymphoid AhR-R expression levels were higher in AW fish (Figure 1D). Gene expression of the two phase II enzymes, GST-alpha and UGT, differed in lymphoid tissues and intestines between the two populations, with AW fish expressing less (Figure 2). Of the three tissues examine, lymphoid GST was expressed at a higher level than for livers and intestines, even in KC fish. There were no differences in expression of UGT between the two populations, though liver expression was higher in both populations than in lymphoid and intestine tissues.

When gene expression data were further analyzed for gender differences, and statistical interactions, there were differences between male and female expression of GST in AW fish; males express much higher GST (Figure 3A), and the same is true for liver AhR-R (Figure 3B). Analysis for statistical interactions show that GST expression is different between AW and KC males; GST expression is higher in KC males (Figure 3A). However, there are also statistical interactions between AW and KC males; AhRR expression is higher in AW males (Figure 3B). Based on previous work in our lab

indicating that COX-2 protein expression is higher in AW vs. KC killifish (Frederick *et al.*, 2007), the expression of COX-2 gene expression was examined in intestine, liver, and lymphoid tissues. Liver COX-2 is nearly three times higher in AW than KC killifish (Figure 4). Of particular note, liver COX-2 expression in AW fish was also nearly three times higher than other tissues, regardless of population.

Upon examination of whole liver S9 fractions, CYP1A protein expression was clearly detected in some, but not all fish examined (Figure 5). Even though King's Creek killifish are considered a reference population for AW, CYP1A is expressed in some individuals as well. Intestine CYP1A expression is fairly uniform in all AW fish, with some individuals exhibiting very high expression (Figure 6). No intestine CYP1A protein was observed in KC fish. These observations seem to contradict the assumption that AW fish are resistant to PAHs, and therefore do not express CYP1A as a means of preventing the toxicity of PAHs that may work through AhR2-related mechanisms. Moreover, and as an extension of CYP1A expression, AhR-R protein in AW livers was expressed in more AW individuals than in the KC samples, and more intensely in females than in males within the AW samples (Figure 7).

Considering that liver CYP1A protein is expressed in at least some adult AW killifish livers, and at both the gene and protein level in all intestines examined, localization of CYP1A was determined by IHC. And because CYP1A activity follows AhR2 activation, this protein was also examined by IHC. Just as was found nearly 20 years ago in AW fish, virtually all AW fish from this study have severe liver lesions,

ranging from altered foci to fully malignant tumors (see lesion summary, Table 2). As can be seen in representative examples provided, CYP1A expression is not readily found in KC fish, but in found at different degrees in liver lesions (Figures 8, 9, and 10), each representing a different histopathological diagnosis. Early tumorogenesis in eosinophilic foci seem to express high CYP1A, while more basophilic (higher protein expression) express AhR2. Neither CYP1A, nor AhR2 is expressed in other cells of the same liver (Figure 8). However, in another individual harboring a hemangiosarcomoa, CYP1A and AhR2 are co-localized in the same tumor cells (Figure 9). For another individual, and in one of the more progressed tumors examined, a pancreatic ascinar carcinoma is void of CYP1A expression other than cells lining one of the major blood vessels (Figure 10). AhR2 expression in that same tumor was modeled. No tissue tumors or gross lesions were found by histological and IHC examination in intestine tissues of either population. However, intestine CYP1A protein expression was observed in all AW fish examined, as presented in Figure 11. None of the intestines from KC fish were positive for CYP1A staining, as presented in Figure 12. Of special note, AhR2 protein expression in both populations was highly expressed (Figure 11, 12). In overview, the IHC data support gene expression data, and especially with CYP1A induction.

Observations that intestinal CYP1A (gene and protein) is clearly induced and expressed in AW fish (but not KC fish), that liver COX-2 gene expression is higher in AW fish than in KC fish, and our previous studies pointing to elevated innate immune functions in AW vs. KC fish, further studies were initiated. Intestines of fish contain high numbers of ECGs, the teleost equivalent of mammalian mast cells(Reite and

Evensen, 2006), and these eosinophil-like cells contain, among other biologically active proteins, preloaded granules harboring major basic protein (MBP). High expression of MBP in fish intestines indicates higher inflammation, and thus may serve as a biomarker of local inflammation. In the study described herein, the 12 kDa major basic protein was expressed at higher levels in intestine lysates of AW fish, as indicated by both immunoblotting and ELISA techniques (Figure 13, Figure 14). Previous work in our lab generated a fish-specific anti-lysozyme antibody that readily recognized the protein in IHC, ELISA, and immunoblotting techniques (Marsh and Rice, 2010). There were no differences in lysozyme protein expression in intestines of AW vs. KC killifish (Figure 15). As noted from gene expression analysis, liver COX-2 gene expression is much higher in AW vs. KC fish, and mAb CX53-1 staining localized to cells (activated macrophages) in AW livers (Figure 16), and these COX-2-positive cells are absent in lesions/tumors.

DISCUSSION

In this study I found that adult killifish adapted to sediments at the Atlantic Wood superfund site have AhR2-related activities in intestines, liver, and lymphoid tissues. Intestinal CYP1A and AhR2 protein expression is very high in AW fish, while expression of this protein in liver varies greatly, from none observed to modeled expression throughout the organ as a result of lesions and tumors. These finding are in contrast to the widely held assumption that killifish have adapted to environments like AW by somehow shutting down the AhR-related pathway to reduced CYP1A expression, and therefore impede metabolism of parent PAHs to carcinogenic, teratogenic, and immunotoxic metabolites. These findings also support earlier findings by others that differential CYP1A expression occurs in AW liver lesions vs. livers without lesions (Van Veld *et al.*, 1992). Information presented in this dissertation does, however, present a conundrum in terms of reconciling with the many publications using AW embryos – AhR-related pathways are blunted in developing AW embryos.

To my knowledge, this study was the first to examine the expression of a battery of genes related to the known toxicity of PAHs on the NPL, and simultaneously the first to describe ranges of expression in intestines, livers, and lymphoid tissues of AW killifish at the same time. The most significant observations were that intestine CYP1A and liver COX-2 gene expression are very high in AW killifish compared to KC fish. In contrast, intestine and lymphoid GST gene expression was much lower than in KC fish. At the time of primer design and application in qPCR assays for this study, GST-alpha was the only killifish sequence available, and therefore the results are interpreted only within the

context of this subclass of GST; the full suite GST subclasses in killifish have not yet been described and characterized. However, multiple classes of GSTs have been identified in other species of teleostean fish, including alpha-, mu-, pi-, and rho-type proteins, each generally being co-expressed, and at high levels (Trute *et al.*, 2007). The difference between classes seems to be related to different oxidized substrates and which types of chemicals induce oxidative stress. In terms of GST activity in AW killifish, previous work demonstrated higher total activity in livers and intestines (Van Veld *et al.*, 1991), and a unique GST protein was isolated from AW killifish not present in KC fish (Armknecht *et al.*, 1998). Unfortunately, the particular class was not identified or further investigated. It is possible that GSTs other than the alpha class are elevated in AW killifish, while alpha is suppressed, or selected against. Now that the genome of killifish has been sequenced, and annotation continues, it will be possible to later revisit this issue of GST forms in AW fish. As with the only available GST sequence available at the time of this study, only UDP-UGT2A (UGT-2A) was available. Thought not statistically significant, there was a trend towards higher UGT expression in lymphoid tissues of KC, indicating the possible presence of phenolic intermediates as substrates in these tissues. It is difficult to explain the mechanisms behind reduced phase II enzymes in AW killifish intestines and lymphoid tissues, and elevated intestine CYP1A, when each of these enzymes are under the direct control of the AhR as part of the battery of genes expressed. Much of what we know about AhR activity is based on either in vitro studies, or on whole animal studies beginning with unexposed animals and following activation of the AhR over time. In a field study like the one described herein, exposures are ongoing, and

fish are exposed to multiple abiotic and biotic factors and stressors, and some factors may activate, while others inhibit gene expression. One clue to understanding the data lies in gender differences in GST activity, with male AW fishing expressing more than females. These fish were caught and tissues collected on the full moon, which coincides with lunar spawning cycles in killifish, when estrogen is highest in females. The cross talk between estrogen receptors and AhR has been studied extensively, with the understanding that activation of one can inhibit the other by co-opting co-activators (Safe and Wormke, 2003; Matthews and Gustafsson, 2006) But ultimately, it is the expressed protein and function that yield the phenotype observed at the time of sampling, and therefore either function or protein expression should be evaluated in future studies. Gene expression for AhR-R was also different by site and gender, with AW fish expressing more, and females expressing more than males – this was supported by protein expression data as well. Why the gender difference is unclear, it may be related to the amount of estrogen in circulation at the time of sampling in that the degradation of AhR-R is inhibited or slowed during estrogen receptor activation.

Upon examination of CYP1A protein expression in livers, it was clear that several fish from both sites had fairly high levels of expression. This is probably due to different reasons; expression in KC fish is more than likely the result of random exposure to motor boat oil, or creosote leaking from a small bridge near the collection site. In AW fish, induction of liver CYP1A is due to both AhR2-related signaling and intrinsic aspects of lesions and tumors (as discussed below). It appears that the incidence of tumor lesions and tumors has not subsided over the twenty years since first described. From multiple

samples, both male and female, AW fish livers are still impacted with multiple lesions, and often at least one cancer type. One could speculate that reduced liver CYP1A activity in these fish (Van Veld and Westbrook, 1995) is due mostly to severe liver damage, and this may be the case in many of the fish sampled. However, several of the tumor types are of non-liver parenchymal origin (e.g., sarcoma, adenoma etc), and do not normally express CYP1A to begin with, so not observing CYP1A by IHC in AW fish livers may not be unexpected. Nearly twenty years ago this same profile was observed (Van Veld *et al.*, 1992), though they did not examine AhR expression at the time. It was speculated that progression of tumor types follows a predictable course of over-expression of CYP1A in early lesions, and reduction or absence in later progressed lesions, as has been well documented in rat liver tumor models (Roomi *et al.*, 1985).

From another perspective, recent studies show that some human breast cancers constitutively express high levels of CYP1A1 (Rodriguez and Potter, 2013), and that knocking down CYP1A1 in breast cancer cells lines results in reduced cell cycling, growth rates, and intracellular signaling related to proliferation. Though not examined in the Rodriquez and Potter study, constitutive expression of CYP1A would suggest a likewise constitutive expression and activity of AhR, which is known to be the case in several tumor types and in inflammation (Moennikes *et al.*, 2004; Tauchi *et al.*, 2005). As noted in this study (Figure 8), early eosinophilic lesions express CYP1A, but little AhR2, while the more progressed, basophilic lesions express little or no CYP1A, but higher levers of AhR2. Other cancers, and especially carcinomas (epithelial origin) express both AhR and CYP1A via a modeled appearance, while other tumor types are

barren of expression, except for blood vessel endothelial cells. However, at the whole organ level, total CYP1A gene and protein may be minimal, which can explain low levels of liver CYP1A protein expression in many individuals.

One of the more interesting findings from this study is there is a confirming correlation between CYP1A/COX-2 gene expression and protein expression. Elevated COX-2 expression in AW livers is a novel finding, in that a correlation between this protein and liver lesions and tumors in fish has not been published. COX-2 expression is known to be directly linked to AhR activity (Vogel *et al.*, 2007b; Degner *et al.*, 2009; Dong *et al.*, 2010), and is a prognostic indicator in colon and liver cancer (Eberhart *et al.*, 1994; Kondo *et al.*, 1999), and may have a role in prostate cancer (Song *et al.*, 2002). The significance of high COX-2 expression in AW fish livers is probably related to a state of chronic inflammation-like conditions. Chronic inflammation in mammals is linked to hepatotoxicity (Luster *et al.*, 2001), and tumor associated macrophages actually promote tumor progression (DeNardo and Coussens, 2007; DeNardo *et al.*, 2008; Sica and Mantovani, 2012).

The mechanisms behind the ability of macrophages to promote tumor growth and progression is related to the state of polarization from M1- to M2-type cells, with M2 cells secreting wound-healing growth factors which then promote the growth of tumor cells (Mantovani, 2006; Mantovani *et al.*, 2008; Biswas and Mantovani, 2010; Mills and Ley, 2014). High COX-2 expression in AW livers may also play a role in PAH metabolism, leading to further toxic metabolite formation, as COX-2 can metabolize

some PAHs to diol-epoxide intermediates (Eling *et al.*, 1990; Parkinson, 2001). Moreover, CYP1A1 may metabolize prostaglandin endoperoxide to the mutagen malondialdehyde (Plastaras *et al.*, 2000), which would further damage local tissues.

No significant COX-2 expression was found in intestines of fish from either population, but higher levels of EGC granule content (presumably major basic protein) in AW vs. KC fish suggest local inflammation. Human eosinophils are implicated in intestinal inflammation, and major basic protein induces intestinal epithelial apoptosis and increased cell turnover (sloughing) (Powell *et al.*, 2010). Thus, sloughing of intestinal epithelial cells is a protective mechanism that can eliminate pathogens, but also prevent intestinal epithelial cancers from forming. More than likely, this is why there is a uniform expression of CYP1A in AW intestinal epithelial cells; this barrier is not exposed to toxicants long enough to be severely damaged (as with livers), and therefore new cells are being exposed and induced on a rapid basis. To further substantiate this hypothesis, a good antibody marker for cell proliferation is needed that will work in fish. For example, anti-PCNA and anti-Ki67 antibodies work well in mammals, but not in fish.

This study leaves a large question: what happens to AW killifish between the time they go through development, via a reduced AhR2/CYP1A activity strategy in the face of high levels of toxic PAHs, hatching, and growth to adults? Do they lose some sort of suppressive mechanism (epigenetic) during transition and growth? If so, then it would be hard to explain how adult AW killifish can spawn F1 and F2 generations that maintain their resistance phenotype even when maintained in unpolluted water away from the

estuary (Meyer and Di Giulio, 2003; Clark *et al.*, 2013a; Clark *et al.*, 2013b). One clue may be related to the role(s) of different AhR forms (AhR1, AhR2) during development and into adult hood, as adult zebrafish are far less sensitive to TCDD than are development embryos and young larvae (Lanham *et al.*, 2012). This in turn may be explained by the expression and activity of both AhR1 and AhR2 in developing embryos, while AhR1 is not expressed or functional in adults (Hahn *et al.*, 1997; Andreasen *et al.*, 2002a; Franks *et al.*, 2005). In comparing the sensitivity of various fish to TCDD, salmon are more sensitive that more recently evolved fish, and this may be related to the multiple forms of AhR2 (Hansson and Hahn, 2008), with each having the ability to bind and respond to ligand. However, at least in red sunbream, both AhR1 and AhR2 can bind ligands, though the affinity for ligand is much higher in for AhR2 (Bak *et al.*, 2013). It is important to point out that most of these comparisons are done with recombinant proteins expressed in COS-1 cells, not whole-live embryos or adults.

CONCLUSIONS AND SUMMARY

From this study, I can conclude that Atlantic killifish inhabiting the waters and sediments of the Atlantic Wood superfund site have active AhR2-related signaling capabilities. This is demonstrated in a uniform CYP1A expression in intestines of AW fish, as well as in some of the livers, though in most cases, this may be related to intrinsic biology of tumor type and state of progression. Also found in this study, is a state of inflammation in AW killifish livers, as demonstrated by high expression of COX-2, and this may be the most novel finding. Altered expression of CYP1A in AW livers was first shown nearly 20 years ago, but this is the first to demonstrate COX-2 expression in livers. Unlike what is seen in many mammalian tissues, where macrophages are associated with tumors, COX-2 expressing macrophages surround, but not invade lesions and tumors in these fish. An addition finding is that AhR-Repressor protein is expressed more in AW fish than in reference KC fish, and more so in females than males.

Unfortunately, mAb 9R1 does not seem to recognize it's epitope in fixed tissues, so at this point I don't know if elevated liver AhR-R in AW fish is primarily in normal tissue, or more heavily expressed in lesions and tumors. However, despite the shortcomings of this particular antibody, from this study an additional three antibodies were added to the growing toolbox of reagents for working with the Atlantic killifish. This toolbox will also aid those working with Gulf killifish, a sister species of the Atlantic killifish, in that all reagents developed for one work well for the other, and the same is true for molecular probes etc.

Future studies with PAHs on the southern branch of the Elizabeth River will need to focus on other sites because Atlantic Wood superfund site is now officially sealed off by a cofferdam, and back-filled with sediments surrounding the site, then covered with a concrete "lid". As recently described (Clark *et al.*, 2013b), another creosote-contaminated site, referred to as "Republic" also has high levels of sediment PAHs, and killifish from this site also exhibit a resistance phenotype, at least with developing embryos. This site may allow researchers to continue with my line of research, though preliminary studies are required to confirm liver pathologies, induced intestine CYP1A, and other aspects described at the AW site. Two very key questions must be answered going forward: 1. What are the GST classes expressed in creosote-adapted killifish, and what are their regulatory restraints, and 2. Are there unique AhR2 nsSNPs in these fish, thus allowing them to not respond to typical PAH ligands? These two questions can be answered with information now available from the killifish genome project, and using approaches recently published from Dr. Mark Hahn's lab.

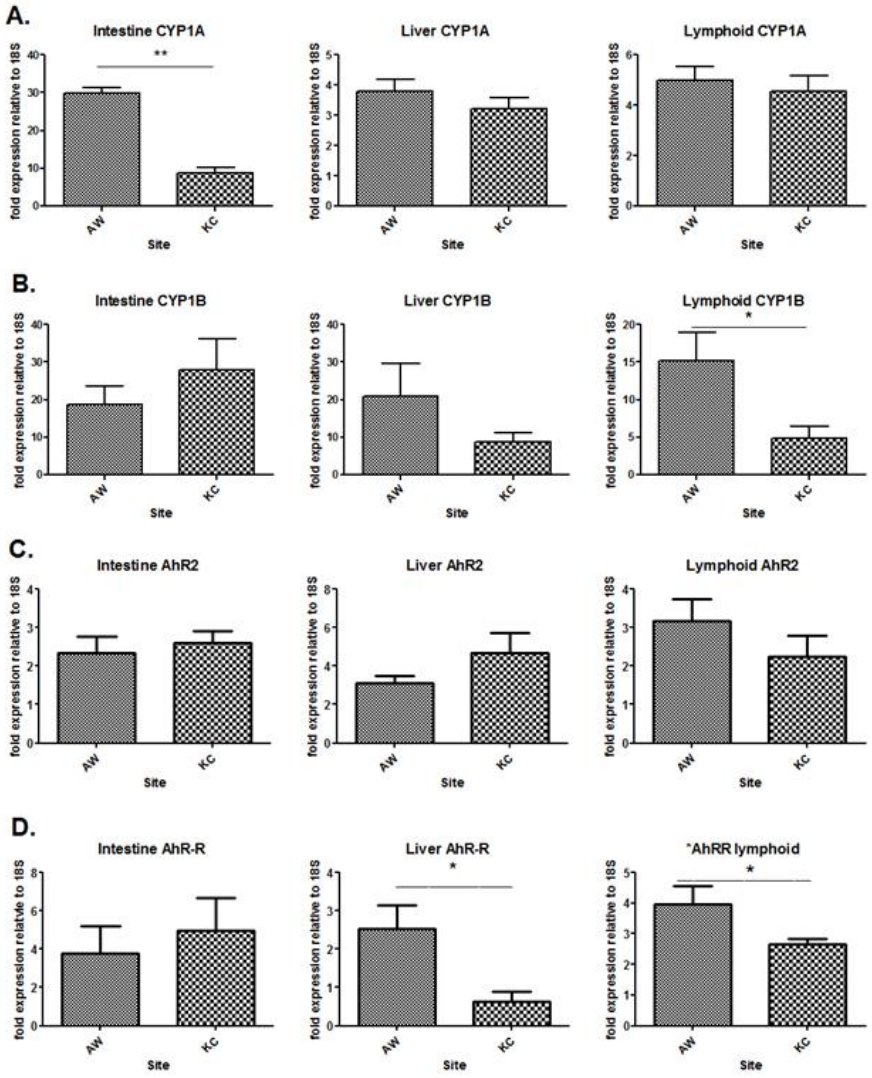

Figure 1. qRT-PCR of relative fold expression of target CYP1A, CYP1B, AhR2, and AhRR expression in the intestines of AW and KC *F. heteroclitus* populations. Fold expression values were calcualted to relative 18S levels using the Pfaffl method which uses the cycle number, Ct, and amplification efficiences. Error bars show ± SEM, standard error of the mean, of the 4 samples. * Indicates statistically significant difference in male and female AW intestines when compared against male and female KC intestines ($p \leq 0.05$).

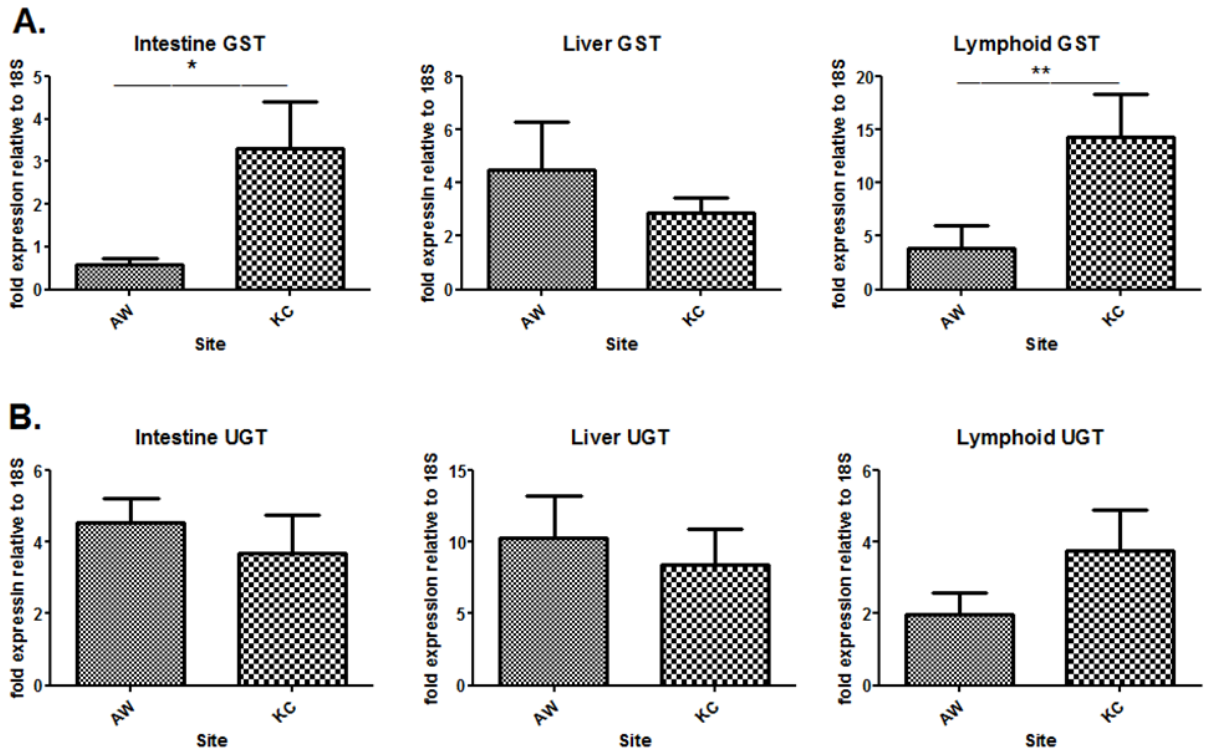

Figure 2. qRT-PCR of relative fold expression of target phase II detoxyfing enzyme genes, GST and UGT *gene* expression in the livers of AW and KC *F. heteroclitus* populations. Fold expression values were calcualted to relative 18S using the Pfaffl method which uses the cycle number, Ct, and amplification efficiences. Error bars show ± SEM, standard error of the mean, of the 4 samples. * Indicates statistically significant difference in male and female AW livers when compared against male and female KC livers ($p \leq 0.05$).

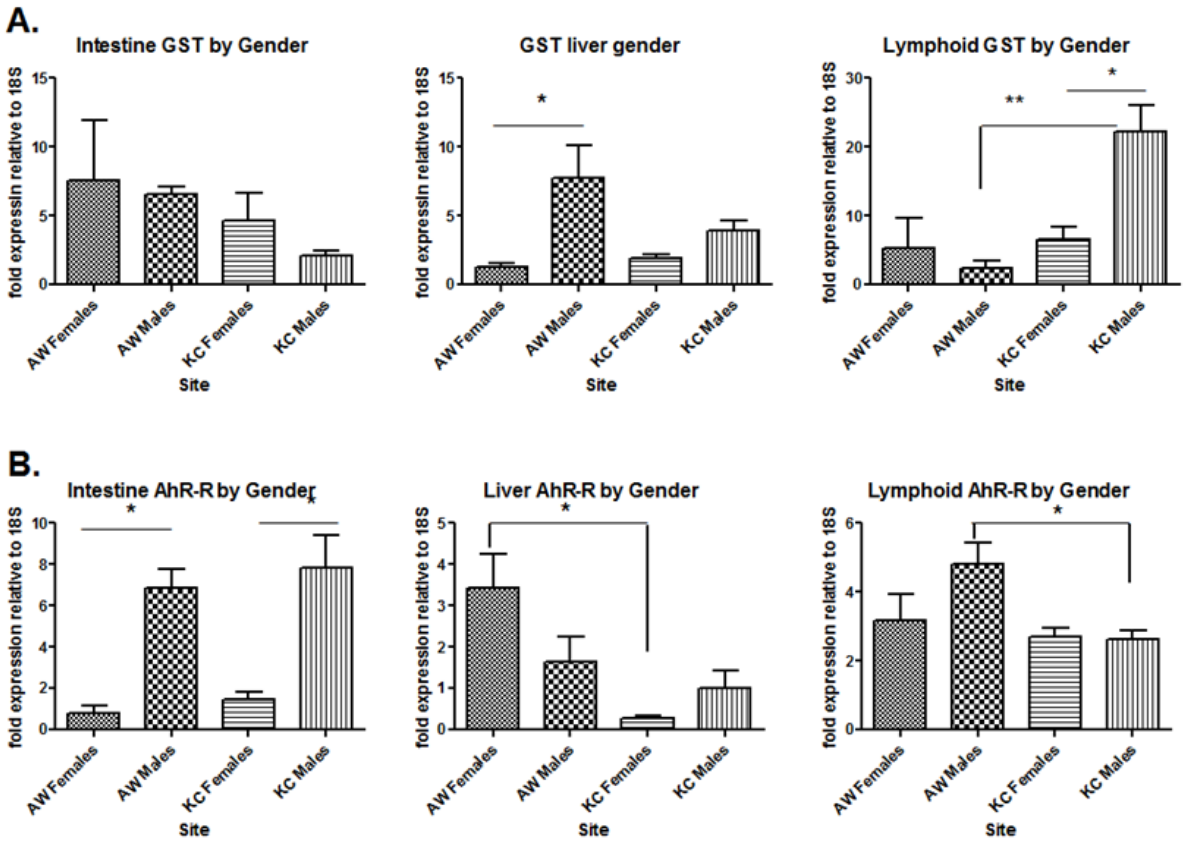

Figure 3. qRT-PCR of relative fold expression of target GST and AhRR by gender in the intestines of AW and KC *F. heteroclitus* populations. Fold expression values were calcualted to relative 18S levels using the Pfaffl method which uses the cycle number, Ct, and amplification efficiences. Error bars show ± SEM, standard error of the mean, of the 4 samples. * Indicates statistically significant difference in male and female AW intestines when compared against male and female KC intestines ($p \leq 0.05$).

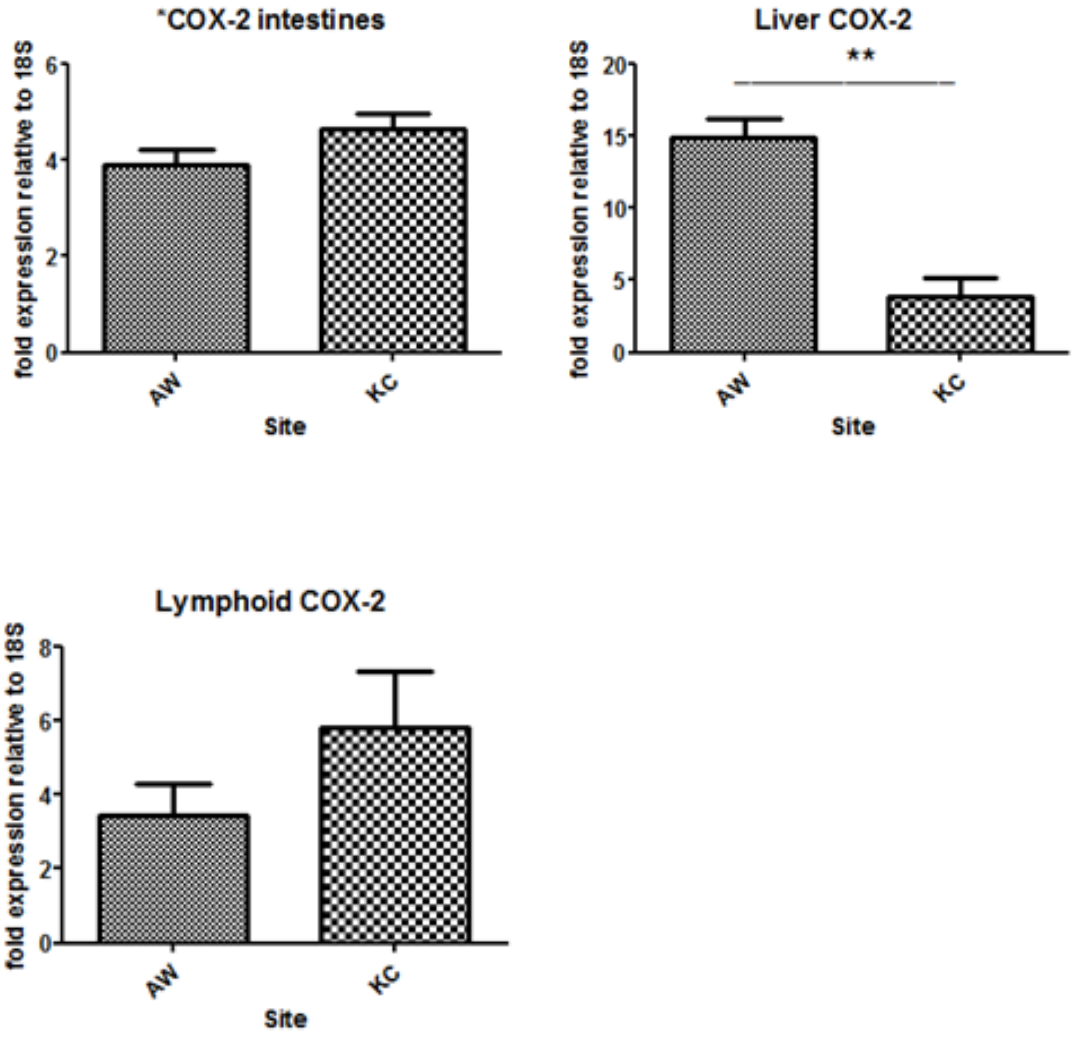

Figure 4. qRT-PCR of relative fold expression of target COX-2 expression in the intestines of AW and KC *F. heteroclitus* populations. Fold expression values were calcualted to relative 18S, 18S ribosomal RNA housekeeping gene, using the Pfaffl method which uses the cycle number, Ct, and amplification efficiences. Error bars show ± SEM, standard error of the mean, of the 4 samples. * Indicates statistically significant difference in male and female AW intestines when compared against male and female KC intestines ($p \leq 0.05$).

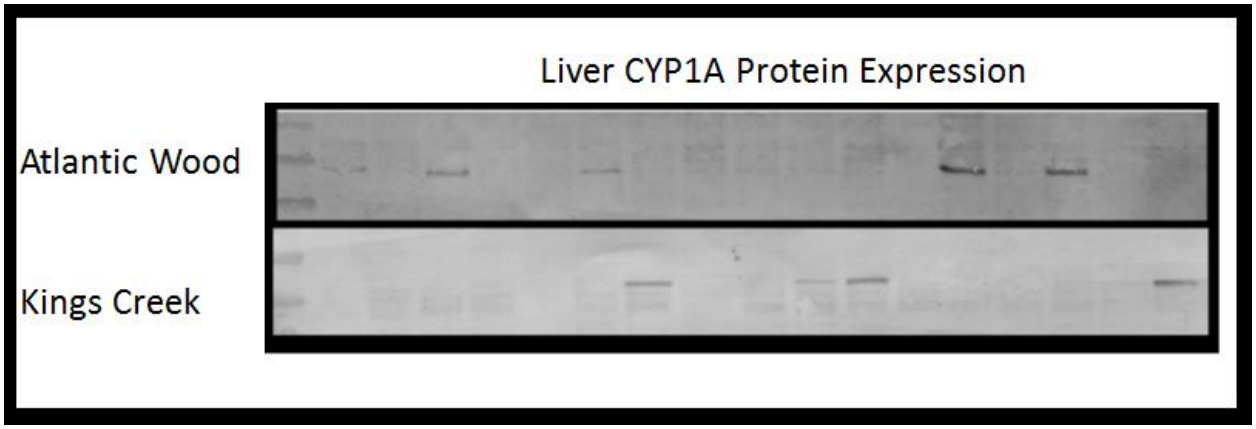

Figure 5. Expression of CYP1A in homogenates from 16 mummichog livers collected from Atlantic Wood (AW) and King's Creek (KC) sites in VA. Liver proteins were probed with mAb C10-7 for CYP1A detection. 30ul of protein were subjected to SDS-PAGE and immunoblotting.

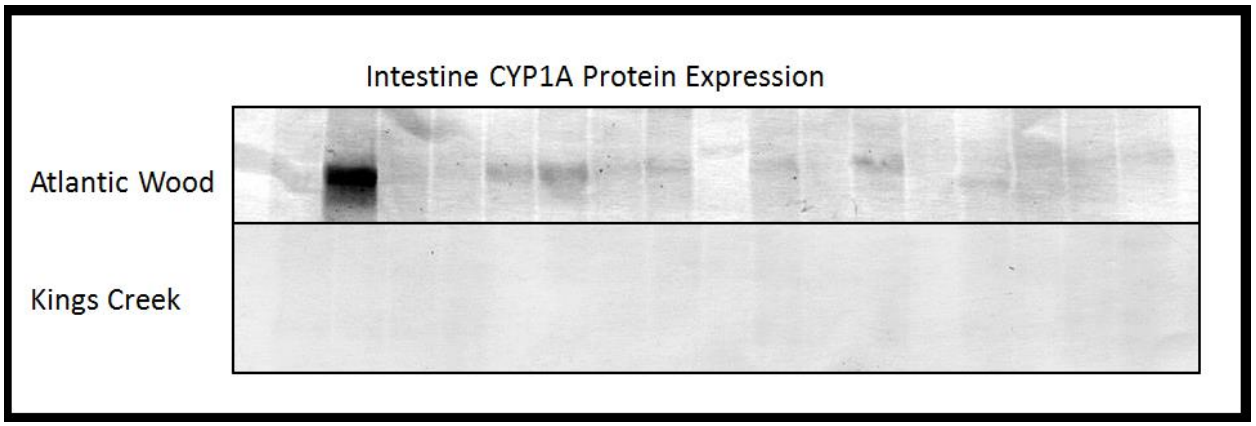

Figure . 6Expression of CYP1A in homogenates from 16 mummichog intestines collected from Atlantic Wood (AW) and King's Creek (KC) sites in VA. Intestine proteins were probed with mAb C10-7 for CYP1A detection from 8 adult males (shown at the left of the molecular weight marker) and 8 females (shown in the further right). 30 µg of protein were subjected to SDS-PAGE and immunoblotting.

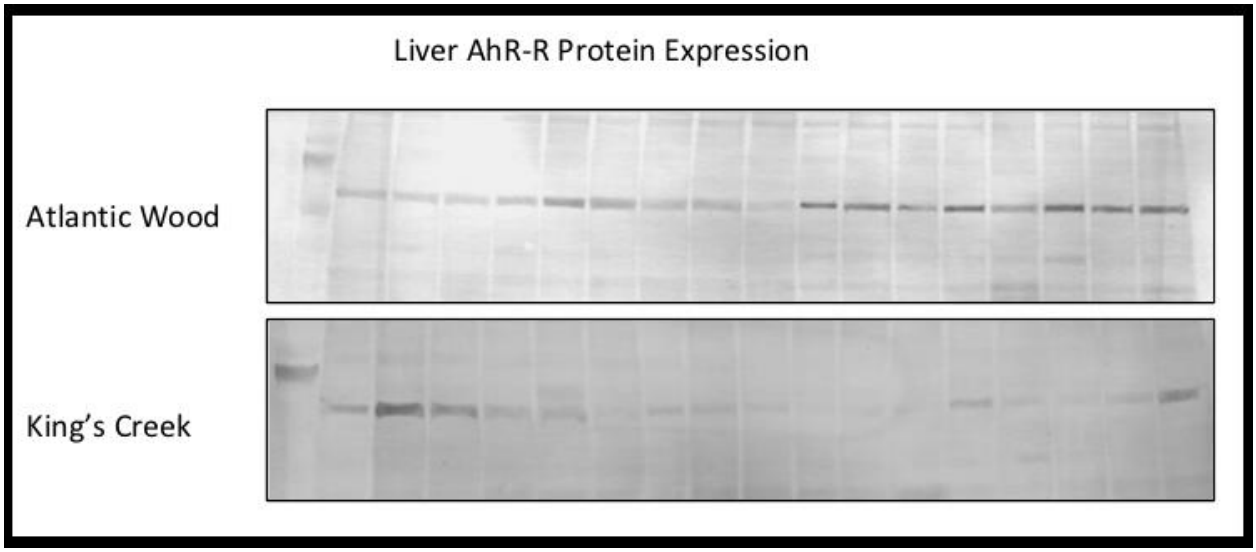

Figure 7. Expression of AhR-R in homogenates from 16 mummichog livers collected from Atlantic Wood and King's Creek sites in VA. Liver proteins were probed with mAb R91 for AhR-R detection from 8 adult males (left to right) and 8 adult females (fartherst 8 samples). 30 µg of protein were subjected to SDS-PAGE and immunoblotting.

Table 2.

Histopathology of Atlantic Wood and King's Creek Livers

Atlantic Wood site (n=94)	King's Creek site (n=90)
▪ Normal – 9%	▪ Normal – 100%
▪ Altered Hepatic Cell Foci – 18%	
▪ Eosinophilic and or Basophilic Foci – 30%	
▪ Tumors – 37%	

Table 2. Summary of histopathological findings in livers from Atlantic Wood superfund site vs King's Creek. Tissues were formalin-fixed and paraffin-embedded, then processed for H&E staining, following by examination by a histopathologist.

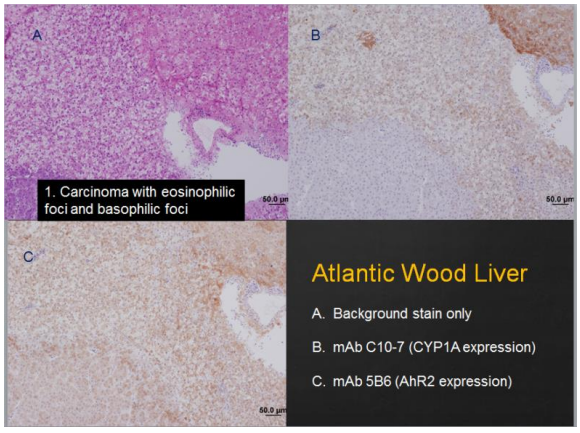

Figure 8. Immunohistochemistry of liver tissue from mummichogs collected at the Atlantic Wood site in VA. 5uM tissue sections were prepared for IHC and probed with backgroumd stain only (A), mAb C10-7 (B), or mAb 5B6 (C). Images were captured using 10x lens. Postive staining is noted by a dark-red intensity.

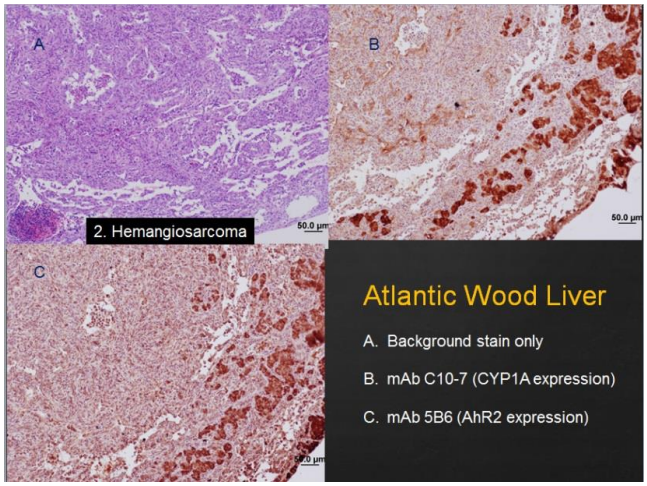

Figure 9. Immunohistochemistry of liver tissue from mummichogs collected at the Atlantic Wood site in VA. 5uM tissue sections were prepared for IHC and probed with backgroumd stain only (A), mAb C10-7 (B), or mAb 5B6 (C). Images were captured using 10x lens. Postive staining is noted by a dark-red intensity.

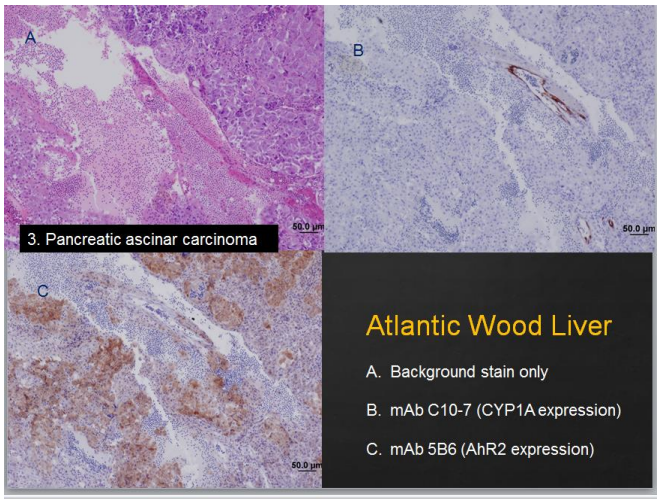

Figure 10. Immunohistochemistry of liver tissue from mummichogs collected at the Atlantic Wood site in VA. 5uM tissue sections were prepared for IHC and probed with backgroumd stain only (A), mAb C10-7 (B), or mAb 5B6 (C). Images were captured using 10x lens. Postive staining is noted by a dark-red intensity.

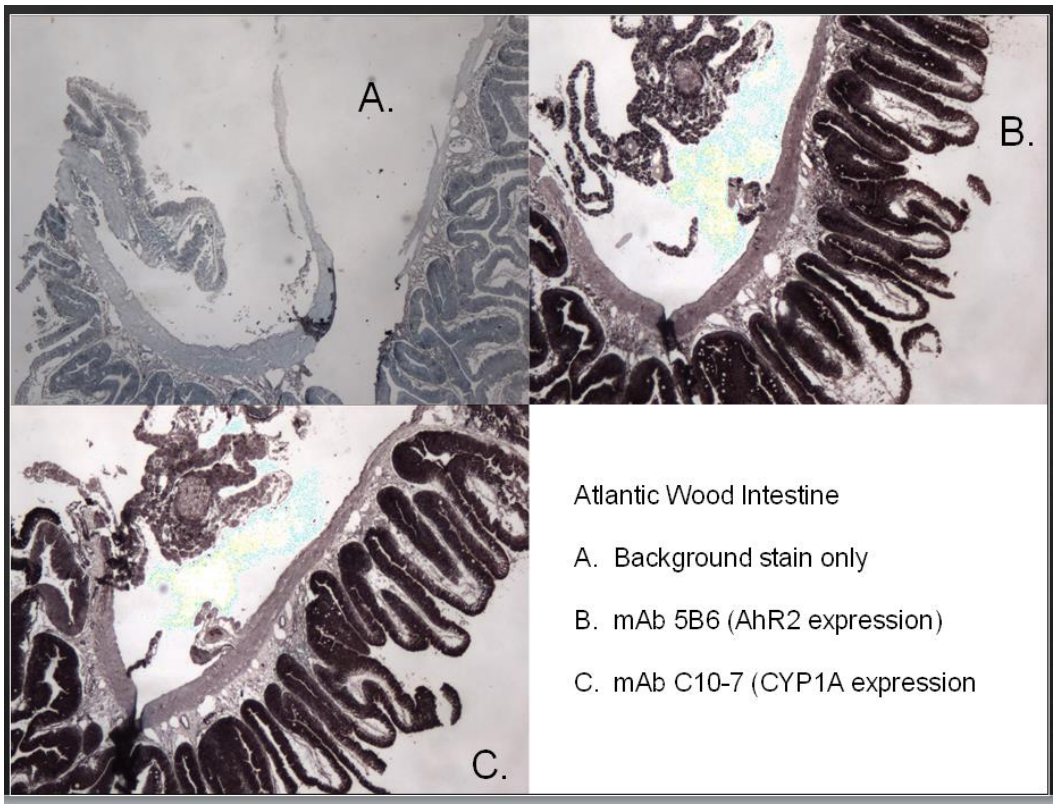

Figure 11. Immunohistochemistry of intestine tissue from mummichogs collected at the Atlantic Wood site in VA. 5uM tissue sections were prepared fro IHC and probed with background stain only (A), mAb 5B6 (B), or mAb C10-7 (C). Images were captured using 4X lens. Positive staining is noted by the dark-red/brown intensity. Note that epithelial cells stain for both AhR2 and CYP1A proteins.

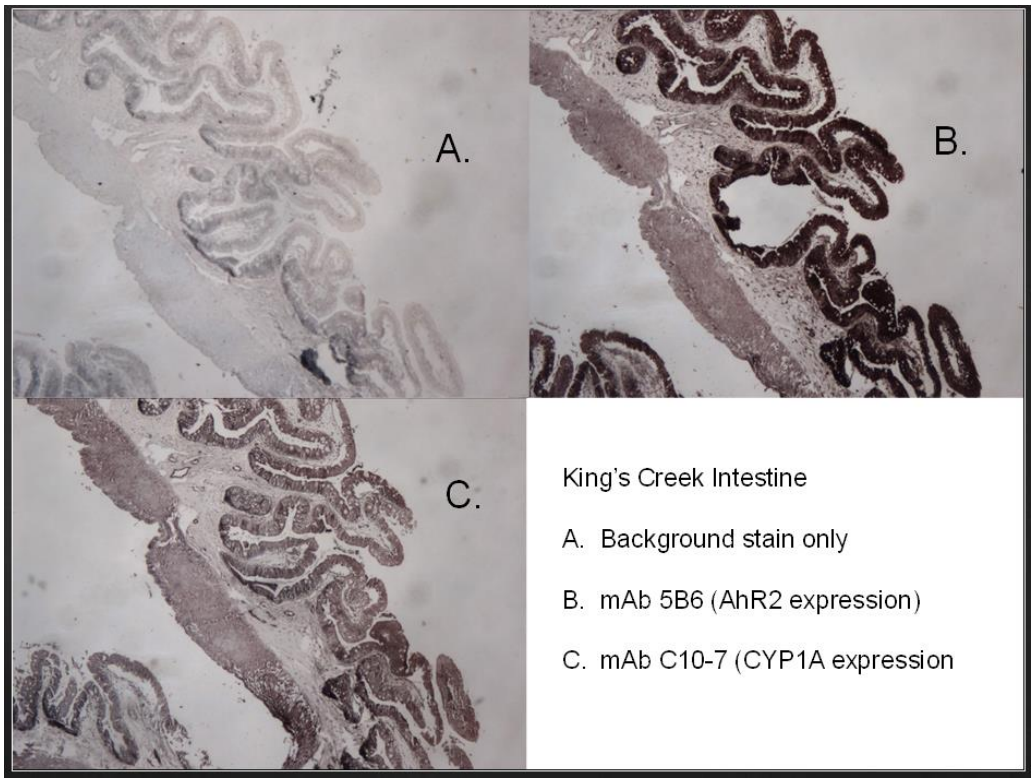

Figure 12. Immunohistochemistry of intestine tissue from mummichogs collected at the King's Creek site in VA. 5uM tissue sections were prepared fro IHC and probed with background stain only (A), mAb 5B6 (B), or mAb C10-7 (C). Images were captured using 4X lens. Positive staining is noted by the dark-red/brown intensity. Note that epithelial cells stain for both AhR2 and CYP1A proteins.

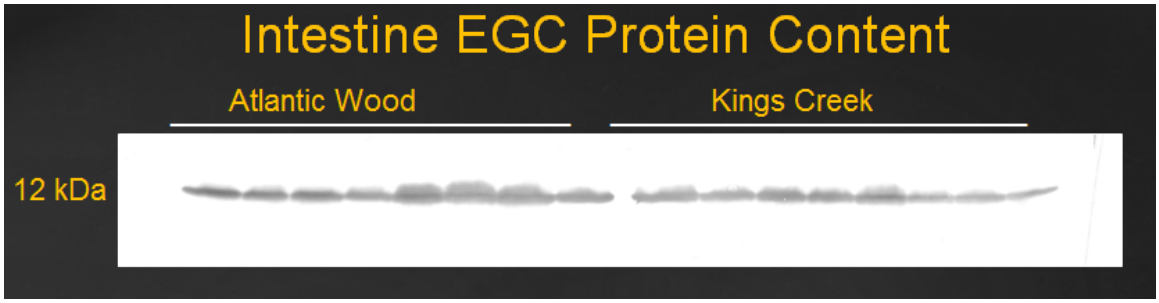

Figure 13. Expression of major basic protein in intestinal lysates from AW and KC mummichog populations. Intestinal lysates from 8 mummichogs from AW and 8 from KC were probed with mAb 2c11 for major basic protein of ECG cell detection. 30ul of protein per sample were subjected to SDS-PAGE and immunoblotting to show increasing band intensity with increasing protein concentration. Antibody picks up the protein at 12kDa.

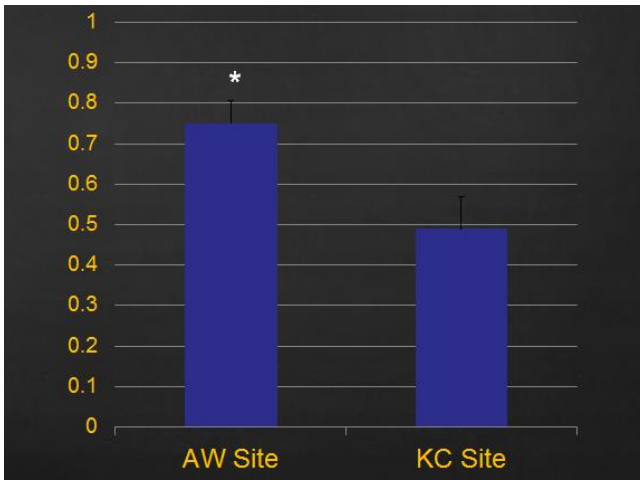

Figure 14. ELISA for major basic protein content in intestine lysates from mummichogs collected at the Atlantic Wood (AW) Superfund site and its historical reference site, King's Creek (KC) in VA. Capture antibody was mAb2c11. Data are O.D. units. Bars represent the average values of n=16 individuals and bars represent the standard error of the means. * P≤.05.

Figure 15. ELISA for lysozyme content in intestine lysates from mummichogs collected at the Atlantic Wood (AW) Superfund site and its historical reference site, King's Creek (KC) in VA. Capture antibody was mAb M24-2. Data are O.D. units. Bars represent the average values of n=16 individuals and bars represent the standard error of the means.

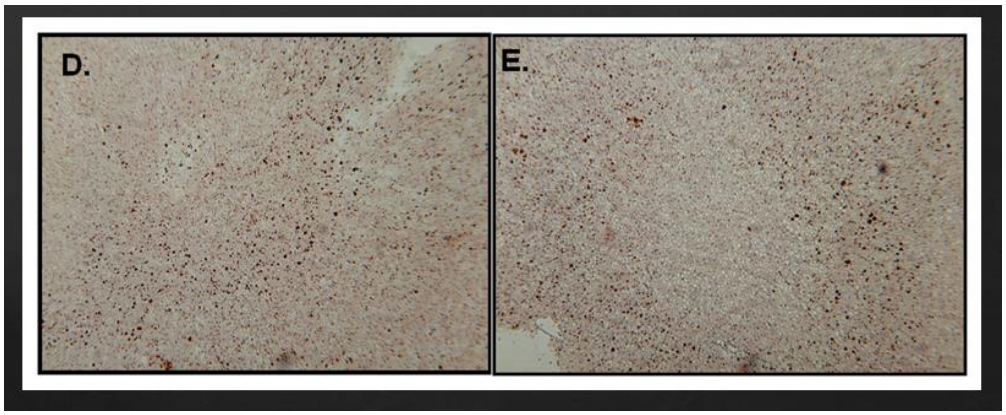

Figure 16. Immunohistochemistry of liver tissue with a pancreatic tumor obtained from an AW mummichogs. 5uM tissue sections were prepared fro IHC and probed with mAb CX53-1 against Cox-2. Images were captured using 10X objective. Positive staining is noted by the dark-red/brown intensity. Note high numbers of COX-2 staining cells outside of the tumor.

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