

12-2010

Development, characterization, and technical applications of an atlantic killifish AhR-2 specific monoclonal antibody (mAb 5B6)

Josephine Wojdylo

Clemson University, jwojdyl@bellsouth.net

Follow this and additional works at: https://tigerprints.clemson.edu/all_theses

 Part of the [Biology Commons](#)

Recommended Citation

Wojdylo, Josephine, "Development, characterization, and technical applications of an atlantic killifish AhR-2 specific monoclonal antibody (mAb 5B6)" (2010). *All Theses*. 1034.

https://tigerprints.clemson.edu/all_theses/1034

This Thesis is brought to you for free and open access by the Theses at TigerPrints. It has been accepted for inclusion in All Theses by an authorized administrator of TigerPrints. For more information, please contact kokeefe@clemson.edu.

DEVELOPMENT, CHARACTERIZATION, AND TECHNICAL APPLICATIONS OF
AN ATLANTIC KILLIFISH AHR-2 SPECIFIC MONOCLONAL ANTIBODY
(mAb 5B6)

A Thesis
Presented to
the Graduate School of
Clemson University

In Partial Fulfillment
of the Requirements for the Degree
Master of Science
Environmental Toxicology

by
Josephine Veronique Wojdylo
December 2010

Accepted by:
Charles D. Rice, PhD, Committee Chair
Thomas R. Scott, PhD
Peter Van den Hurk, PhD

ABSTRACT

The aryl hydrocarbon receptor (AhR) functions as a ligand-activated transcription factor normally found in the cytoplasmic compartment of cells held by chaperones and immunophilin-like proteins. Ligand binding dissociates the AhR/ligand from chaperone proteins, allowing translocation to the nucleus with subsequent transcription of a suite of responsive genes, most notably Phase I, II, and III drug metabolism genes. Select environmental contaminants such as co-planar PCBs, planar polyaromatic hydrocarbons (PAHs), and halogenated aromatic hydrocarbons (HAHs) are potent AhR agonists, with 2,3,7,8-tetrachlorodibenzodioxin (TCDD) being one of the most potent. Adverse effects of exposure to these potent environmental AhR ligands include immune suppression, reproductive, and developmental disorders. Mammals express a single AhR protein, while fish express both AhR1 and AhR2. However, to date only the AhR2 protein appears to be involved in mediating the toxic effects of known xenobiotic AhR ligands.

Using bacterial expression plasmid systems, a recombinant Atlantic killifish, *Fundulus heteroclitus*, AhR2 protein was expressed and used to produce a monoclonal antibody (mAb 5B6) that does not cross-react with AhR1. AhR2 expression can be detected with abundance in the cytosol of individual cells and in select organs. By testing the antibody against paraffin-embedded tissues, it was found that mAb 5B6 requires microwaving tissues under high pH conditions to properly recognize its epitope. High levels of AhR2 protein are detected in the liver, spleen, intestine, and anterior kidney. Experimental exposures to the potent AhR2 ligand PCB-126 induce

expression of both AhR2 and CYP1A proteins in most tissues, especially the intestine and liver. Basal expression of AhR2 protein was determined in livers of Atlantic killifish collected at the US-EPA Superfund site in Portsmouth VA, a site heavily contaminated with creosote and containing very high levels of PAHs. Several livers from the Superfund site harbored aggressive tumors and other hepatic lesions. AhR2 protein expression was high in normal tissue, but not cells within lesions. Overall, CYP1A protein expression patterns mirror those of AhR2 protein. This is the first study to examine AhR2 expression in tissues isolated from fish collected in the field, and like CYP1A, this protein may be a sentinel biomarker in future studies.

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, Vixey, Skrat, and Constantine. I could not have done any of this without their love and support.

ACKNOWLEDGMENTS

I would like to thank Dr. Rice, my advisor, for all of his support, guidance, and encouragement during my research. I appreciate all the faith he has put in me especially during the many trials and tribulations of scientific research. I am grateful for all the knowledge he has shared with me about immunology and for all the advice he has given me about life in general. I feel privileged to have been given the opportunity to work on this project and the direction it has taken. I would also like to thank my other committee members, Dr. Thomas Scott and Dr. Peter Van den Hurk, who have also supported and guided me through my research. Additionally, I would like to thank Nancy Korn for her support and guidance in the preparation of many of my slides. I would also like to thank my friends and lab mates, Abby Babcock and Amy Anderson for all their support. I would also like to thank my friends who made me laugh during the hard times: Julia Wojdylo, Beth Farwell, Stephanie Riffle, Farzana Federaous, Amanda Steffens.

TABLE OF CONTENTS

	Page
TITLE PAGE	i
ABSTRACT	ii
DEDICATION	iv
ACKNOWLEDGMENTS	v
LIST OF FIGURES	vii
CHAPTER	
I. LITERATURE REVIEW	1
Introduction to <i>Fundulus heteroclitus</i>	1
<i>Fundulus heteroclitus</i> as research animals	2
Introduction to aryl hydrocarbon receptor (AhR)	2
Ligands of AhR	3
Activation of AhR	4
AhR1 and AhR2	7
Location and expression of AhR	9
Effects of AhR activation	10
mAb for AhR2 in <i>Fundulus</i>	11
II. DEVELOPMENT, CHARACTERIZATION, AND TECHNICAL APPLICATIONS OF AN ATLANTIC KILLIFISH AHR-2 SPECIFIC MONOCLONAL ANTIBODY (mAb 5B6)	13
Introduction	13
Materials and Methods	17
Results	27
Discussion	40
Literature Cited	44

LIST OF FIGURES

Figure	Page
1. Aryl Hydrocarbon Receptor Pathway.....	6
2. Purification of his-tagged rAhR2 from DE3 <i>E. coli</i> cells.....	27
3. Immunoblot with mAb 5B6 against rAhR2, COS-Expressed AhR2 and tissue AhR2.....	28
4. Intracellular expression of AhR2 proteins in lymphoid cells of mummichogs.....	29
5. ELISA for AhR2 in liver S-9 fraction preparations in mummichogs collected at the Atlantic Wood (AW) Superfund site and a reference site, King's Creek (KC).....	30
6. Liver homogenates from lab-reared Belle Baruch, SC (GT), King's Creek, VA (KC), and Atlantic Wood site, Elizabeth River VA (AW).....	31
7. Expression of CYP1A and AhR-2 in liver S-9 fractions from 8 mummichogs collected from the Atlantic Wood and Kings Creek sites in VA.....	32
8. Immunohistochemistry of anterior kidney tissue from mummichogs collected at King's Creek site in VA.....	33
9. Immunohistochemistry of anterior kidney tissue from mummichogs collected at the Atlantic Wood site in VA.....	34
10. Immunohistochemistry of intestine tissue from mummichogs collected at King's Creek site in VA.....	35
11. Immunohistochemistry of intestine tissue from mummichogs collected at the Atlantic Wood site in VA.....	36

List of Figures (Continued)

Figure	Page
12. Immunoblot with mAb 5B6 against rAhR2, COS-Expressed AhR2 and tissue AhR2	37
13. Immunohistochemistry of anterior kidney tissue from mummichogs collected at the Atlantic Wood site in VA	38
14. IHC for AhR2 and CYP1A proteins in intestines from mummichogs given i.p. injection of either corn oil carrier, or carrier containing 1 mg/kg 3MC	39

CHAPTER ONE

REVIEW OF LITERATURE

Introduction to *Fundulus heteroclitus*

The estuarine killifish, *Fundulus heteroclitus*, (Linnaeus) also known as the mummichog or Atlantic killifish is a small, ubiquitous, teleost fish that is distributed along the east coast of North America, extending from Newfoundland to northern Florida (Bigelow and Schroeder, 1953; Hardy, 1978). These fish normally inhabit saltwater marshes, estuaries, tidal creeks (Teo and Able, 2003; Kneib, 1986) and due to their limited migration (Duvernell et. al., 2008) these fish have a high home range fidelity throughout their entire life cycle (Kneib, 1986). Mummichogs are hardy euryhaline fish that can occupy bodies of water with ranges in salinity from freshwater, 0 parts per thousand (ppt), to salinities of upwards to 120.3ppt (Abraham, 1985; Griffith, 1974).

The mummichog is a sexually dimorphic fish. The males are easily recognized by having horizontal stripes and colorful spots compared to the females, which lack stripes and are pale in comparison (Bigelow and Schroeder, 1953). These fish spawn by lunar cues during high tide on the new and full moon from spring to fall (Cochran *et al.*, 1988; Taylor and DiMichele, 1980). Female mummichogs are oviparous egg layers. During spawning, females are capable of producing several hundred eggs, which remain transparent throughout embryonic development (Armstrong and Child, 1965).

***Fundulus heteroclitus* as research animals**

Due to the mummichog's small size and hardiness it is simple to culture them in captivity (Bigelow and Schroeder, 1953; Overstreet *et al.*, 2000). Since mummichogs live along the coast of embayments and waterways, which are often exposed to a myriad of anthropogenic contaminants from nearby factories and production plants that produce by-products such as PCBs (Polychlorinated biphenyls) and PAHs (polycyclic aromatic hydrocarbons), these fish have adapted to their surroundings and are abundant in pristine as well as polluted environments. Because of this, these *Fundulus* fish are often used as an indicator species to monitor the overall health of its ecosystem and have therefore been used in a plethora of toxicological studies (Frederick *et al.*, 2007; Nacci *et al.*, 2010; Vogelbein *et al.*, 1990; Wassenberg *et al.*, 2002; Munns *et al.*, 1997; van den Hurk *et al.*, 2000; Weis, 2002; Nevid and Meir, 1993; Nevid and Meier, 1994; Roszell and Rice, 1998; Rice and Xiang, 2000).

Introduction to aryl hydrocarbon receptor (AhR)

The aryl hydrocarbon receptor, AhR, belongs to a multigene family of transcription factors that has a signature PAS domain. The AhR's function is to play a role in detecting and adapting to environmental pollutants by regulating drug-metabolizing enzymes. In the PAS family of proteins, the PAS domain is named after the first letter of the first three founding proteins in the family, PER, ARNT, and SIM, respectively. The PAS domain within AhR allows for either the homotypic interaction

between another PAS protein or a heterotypic interaction, such as with a chaperone protein or ligand. Within the AhR protein, there is a second domain which contains 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 (reviews by Gu *et al.*, 2000; Kewley *et al.*, 2004).

The AhR is a ligand-activated transcription factor that primarily activates genes which elicit biochemical and toxic responses in the presence of ligands. When AhR is in its latent form, unbound to its ligand, it is normally found in the cytoplasm of cells and is stabilized by chaperone proteins including two 90-kDa heat shock proteins, Hsp90 (reviews by Kewley *et al.*, 2004; Hahn, 1998; Merson *et al.*, 2006), p23, and hepatitis B virus X-associated protein (also known as XAP2 or AIP or Ara9) (Denison *et al.*, 2002).

Ligands of AhR

A large range of ligands can bind to the AhR binding site. These ligands are categorized into two basic types: exogenous compounds, such as xenobiotics and other anthropogenic compounds, and endogenous compounds, which are dietary or biologically derived (reviewed by Denison and Nagy, 2003). The most common and highest affinity ligands of 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 (Cherng *et al.* 1996). These exogenous compounds include

halogenated aromatic hydrocarbons, HAHs (for instance TCDD - 2, 3, 7, 8-tetrachlorodibenzo-p-dioxin) and planar aromatic compounds (reviewed by Hahn, 1998). The planar aromatic compounds include the polyaromatic hydrocarbons, PAHs, such as benzo(a)pyrene and 3-methylcholanthrene, and polychlorinated biphenyls, PCBs, like 3,3', 4, 4', 5-pentachlorobiphenyl PCB-126 (Quattrochi, *et al.*, 1994; reviewed by Hahn, 1998).

Different types of ligands have different binding affinities for AhR. For instance, ligands such as HAHs have relatively high binding affinities for AhR that range in the neighborhood of pM to nM, whereas ligands such as PAHs have significantly lower binding affinities to AhR, being in the nM to μ M scale (reviewed by Whitlock, 1999; Denison and Nagy, 2003; Mitchell and Elferink, 2009).

While no high affinity endogenous ligands have been found, low affinity ligands from plant products such as indole-containing compounds, sterols, and tryptophan photoproducts, flavonoids, carotenoids, to name a few, exist (Denison *et al.*, 2002; Mitchell and Elferink, 2009).

Activation of AhR

Upon ligand binding, a conformational change within the ligand:AhR complex results in the exposure of the NLS, nuclear localization sequence, followed by nuclear translocation (Denison *et al.*, 2002). Once inside the nucleus, AhR will dissociate from its protein complex (Denison *et al.*, 2002; Mitchell *et al.*, 2008). Since AhR belongs to a Class I group of bHLH/PAS proteins that neither heterodimerise nor homodimerise with

other Class I proteins, AhR must dimerise with a ubiquitous Class II bHLH/PAS protein, known as aryl hydrocarbon receptor nuclear translocator, ARNT. ARNT belongs to a promiscuous class of bHLH/PAS proteins that can homodimerise or heterodimerise with other bHLH/PAS proteins (reviewed by Kewley *et al.*, 2004).

Once AhR and ARNT form a heterodimer, an active transcription complex in the nucleus of the cell is formed. The ligand bound complex will then bind to xenobiotic response elements (XREs), also known as dioxin responsive elements (DREs) or aryl hydrocarbon responsive elements (AHREs), on the nuclear DNA. The consensus sequence for these elements is 5'-(T/G)NGCGTG-3' (ZeRuth and Pollenz, 2007; reviewed by Whitlock, 1999). This interaction will cause a transcriptional activation of a variety of many target genes such as *CYP1A1*, *CYP1A2*, *CYP1B1*, flavin monooxygenases (FMOs), glutathione S-transferases (GSTs), and diphosphate glucuronosyltransferases (UGTs) which will then translate into enzymes that will be involved in xenobiotic metabolism as shown in Figure 1 (reviewed by Whitlock, 1999; Hahn *et al.*, 2005).

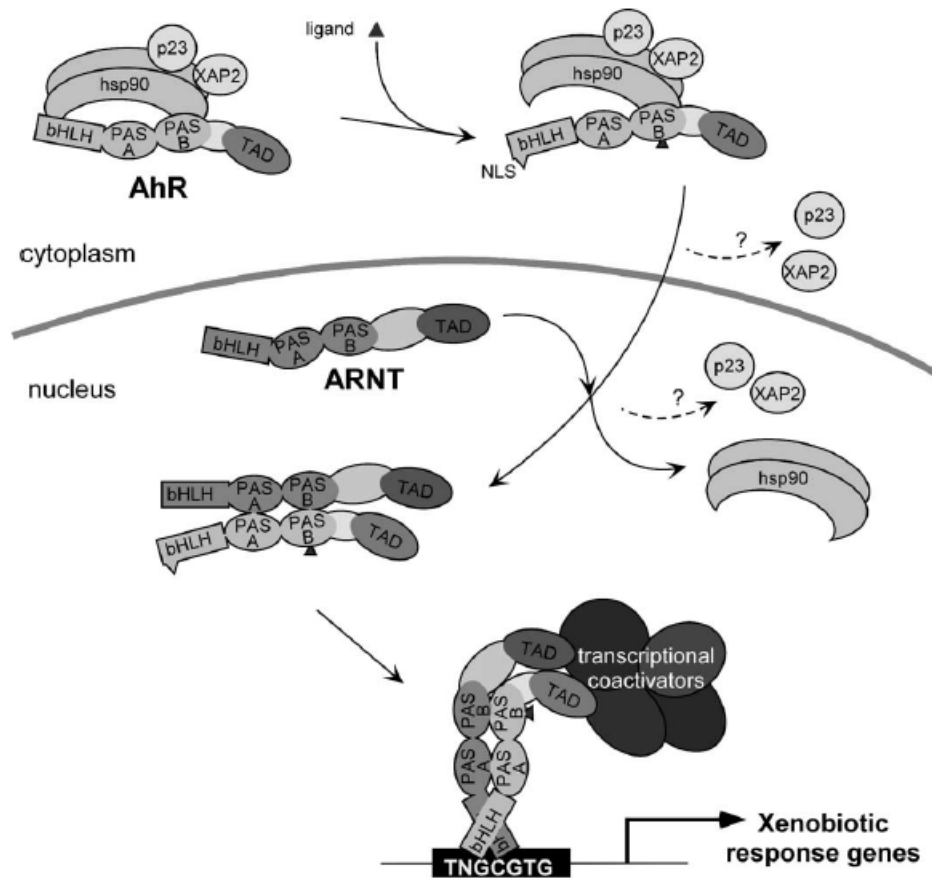


Fig 1. The activation of the aryl hydrocarbon receptor through ligand binding.
(reviewed by Kewley *et al.*, 2004)

One of the most characterized genes that shows the downstream effects of AhR induction, *Cyp1A*, also known as cytochrome *P4501A1* (reviewed by Whitlock, 1999). This classically studied gene belongs to the cytochrome P450, also known as CYP, family of phase I enzymes that primarily function as monooxygenases, meaning they insert one oxygen atom into a substrate. There are over 400 genes that have been identified for the CYP family, and each CYP's gene activation is controlled by different transcription factors that are present in distinct mechanistic pathways (Boelsterli, 2007).

CYPs are named according to their genetic relationships with each other. For instance, CYP1A1, the first 1 denotes to which gene family the enzyme belongs to where there is a forty percent or greater homology among members, the letter A stands for the subfamily the enzyme belongs to, and 1A1 denotes the individual gene of the protein. *CYP1A1*, *CYP1A2*, *CYP1B1* genes happen to become activated through ligand activation of AhR. Once these particular genes are induced, the enzymes of those translated genes will often reduce the biological half life of the parent compound, the initial ligand, or alter the response of the compound (Denison, *et al.*, 2002).

Besides activating the transcription of cytochrome P450 genes, the AhR/ARNT complex can also activate the transcription of AhR repressor (AhR-R) genes which are then translated into their protein products. Aryl hydrocarbon receptor repressor is a member of the bHLH/PAS family of proteins that down regulates the induction of AhR. AhRR is a negative regulatory loop in AhR mediated pathway that will compete with AhR for dimerization with ARNT, which will consequentially inhibit AhR function and resulting effects (Mimura *et al.*, 1999; Hahn *et al.*, 2005). Mechanistically, it thought that the AhR-R targets the AhR for ubiquination and subsequent degradation of the AhR.

AhR1 and AhR2

In mammals, there has only been one AhR receptor discovered thus far, while in bony fish, there have been up to six AhR paralogs discovered (Merson *et al.*, 2006). In *Fundulus heteroclitus*, there have been two highly divergent AhRs identified, AhR1 and AhR2 (Hahn *et al.*, 2006; Karchner *et al.*, 1999; Merson *et al.*, 2006). Hahn (2002)

speculates 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, 2002).

Fundulus AhR1 is more closely related to mammalian AhR and is considered its ortholog. The bHLH domain of AhR1 shares 83% amino acid identity compared to the domain of mammalian AhR. The bHLH domain of AhR2 shares a 73% amino acid identity compared to the domain of mammalian AhR. Phylogenetic analysis suggests that AhR2 may have evolved either after the divergence of bony fish from vertebrates or that this gene could have become lost in mammals long after they diverged from bony fish (Karchner *et al.*, 1999).

It has been shown that slight differences in amino acid sequences of mouse AhR strains can have substantial impacts on their ligand affinity which may in turn cause different physiological effects such as ligand sensitivity and toxicity (Hahn, 2002). Since AhR1 and AhR2 have different structures, binding affinities to ligands, and different levels of expression in tissues and organs, this suggests that they also have different functions from one another (Karchner *et al.*, 1999).

Fish larvae are very sensitive, especially during early development, to dioxin-like compounds, which are agonists for AhR (Hahn, 2002; Andreasen *et al.*, 2002; Belair *et al.*, 2001; Teraoka *et al.*, 2002). Based on expression studies, AhR2 appears to be the predominant form of AhR expressed in fish and is therefore thought to be the main mediator in ligand induced toxicity in fish, especially in larval fish and embryos

(Hahn, 2002; Karchner *et al.*, 1999; Andreassen, *et al.*, 2002; Prasch, *et al.*, 2003; Hahn *et al.*, 2005). In gene knock-down methods 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).

Location and expression of AhR

The level of expression of AhR varies in organs, tissues, cell types and 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 (reviewed by Kewley *et al.*, 2004; Hahn, 1998). Analyzing the varying degrees of AhR expression can be vital in determining the effects of different ligands (reviewed by Hahn, 1998).

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*. AhR2 is expressed in most tissues in *Fundulus*, whereas, AhR1 is most often expressed in the heart, brain, and gonads (Karchner *et al.*, 1999; Hahn, *et al.*, 2006).

Effects of AhR activation

The AhR comes from a family of bHLH/PAS family of proteins that are involved in developmental and environmental signaling (Hahn *et al.*, 2006; Gu *et al.*, 2000; Kewley *et al.*, 2004). AhR is considered to be involved with cell cycle control and controlling growth factor transduction pathways (Karchner, et al., 1999). Early exposures to AhR agonists, such as 2, 3, 7, 8-tetrachlorodibenzo-p-dioxin in larval zebrafish, *Danio rerio*, has shown to cause development toxicity such as stunted growth, impaired swim bladder, impaired jaw development and even mortality (Andreasen et al., 2002; Belair et al., 2001; Teraoka et al., 2002). AhR is also involved in vascular development and cell proliferation, as AhR knockout mice experience defects in vascular development and have a slower rate of cellular proliferation (Mitchell and Elferink, 2009).

Studies with AhR knockout mice have also shown that TCDD toxicity is caused mostly in the presence of AhR, suggesting that AhR is responsible for most if not all of the toxic effects caused by TCDD exposure such as cardiovascular dysfunction, edema, hemorrhages, jaw malformations, inhibition of growth and mortality (Prasch, *et al.*, 2003). Gene knock-down methods using morpholino-modified oligonucleotides (MO) have been used in AhR2 MO zebrafish which have shown to reduce the effects of TCDD toxicity (Hahn, *et al.*, 2005). Like *Fundulus*, zebrafish also have two AhRs, zfAhR1 which is similar to its mammalian counterpart, and zfAhR2 which is different from mammalian AhR. In an experiment where zebrafish were exposed to TCDD, the zfAhR1 seemed to be inactive, while zfAhR2 led to CYP1A2 induction and TCDD mediated toxicity (Andreasen, *et al.*, 2002; Hahn *et al.*, 2005).

In the mammalian AhR pathway there is only one AhR and it mediates a toxic response in TCDD and HAH exposure. In *Fundulus*, while the two AhRs have the capability to bind to TCDD, it has been discovered within tissues that expressed CYP1A (caused by PHAH exposure and a biochemical indicator of aryl hydrocarbon receptor induction) that there were nearly undetectable amounts of AhR1 mRNA in comparison to mRNA of AhR2 indicating that PHAH toxicity is mainly mediated through AhR2 and that above normal AhR1 expression is not a physiological response to a contaminant exposure (Powell, et al., 2000).

mAb for AhR2 in *Fundulus*

Information showing how conserved the AhR is among different taxa can be used as a guide for the selection of animal models to be used for research and how the generated data can be extrapolated in terms of their physiological responses to AhR induced xenobiotics and pollutants in order to predict similar results in other species such as humans. Knowing the mechanisms of action or how strongly xenobiotics bind to the AhR can help predict its downstream induction and can prove to be vital in predicting environmental risks (Hahn, 1998; Hahn *et al.*, 2005; Hahn *et al.*, 2006). Specific monoclonal antibodies that bind to key protein players in the pathway, such as AhR2, need to be developed in order to better understand the mechanism and physiological effects of AhR induction and overall function (Hahn, 2002; Merson *et al.*, 2006).

Polyclonal antibodies against *Fundulus heteroclitus* proteins involved in the AhR pathway have been made, such as anti-AhR 1, anti-AhR2, and anti-AhRR (Merson *et al.*, 2006). However, polyclonal antibodies have some drawbacks such as having limited quantities, as well as differences in activity from one animal to another as a result of genetic variability in antigen processing and presentation. Genetic differences in processing and presentation can result in the production of different antibodies (Rice *et al.*, 1998). The work in this paper shows the development, characterization, and technical applications of a monoclonal antibody, mAb 5B6, which detects AhR2 in *Fundulus heteroclitus*.

CHAPTER TWO

INTRODUCTION

The aryl hydrocarbon receptor (AHR) is a member of the basic helix-loop-helix PER-ARNT-SIM (bHLH-PAS) superfamily of proteins and functions as a ligand-activated transcription factor (Gu et al., 2000). It is normally found in the cytoplasmic compartment of cells held by chaperones p23/Hsp90 and the immunophilin-like XAP2 protein (Chen *et al.*, 1994; Elferink, 2003; Cox and Miller, 2004). Following the events of ligand binding and nuclear translocation, the chaperone proteins dissociate from the AhR/ligand complex allowing the ligand/AhR complex to form a heterodimer with aryl hydrocarbon receptor nuclear translocator (ARNT). This complex then binds to *cis*-acting response elements that in turn induce the transcription of a suite of responsive genes. Most notably of these responsive genes include phase I and II drug-metabolizing enzymes such as CYP1A1, CYP1A2, NQO1, ALDH3A1, UGT1A6 (Hahn, 1998; Nebert *et al.*, 2000; Merson *et al.*, 2009). Select environmental contaminants such as co-planar PCBs, planar polyaromatic hydrocarbons (PAHs), and halogenated aromatic hydrocarbons (HAHs) are potent AhR agonists, with 2,3,7,8 –tetrachlorodibenzodioxin (TCDD) being one of the most potent environmental ligands characterized to date.

Adverse effects of exposure to these potent environmental AhR ligands include immune suppression, reproductive, and developmental disorders (Holsapple *et al.*, 1996; Kaminski *et al.*, 2008). Furthermore, constitutively expressed AhR is associated with several cancers (Moennikes *et al.*, 2004) and mimics the effects of long term exposure to

environmental AhR ligands (Brunnberg *et al.*, 2006), namely chronic expression of CYP1A1 and reduced organ weights.

In addition to an association between the AhR and toxicity of planar HAHs and PAHs, signaling through the receptor regulates developmental cues and cell cycle progression through the induction of key regulatory proteins in the cell cycle pathway (Elferink *et al.*, 2001; Hines *et al.*, 2001; Reviewed in Elferink, 2003 and Nguyen *et al.*, 2008). Mechanistically, ligand-activated AhR results in the expression of P27^{Kip1}, which in turn represses CDK2 thereby inhibiting phosphorylation of pRb. Thus, a potent AhR ligand like TCDD will arrest cells in the G₀ – G₁ stage of the cell cycle. In the absence of ligand, E2F expression leads to presence of cyclins A and E, and thus increased activity of CDK2 and suppression of P27^{Kip1}.

Mummichogs, *Fundulus heteroclitus*, (a.k.a. Atlantic killifish) are hardy teleostean fish often used as an indicator species in environmental health monitoring and developmental toxicology (Frederick *et al.*, 2007; Nacci *et al.*, 2010 Vogelbein *et al.*, 1990; Wassenberg *et al.*, 2002; Munns *et al.*, 1997; van den Hurk *et al.*, 2000; Weis, 2002). Mummichogs live along the east coast high marshes of North America which are often exposed to a myriad of anthropogenic contaminants, especially near harbor estuaries. These fish spawn on the new and full moons throughout the warmer months, and have a small home range, thus multiple generations with home-site fidelity have lead to populations adapting to their surroundings and are abundant in pristine as well as polluted environments. Many of the contaminants that *Fundulus* are exposed to induce their toxicities through AhR-mediated pathways: the most notable are planar halogenated

aromatic hydrocarbons (HAHs), such as 2, 3, 7, 8-tetrachlorodibenzo-p-dioxin (TCDD) and co-planar PCBs (i.e., PCB-77, PCB-126, and PCB-169) and various polyaromatic hydrocarbons (PAHs), like benzo(a)pyrene and benzo(k)fluoranthene (Quattrochi, *et al.*, 1994; Hahn, 1998).

While there is only one mammalian AhR, which mediates toxic cellular responses to environmental contaminants, studies on teleostean fish have found two aryl hydrocarbon receptors: AhR1 and AhR2 (Hahn *et al.*, 1997; Roy and Wirgin, 1997; Abnet *et al.*, 1999; Tanquay *et al.*, 1999; Merson *et al.*, 2006). Both receptors are functional in that ligand binding leads to translocation to the nucleus, but the binding strength of AhR1 to the ligand is much less than what is observed with AhR2 (Karchner *et al.*, 2002; Andreason *et al.*, 2002). Furthermore, the expression of AhR1 appears to be high early on in embryonic development, but low in juvenile and adult fish (Andreason *et al.*, 2002). Functional studies involving inhibition of AhR1 and AhR2 mRNA expression via morpholinos show that embryonic toxicity, including developmental abnormalities, teratogenesis, and induction of CYP1A gene and protein expression is inhibited with AhR2 knockdown, but not with AhR1 (Clark *et al.*, 2010). Taken together, most molecular toxicologists agree that AhR2 is the primary receptor involved in the toxicity of compounds historically associated with AhR binding in mammals.

One of the missing pieces of the puzzle in understanding the role of AhR(s) in the myriad of toxicities resulting from ligands is tissue-level expression in unexposed animals as well those animals exposed to ligands in the environment. Whole tissue mRNA or protein expression of AhR does not yield specific cell types and/or location of

cells expression the AhR. Specific antibodies to the AhR would allow for such studies and the elucidation of cell and tissue expression. To date, rabbit polyclonal antibodies were developed against recombinant mummichog AhR1 and AhR2, but these antibodies are in limited supply, there seems to be a limited degree of specificity in terms of how clean immunoassays turn out (westerns, ELISAs, IHC, etc), and, as with all polyclonal antibodies the quality can vary from rabbit to rabbit. Monoclonal antibodies circumvent these issues, and can be generated in large quantities. Moreover, during the process of hybridoma selection and cloning, only those clones giving the best results in immunoassays are chosen. The work described herein details the development, characterization, and technical applications of a mummichog AhR2-specific monoclonal antibody. To the author's knowledge, this is the first study to show cellular-level expression of the AhR2 via immunohistochemistry in any species of fish.

MATERIALS AND METHODS

Expression and purification of rAhR2, and generation of mAb

A C-terminus portion of *Fundulus heteroclitus* AhR2 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). This expression plasmid system was kindly provided by Dr. Mark Hahn, Woods Hole Oceanographic Institute, Woods Hole, MS USA, and used for subsequent steps as outlined herein. Levels of protein expression using the strain of *E. coli* produced minimal amounts of rAhR2 at 37 °C for 3 hr in the presence of IPTG as described by Merson et al., 2006, therefore the pQE80/82 plasmid was isolated from BL21-CodonPlus (RP) cells and used to transfect the DE3 strain of *E. coli* (Stratagene). DE3 strain of *E. coli* cells harbor chaperone proteins designed to express proteins at cold temperatures (Arctic Express™, Stratagene). According to procedures outlined by Stratagene, cells expressing both rAhR2 and chaperone proteins are selected under ampicillin, gentamycin, and chloramphenicol induced-pressure during culture.

DE3 cells transfected with the pQE80/82 plasmid containing mummichog rAhR2 were grown at 37 °C overnight in 25 ml LB broth, then added to 500 ml of LB broth containing antibiotics and grown for 3 hr at 30 °C. Batches of transformed DE3 cells were then treated with 1 mM IPTG and grown for 48 hr at 15 °C, collected by centrifugation, and lysed using reagents provided in a commercially available kit from Qiagen (Quick Start, Ni-argarose). This kit provides reagents for determining if isolation of HIS-tagged recombinant proteins is optimal under native or denaturing conditions.

Preliminary studies demonstrated that denaturing conditions are required for optimal recovery of the HIS-tagged rAhR2 protein (data not shown). Though the ingredients of the lysis buffer are proprietary, and therefore unknown to end users, this lysis buffer most likely contains high concentrations of urea. Recombinant AhR2 protein used for immunizations was isolated over Ni-agarose columns provided in the kit, and all wash and elution buffers were provided in the kit as well.

The purity of recombinant 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-naphthol as a substrate. Only the most visually pure elutions were used to immunize mice.

Female balb/c mice, 6-8 wks of age, were obtain from Harlan and housed in Godley-Snell facilities at Clemson University, and under ALAC approved conditions. Mice were immunized s.c. with 50 ug rAhR2 using TiterMax as an adjuvant. Two weeks later mice were re-immunized s.c. using Freund's incomplete adjuvant, followed by 2 more immunizations at 21 day intervals without adjuvant. The final immunization was administered i.p. and mice were euthanized using CO₂, and the spleens quickly removed. Spleen cells were isolated and fused with SPO/14 myeloma cells in the presence of PEG

4000, and subsequent hybridomas screened for reactivity by ELISA against rAhR2 using techniques previously described by (Rice *et al.*, 1998).

Supernatants from positive primary hybridomas were further evaluated by SDS-PAGE/immunoblotting steps as follows. Two μg rAhR2 were applied to lanes of a 4-20% acrylamide gel and subjected to SDS-PAGE, and the separated proteins transferred to a methanol-treated a 0.45 μM Immulon (PVDF) membrane (Fisher Scientific) in transfer buffer.

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 cut into strips and incubated 1 hr with supernatants from primary hybridomas. Strips were wash 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 alkaline phosphatase buffer, hereafter referred to as AP buffer.

Based on the results of screening by SDS-PAGE/immunoblotting, one primary hybridoma (5B6) was chosen for two rounds of cloning by limiting dilution to yield mAb 5B6. Using a commercially available isotyping kit (Southern Biotechnology), it was determined that mAb 5B6 is an IgG₁ immunoglobulin expressing kappa light chains. Hybridoma 5B6 was maintained in Dubelco's Modified Eagles Medium (DMEM, Cellgrow™ - Fisher Scientific) supplemented with P/S antibiotics, L-glutamine, sodium

bicarbonate, sodium pyruvate, non-essential amino acids, HEPES, and 4/5 g/L glucose, and grown to confluence in T-150 Corning flasks 95% humidity and 5% CO₂ at 37 ° C. Supernatants were collected by centrifugation and treated with 0.05% NaN₃ to prevent bacterial contamination, then stored at 4 ° C until needed. Hybridomas were frozen in 95% FBS/10% DMSO at -80 ° C.

The monoclonal antibody, 5B6, was further examined for its ability to recognize only AhR2, and not AhR1, as well as for the ability to recognize native protein in tissue homogenates. Full length AhR1 and AhR2 proteins individually expressed in African green monkey kidney COS cells were generously provided by Dr. Mark Hahn, WHOI, Woods Hole MA USA. Adult mummichogs were collected using baited minnow traps at the Belle Baruch Marine Lab, Georgetown SC (33 °, 19 min, 46 sec North/79 °, 10 min, 12 sec West), a site previously used by the lab (Frederick *et al.*, 2002; Hunt and Rice, 2008; Marsh and Rice, 2009), and transported to the Aquatic Animal Facility at Clemson University, maintained at 25 ° C in aerated artificial sea water (15 ppt), and fed a Tetramin fish food diet twice daily. To obtain tissue cytosolic protein, adult mummichogs were euthanized in Tricaine (MS-222) and livers quickly removed and homogenized in homogenation buffer [25 mM MOPS (pH 7.5), 1 mM EDTA, 5 mM EGTA, 0.02% NaN₃, 20 mM Na₂MoO₄, 10% glycerol (v/v), 1 mM DTT), containing protease inhibitors (20 uM TLCK, 5 ug/ml leupeptinin, 13 ug/ml aprotinin, 7 ug/ml pepstatin A, 0.1 mM PMSF], hereafter referred to as HB. The homogenate was centrifuged at 1,000 x g for 10 min to remove organelles, and the supernatant centrifuged at 12,000 x g to obtain S-9 fraction containing AhR2 protein. Thirty µg of liver protein,

1 µg each of COS-expressed AhR1 and AhR2, and rAhR2 were subjected to SDS-PAGE/immunoblotting on 10% gels using the above described procedures with mAb 5B6 diluted 1:10 with PBS-Tw.

Detection and localization of AhR2 in *F. heteroclitus* cells:

The presence and localization of cytosolic AhR2 in intact cells was evaluated using lymphoid cells isolated from head kidney and spleens using previously described procedures (Rosell and Rice, 1998; Frederick et al., 2002; Marsh and Rice, 2009). Cells were then adhered onto poly-L-lysine treated glass slide cover slips (Marsh and Rice, 2009) over a 60 min period, then fixed with a 1% solution of methanol-free formalin in PBS. Cells were permeabilized with 0.1% triton-x100 in PBS over a 30 min period, washed extensively with PBS-Tw, then covered with blocking buffer (10% FBS in PBS-Tw) for 1 hr, washed extensively with the same, and probed with a 1:20 dilution of confluent hybridoma supernatants in PBS for 1 hr. After washing x 3 with PBS-Tw the cells were probed with AccuFlour 488 Fluorocein-conjugated goat anti mouse IgG (1:100 in PBS-Tw) for 1 hr. Following washing x 3, the cells were probed with DAPI in PBS to label DNA as marker for the nucleus. After another round of washings, the cover slip with cells was mounted on glass slides with 50% glycerol in PBS and sealed. The presence and localization of AhR2 was detected by Epifluorence microscopy at the Jordan Hall imaging facility, Clemson University.

Ahr2 protein expression in tissues collected from a population of creosote-adapted

***F. heteroclitus*:**

Adult Atlantic killifish were collected from the Atlantic Wood (AW) Superfund Site located on the southern branch of the Elizabeth river near Norfolk and Portsmouth VA, as well as the historical reference site at King's Creek (KC), VA, using baited minnow traps. Fish were transported to the Virginia Institute of Marine Science, School of Marine Science, College of William & Mary, Gloucester Point VA. Fish were held in York River sea water under constant aeration, and then euthanized by sedation with MS-222 and decapitation. Livers were quickly removed and sectioned into two parcels: one was flash frozen in liquid nitrogen, then stored at -80 °C until overnight express delivery to Clemson University. The other parcel was immediately placed in 9% methanol-free formalin diluted in HBSS, and stored on ice before transport to Clemson University. Intestines and head kidneys were also collected and formalin-fixed. Formalin-fixed tissues were embedded in paraffin and processed for 5 µM sections by the Clemson University Histology Core Facility, then de-paraffinized and processed for H&E staining or immunohistochemistry using mAb 5B6.

Frozen liver tissue samples from the two VA sites, as well as liver tissues from laboratory-raised fish collected near Georgetown, SC were thawed on ice and homogenized with HB containing protease inhibitors, differentially centrifuged as described above, and the resulting S-9 fraction protein concentration determined. Fifty µg of cytosolic protein representing tissues from 3 - 5 individuals from the three groups of fish were subjected to SDS-PAGE/immunoblotting steps as described above using 5B6

hybridoma supernatants diluted 1:10 in PBS-Tw. Upon examination of H&E-stained slides by marine histopathologist Dr. Wolfgang Vogelbein (VIMS), it was determined that livers from the AW site harbored either advanced tumors of various sorts and stages, or pre-cancerous lesions. Previous studies show that hepatic tumors from the AW site show differential CYP1A staining patterns between tumor cells and normal tissue (Vogelbein et al., 1990). Therefore, select clinical “cases” were used to probe with mAb 5B6 and mAb C10-7 (Rice et al., 1998), the latter being a pan-fish CYP1A-specific mAb, and serves as a prototype biomarker reagent for determining exposure to AhR2-binding ligands. Eight individual livers from the AW site and 8p from the KC site were prepared as described above and 50 µg of the cytosolic S-9 fraction from each animal were subjected to SDS-PAGE/immunoblotting using mAb C10-7 and mAb 5B6.

Capture ELISA for quantifying AhR2 protein in Atlantic Wood vs. King’s Creek Livers

Monoclonal antibody 5B6 was coated onto high bond ELISA plates (NUNC plates, high bond) overnight at 4⁰ C as undiluted confluent hybridoma supernatants (2.5 – 5.0 ug/well. The following morning the 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 50 ug liver S-9 fraction protein in 100 ul bicarbonate buffer were added to wells in duplicate. The plates also received a standard sample of rAhR2 to be used as

a reference for comparing plate to plate variability in O.D readings. Plates were incubated overnight at 4 °C. The following morning plates were emptied and washed x 4 with PBS-Tw. The plates then received 100 ul per well of a mouse polyclonal anti-AhR2 (diluted 1:2000 in blocking buffer) collected from the mice originally used to produce mAb 5B6. Plates were incubated at RT for 1 hr, and then washed x 4 with PBS-Tw. Plates 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 chromagen in AP buffer, incubated for 30 minutes, and the optical density at 405 nM recorded. The O.D. values for duplicate wells for each sample were averaged, as were the duplicated wells for the standard. The ratio of sample O.D. to that of the standard was determined in order to give a relative Unit of expression for each sample.

Immunohistochemistry in paraffin

Fixed tissues were dehydrated through a graded series of 50% ethanol to 100% ethanol and then to xylene. Once in xylene, the tissue samples were then processed and embedded in paraffin and then cut into 5 µm sections and transferred onto superfrost® plus coated slides (VWR Scientific). To view the structural integrity of the tissues, the first slide from each organ set was hematoxylin and eosin stained.

Vectastain Elite ABC kit (Vector Laboratories; Burlingame, CA) was used for immunohistochemical (IHC) staining following the manufacturer's instructions. The

slides containing the tissues were first deparaffinized and hydrated through a graduated series of xylene followed by decreasing concentrations of ethanol and finally de-ionized water. Antigen retrieval was then performed using Vector Antigen Unmasking Solution, high pH 9, in a microwave and heated for 5 minutes at 100% power, followed by 5 minutes of 0% power, followed by another 5 minutes at 100% power. After the slides cooled at room temperature for 30 minutes, they were washed in PBS (.01M, pH 7.4) and then quenched in 3% H₂O₂ in PBS for 30 minutes. The slides were then washed in PBS and then were blocked with serum provided by the kit. Next, the slides were blocked with Avidin D for 15 minutes followed by Biotin for 15 minutes (Vector Laboratories). Next, each section of the slide was covered in mAb 5B6, against AhR-2, or a control mAb HB-145, against human MHC II (major histocompatibility complex) for one hour at room temperature. Other sections were covered with mAb C10-7 to stain for CYP1A protein. In each case the antibodies were used as 1:20 dilutions of confluent hybridoma supernatant. After washing the slides with PBS, the slides were then incubated with a secondary antibody, provided by the kit, for one hour at room temperature. Following incubation, the slides were again washed in PBS, followed by an application of Vectastain ABC Reagent, provided by the kit, for 30 minutes. A peroxidase substrate, NovaRed (Vector Laboratories), stain was then applied for approximately 5 minutes. The slides were then rinsed in water and a counterstained with 0.5% methyl green, applied for one minute and then rinsed in water. Next, the slides were dehydrated and mounted, using PolyMount (Polysciences).

The Effects of 3-Methylcholanthrene on AhR2 Protein Expression in Intestines: a comparison to CYP1A expression.

Single adult male mummichogs (over 10 g each) collected from the Belle Baruch Marine Lab, near Georgetown SC, were injected i.p. with either corn oil as a carrier control, or 1 mg/kg 3-MC in corn oil to induce CYP1A. Seventy two hours later the two fish were euthanized by lethal MS-222 sedation and cervical dislocation. The intestines were removed, washed in HBSS, then fixed in 9% buffered formalin and processed for IHC using mAb 5B6 and mAb C10-7 as described above.

RESULTS

Using a commercial kit for purifications of his-tagged rAhR2, it was clear that denaturing conditions were required to obtain the protein from cellular lysates. The results of column washes and elution quality are shown in Figure (2a). rAhR2 eluted off the columns with relative easy to give clean bands. These same fractions were then subjected to SDS-PAGE steps and transferred to PVDF membranes, then probed with HRP-Ni to specifically detect his-tagged proteins. HRP activity was detected only in the bands corresponding to expected molecular wt of rAhR2 (Figure 2b).

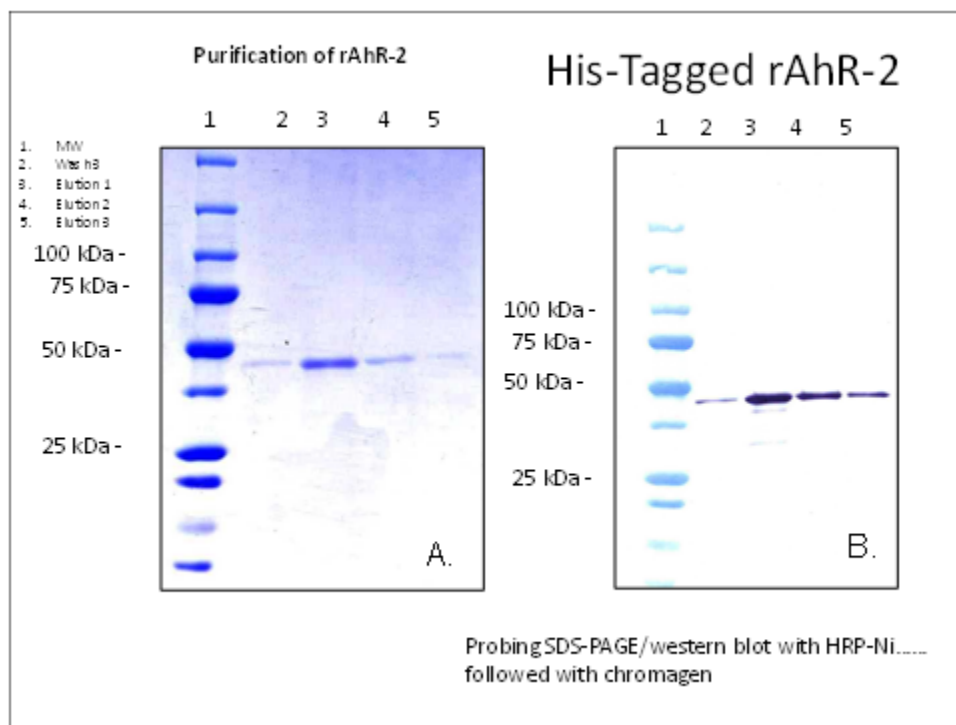


Figure 2. Purification of his-tagged rAhR2 from DE3 E. coli cells. Eluted fractions were subjected to SDS-PAGE analysis on 4-20% gels stained with Coomassie Blue and shown to be relatively pure (A). Eluted fractions were again subjected to SDS-PAGE, then transferred to PVDF membranes and probed with Ni-HRP and examined for HRP activity. The eluted samples were shown to be his-tagged (B), and subsequently used to immunize mice.

Efforts to generate a monoclonal antibody specific to rAhR2 and recognizing native AhR2, and not AhR1, were successful. The hybridoma, named 5B6, produces an IgG₁ immunoglobulin with *kappa* light chains. As can be seen in Figure 3, mAb 5B6 recognizes not only recombinant AhR2, and full length COS-expressed AhR2, but the protein in freshly isolated liver cytosol preparations.

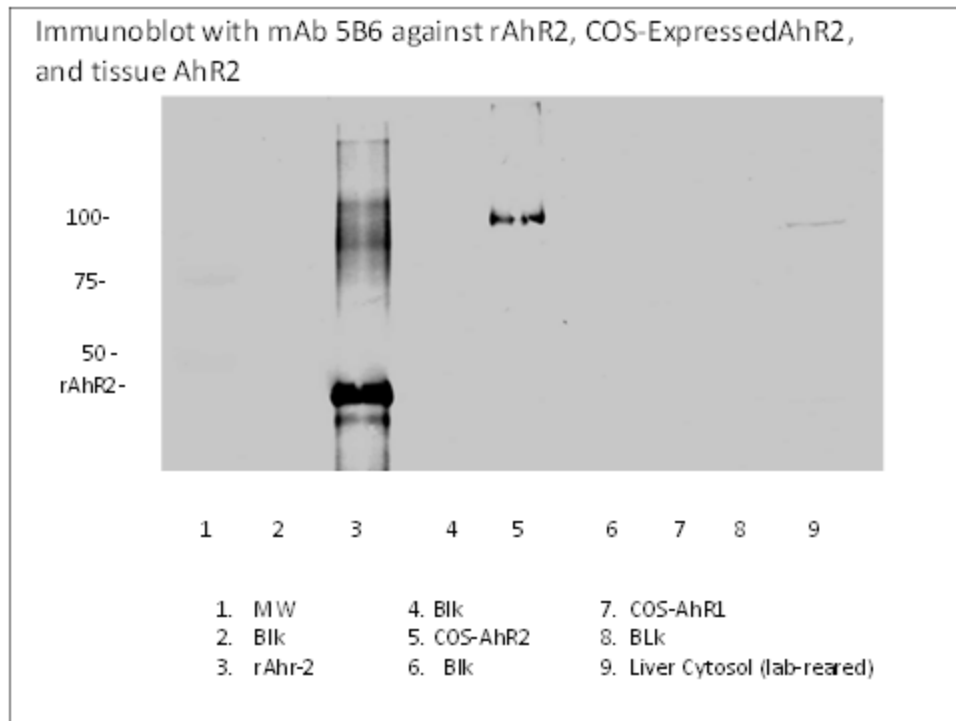


Figure 3. mAb 5B6 recognizes rAhR2, full length COS-expressed rAhR2, and freshly isolated liver S-9 fraction protein AhR2, but not full length COS-expressed rAhR1. Samples were subjected to SDS-PAGE using 10% gels, then transferred to PVDF membranes. The membrane was then probed with mAb 5B6.

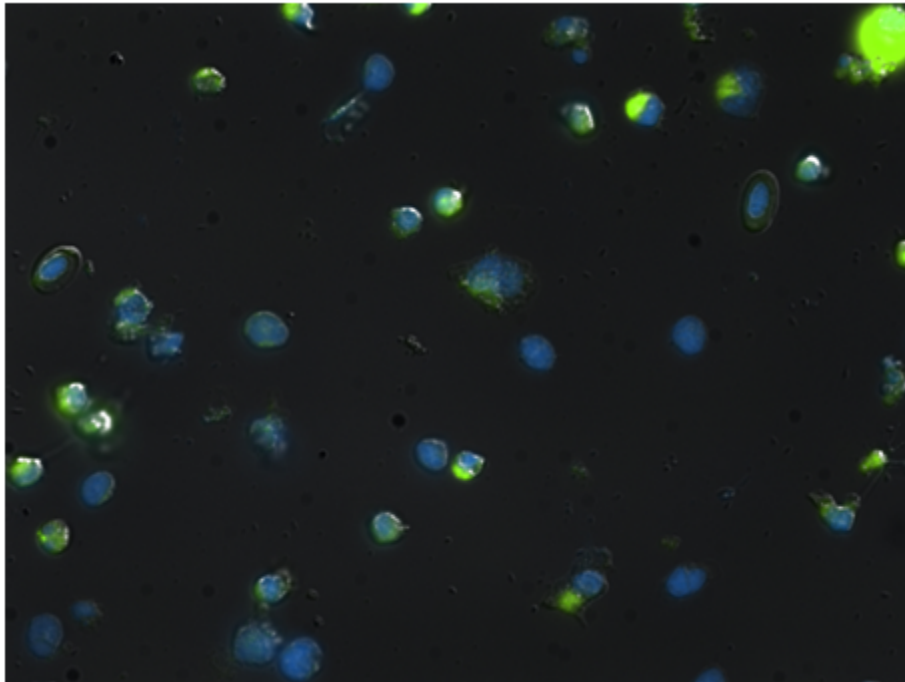


Figure 4. Intracellular expression of AhR2 proteins in lymphoid cells of mummichogs. Lymphoid cells were isolated and allowed to adhere to glass cover slips, fixed in 1% formalin, permeabilized, and probed with mAb 5B6 followed by labeling with FITC-conjugated anti-mouse IgG antibody. Cells were counterstained with DAPI to identify nuclei.

The availability of mAb 5B6 allows for the development of quantifiable techniques to determine how much AhR2 is present in mummichogs under various conditions. Mummichogs from the US EPA Superfund site at the Atlantic Wood site on the Elizabeth River near Portsmouth VA are known to be recalcitrant to the CYP1A inducing effects of PAHs that are so abundant at that site. While CYP1A protein has been examined in those fish, to date there have been no studies on the levels of AhR2. Using a crude and first tier capture ELISA system, it can be seen that slightly more AhR2 is present in whole liver cytosol preparations from the AW site compared to fish from the

KC site ($P \leq 0.05$: Students t-test) (Figure 5). Of particular note, the variability between individuals at each site is very low.

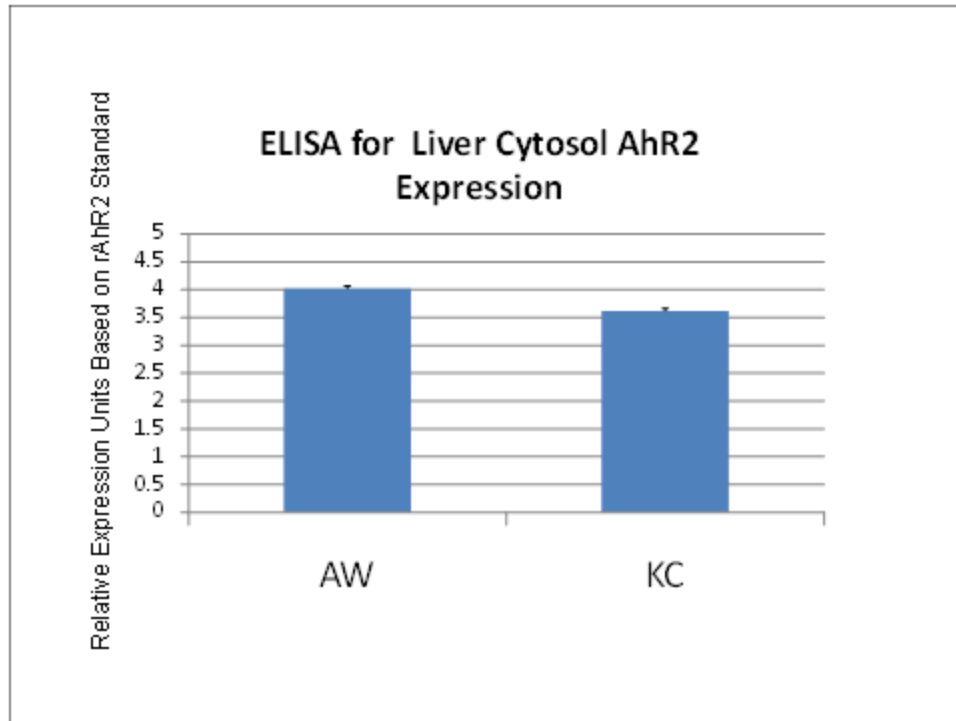


Figure 5. ELISA for AhR2 in liver S-9 fraction preparations in mummichogs collected at the Atlantic Wood (AW) Superfund site and its historical reference site, King’s Creek (KC) in VA. Capture antibody was mAb 5B6 and tracer antibody was mouse polyclonal anti-rAhR2. Data are O.D. units relative to a rAhR2 standard as a reference. Bars represent average values of n-16 individuals, and bars represent standard error of the means.

Since cytosol preparations contain hundreds, if not thousands of proteins, the next logical step was to examine SDS-PAGE/immunoblotting profiles of pooled liver cytosols from each population, and compare to a standard lab-raised population of fish collected from SC. Confirming the ELISA data, there is little, if any difference in cytosolic AhR2 protein between KC and AW populations (Figure 6). However, both populations have much higher levels of AhR2 than found in a long-term lab population from SC.

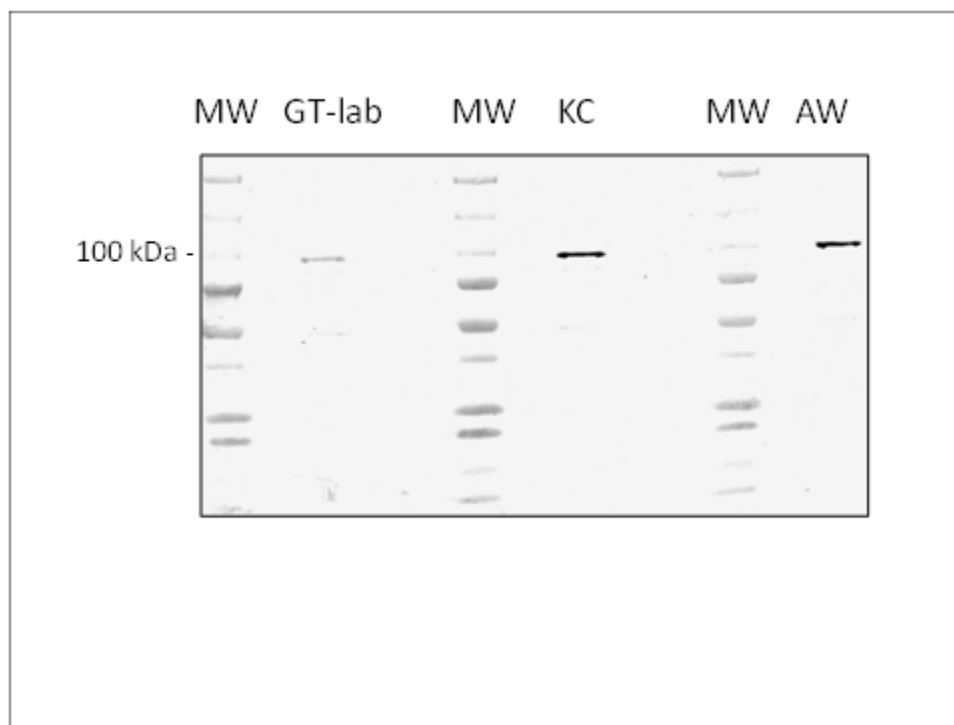


Figure 6. Liver homogenates from lab-reared Belle Baruch, SC (GT), King's Creek, VA (KC), and Atlantic Wood site, Elizabeth River VA (AW). 50 ug of liver S-9 fraction protein loaded onto 4-20% SDS-PAGE gels and transferred to PVDF membranes. Membranes were probed with mAb 5B6.

Given that whole tissue cytosol AhR2 does not differ between AW and KC mummichog populations, the next logical step was to determine if localization of AhR2 differed, and if CYP1A expression differed as well. Three different tissues were examined by IHC. Anterior kidney of KC tissues contained a high degree of AhR2 protein throughout the tissue, including renal epithelial cells (Figure 8a). Most of the anterior kidney was void of CYP1A expression, except for renal epithelial cells, which stain brightly for CYP1A (Figure 8c). AhR2 expression in AW anterior kidney tissues was also highly expressed (Figure 9b) and appeared to be slightly more expressed than in

the representative KC sample. CYP1A expression was pronounced throughout the tissue, indicating induction as a result of exposures to the PAHs at that site (Figure 9c).

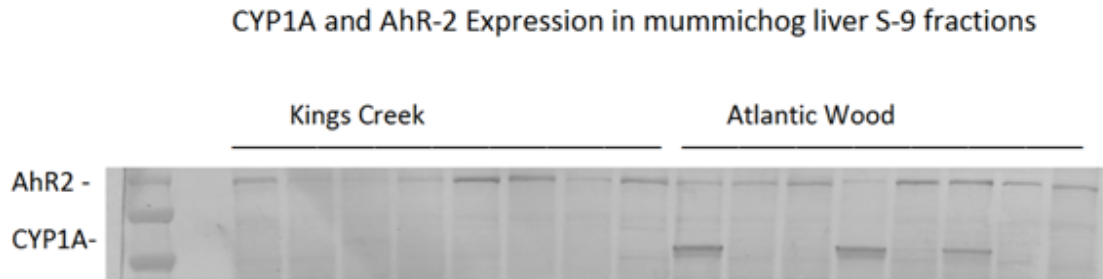


Figure 7. Expression of CYP1A and AhR-2 in liver S-9 fractions from 8 mummichogs collected from the Atlantic Wood and Kings Creek sites in VA. Liver proteins were probed with mAb C10-7 for CYP1A detection and mAb 5B6 for AhR-2 detection. Fifty μ g of protein were subjected to SDS-PAGE and immunoblotting. Note: CYP1A expression only in AW fish. AhR-2 expression is variable between fish and between collection sites.

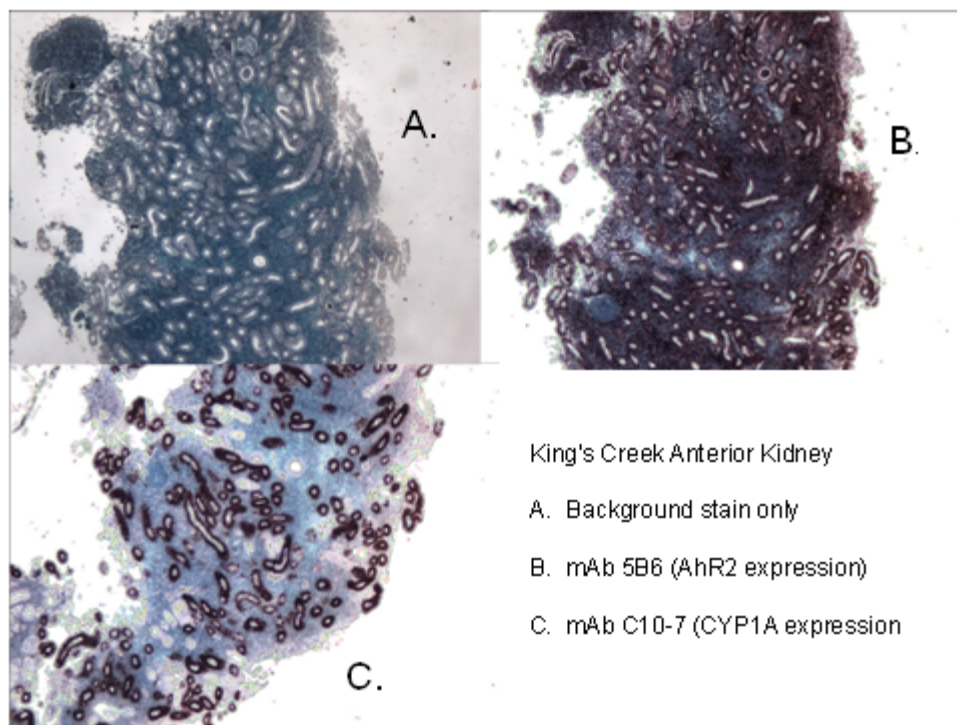


Figure 8. Immunohistochemistry of anterior kidney tissue from mummichogs collected at King's Creek site in VA. 5 μ M tissue sections were prepared for IHC and probed with background stain only (A), mAb 5B6 (B), or mAb C10-7 (C). Images were captured using 4X lens. Positive staining is noted by dark-red intensity. Note that renal epithelial cells stain for CYP1A protein.

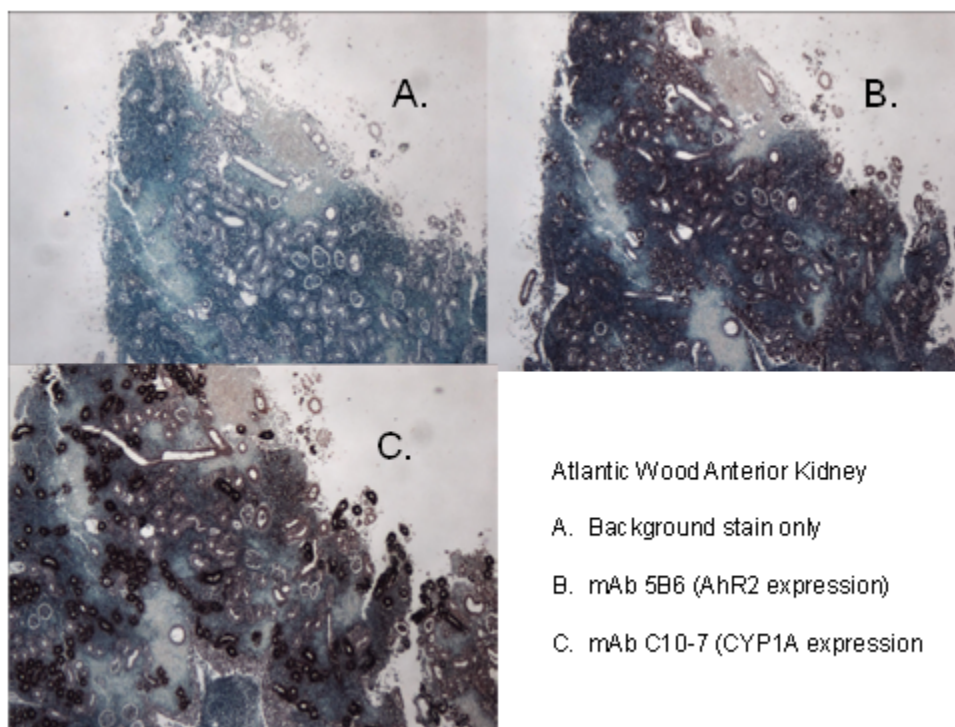


Figure 9. Immunohistochemistry of anterior kidney tissue from mummichogs collected at the Atlantic Wood site in VA. 5 μ M tissue sections were prepared for IHC and probed with background stain only (A), mAb 5B6 (B), or mAb C10-7 (C). Images were captured using 4X lens. Positive staining is noted by dark-red intensity. Note that renal epithelial cells stain for CYP1A protein. Also note dark regional staining with anti-AhR2.

Since dietary exposures to AhR2 ligands is a key means to toxicity of PAHs, it was logical to examine expression of AhR2 and CYP1A proteins in intestinal tissue from the two sites. As can be seen in Figure 10b, intestinal epithelial cells of KC fish express high levels of AhR2, but low levels of CYP1A (Figure 10c). In contrast, CYP1A is highly expressed in AW fish (Figure 11b), while AhR2 is expressed at about the same level, if not slightly higher (Figure 11a), though pixel strength of the staining was not quantified.

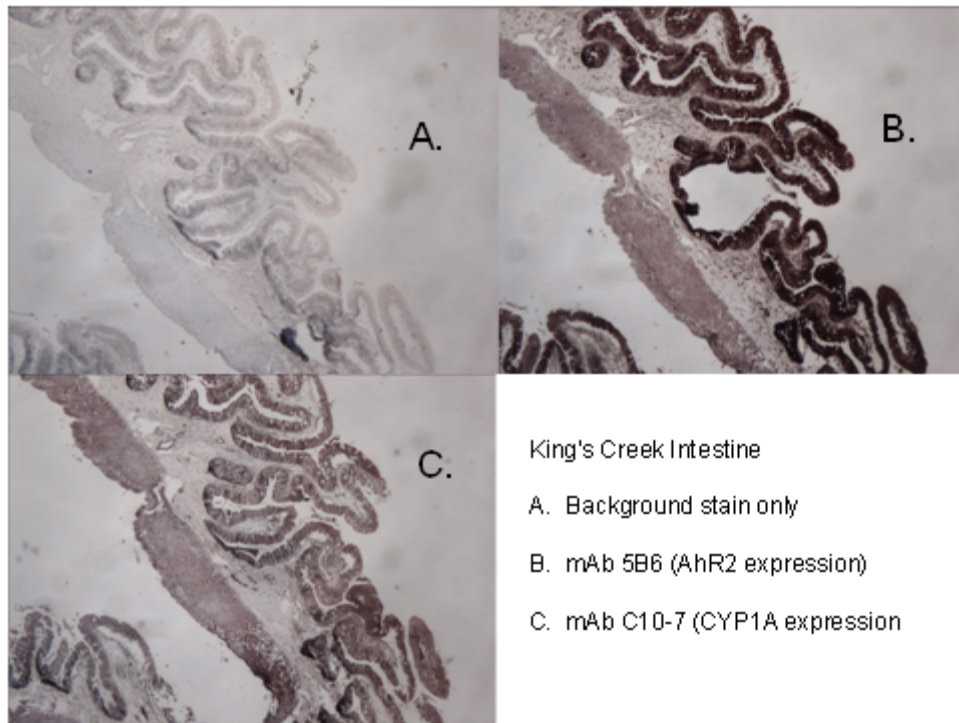


Figure 10. Immunohistochemistry of intestine tissue from mummichogs collected at King's Creek site in VA. 5 μ M tissue sections were prepared for IHC and probed with background stain only (A), mAb 5B6 (B), or mAb C10-7 (C). Images were captures using 4X lens. Positive staining is noted by dark-red intensity. Note that epithelial cells stain bright for AhR2.

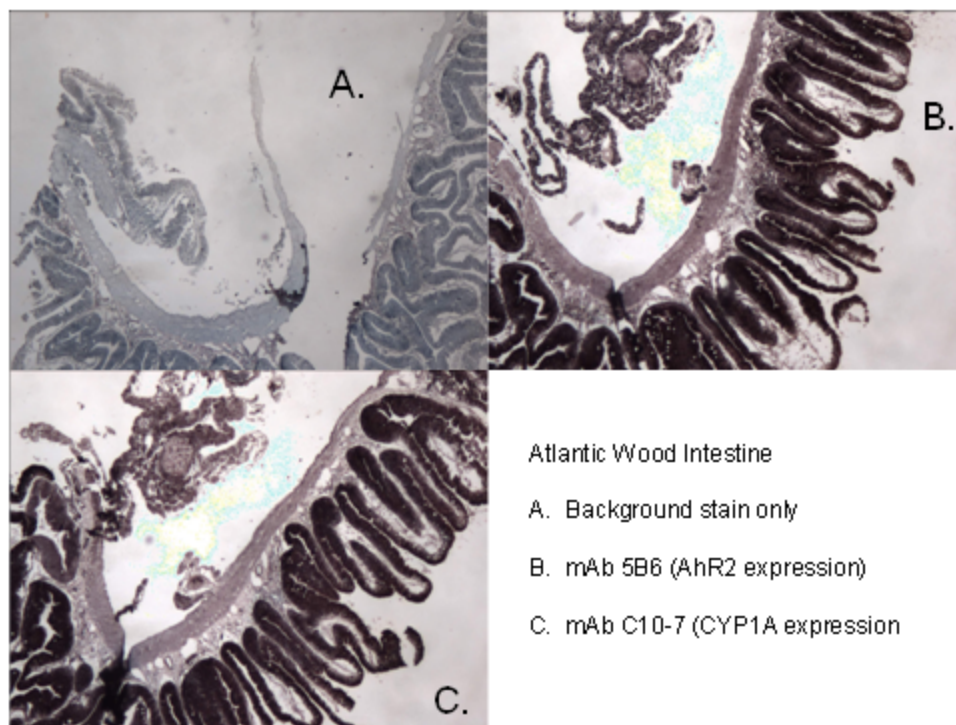


Figure 11. Immunohistochemistry of intestine tissue from mummichogs collected at the Atlantic Wood site in VA. 5 μ M tissue sections were prepared for IHC and probed with background stain only (A), mAb 5B6 (B), or mAb C10-7 (C). Images were captures using 4X lens. Positive staining is noted by dark-red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

Because of the role of the liver in detoxification of most drugs and pollutants, this organ is the most frequently examined in terms of target organ toxicity in environmental studies. In KC fish, the expression of AhR2 appears to be low in most areas, with zones of higher expression (Figure 12b), while CYP1A is relatively non-expressed (Figure 12C). In AW fish, however, both AhR2 and CYP1A are highly expressed (Figures 13b, 13c), indicating induction patterns in response to exposure to PAHs at this heavily polluted site.

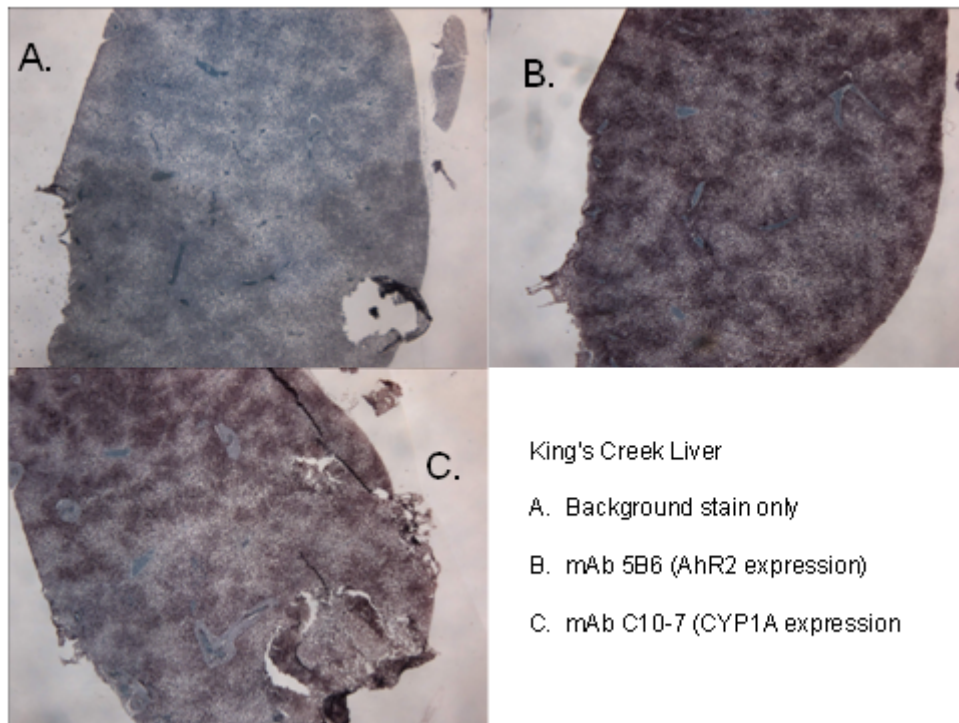


Figure 12. Immunohistochemistry of liver tissue from mummichogs collected at King's Creek site in VA. 5 μ M tissue sections were prepared for IHC and probed with background stain only (A), mAb 5B6 (B), or mAb C10-7 (C). Images were captures using 4X lens. Positive staining is noted by dark-red intensity. Note that cells stain for AhR2 in regions surrounded by the vascular tissues, but in general the staining is light.

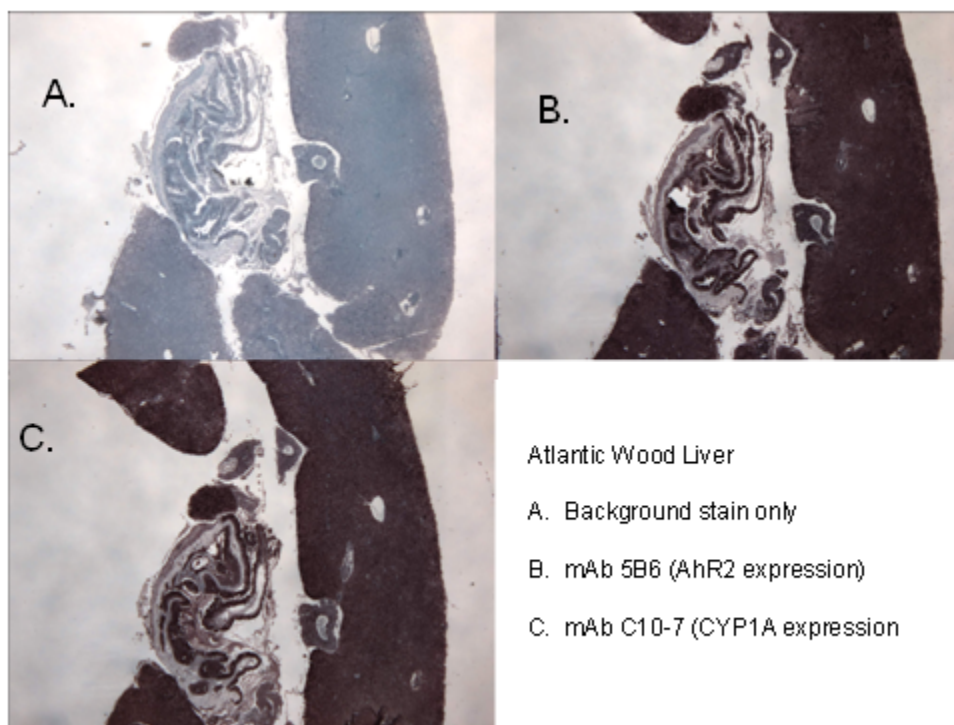


Figure 13. Immunohistochemistry of anterior kidney tissue from mummichogs collected at the Atlantic Wood site in VA. 5 μ M tissue sections were prepared for IHC and probed with background stain only (A), mAb 5B6 (B), or mAb C10-7 (C). Images were captured using 4X lens. Positive staining is noted by dark-red intensity. Note the intensive staining for both AhR2 and CYP1A.

One of the most common experimental systems in environmental toxicology is to expose animals to AhR ligands and follow the toxicity of a particular organ system, including liver toxicity, immunotoxicity, and endocrine toxicity. 3-methylcholanthrene (3-MC) is a model AhR-binding PAH, and is often used to model environmental PAHS, though this particular compound is not found in the environment. Following the clarity of AhR2 expression in intestines of AW vs KC mummichogs, it follows that intestines would be a good target organ to examine in the inducibility of AhR2 by 3-MC. Three days after receiving an i.p. injection of 3-MC or carrier control, mummichog intestines were processed for IHC using mAb 5B6 and mAb C10-7. As expected, very little

CYP1A was detected in the intestine of the carrier-control treated fish, while intestine CYP1A was highly expressed in the 3-MC treated fish (Figure 12). There does not seem to be a difference in expression of AhR2 between the two animals.

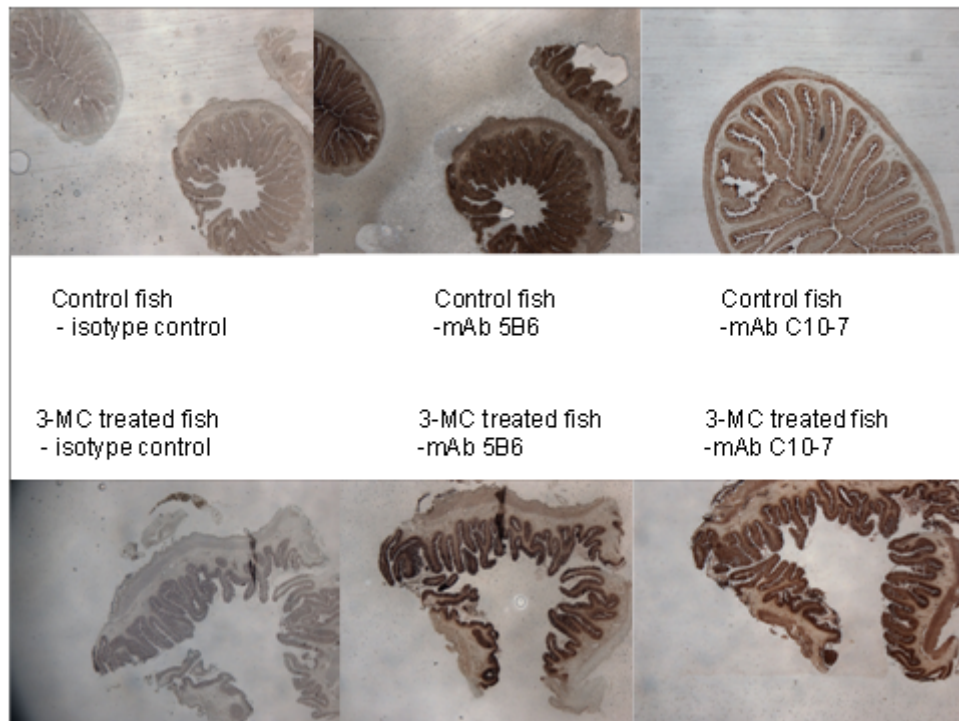


Figure 14. IHC for AhR2 and CYP1A proteins in intestines from mummichogs given i.p. injection of either corn oil carrier, or carrier containing 1 mg/kg 3MC. 72 hr after exposure tissues were removed and fixed in 9% formalin. 5 uM tissue sections were prepared for IHC and probed with background stain only (left panel), mAb 5B6 (middle panel), or mAb C10-7 (far right panel). Images were captures using 4X lens. Positive staining is noted by dark-red intensity. Note dark staining for CYP1A in 3-MC treated fish, but not controls. AhR2 expression is high in both control- and treated-fish tissues

DISCUSSION

The work described herein provides the first monoclonal antibody specific to a fish AhR, and in this case the AhR2 protein in mummichogs, *Fundulus heteroclitus*. While Merson et al., (2006) demonstrated the production of rabbit pAbs against this same recombinant and native protein, those antibodies are in very limited supply and generally not available to the scientific community. Furthermore, the antibody generated herein (mAb 5B6) gives very clean bands on SDS-PAGE/immunoblots and crisp/sharp reactivity in formalin-fixed and paraffin-embedded tissues.

As with virtually all mAbs, tissues slices from formalin-fixed tissues required antigen retrieval steps, which was a major part of the antibody characterization process. Three different techniques were explored; and included microwaving the tissues in the presence of low pH buffer (Tris EDTA, pH 6.0), high pH (Carbonate buffer, pH 9.6), and steam conditions using both buffers in a vegetable steamer (www.abcam.com/technical). All three techniques are fairly common, depending upon the nature of specific antibodies in use. While mAb C10-7 detects its epitopes at both high and low pH conditions using a microwave (personal experience), mAb 5B6 requires a high pH buffer during the microwave process.

Although one would expect very little expression of AhR2 in the cytosol of ligand-exposed fish, and that the AhR2 protein would be located in the nucleus and bound to its specific response element, one of the most significant discoveries using mAb 5B6 is that the AhR2 is abundantly expressed in fish, regardless of exposure to AhR-binding ligands such as PAHs at the AW Superfund site, or after exposure to 3-MC,

which is a very potent inducer of CYP1A, and mediated through the AhR. Since the AhR is also part of the inducible cassette of genes activated by the binding of AhR/ligand to its response unit (Powell et al., 2000), it is possible that what was detected in tissues and liver S-9 fraction proteins was a product of induction in response to the ligand(s). If this is true, then the expression of AhR2 in control or reference fish may reflect normal tissue levels before induction. In both scenarios of exposure to PAHs (AW fish and 3-MC treated fish), tissues were collected long after exposure. It is possible that what is noted in PAH-treated/exposed fish is due to subsequent induction of AhR2.

Also of particular note was the strong labeling of CYP1A in AW mummichogs. Even though these fish are constantly exposed to PAHs, they are recalcitrant to CYP1A induction by these same ligands once the fish are brought the lab. Many in the field of environmental toxicology consider AW fish to be “resistant” to those PAHs (Ownby et al., 2002; Frederick et al., 2007). However, the observations described herein indicate that either such resistance has been lost since, or that previous conclusions were flawed.

The ELISA developed in this study for quantifying liver AhR2 content may be flawed in that a goat anti-mouse IgG-AP antibody was used as the final detection reagent. Both the capture and tracer antibodies for the AhR2 protein were mouse antibodies, therefore the detection antibody may have reacted with both. Ideally, the tracer antibody should have been from a different species, such as rabbit, and the detector reagent would have been an anti-rabbit-AP system. At the time of these studies a rabbit pAb against mummichog AhR2 was not available, at least to our lab. However, future studies may involve the generation of a second (tracer) mAb for AhR2 which can be directly

conjugated with biotin. Alternatively, the mouse pAb anti-AhR2 in this study could have been directly labeled with biotin. In both situations, an avidin-HRP system could be employed to give a sensitive signal. Nonetheless, the values obtained from the ELISA developed during the process are more or less confirmed by western blotting with mAb 5B6 in that there were essentially no differences between AW and KC liver S-9 fraction levels of AhR2.

One of the more exciting future applications of mAb 5B6 will be to conduct cytosol-to-nuclear translocation observations. While this thesis work clearly demonstrates the localization of AhR2 to the cytosol, translocation in response to exposure to prototype AhR2 remains to be observed in any fish model. For example, in mammalian cell cultures it can be easily demonstrated that ligands such as planar HAHs and indirubins induced the translocation of AhR within a 5 hr period. Preliminary studies in the lab with mummichog primary liver and leukocyte cultures attempted to repeat such observations from mammalian cells, but a 5 hr period may be too short. Anything beyond 5 hrs in fish primary cultures quickly become contaminated with bacteria. Future studies will use tissues collected under sterile/aseptic conditions. Alternatively, a mummichog cell line could be developed.

In summary, a novel mAb for detecting AhR2 in the mummichog has been developed and characterized, and its technical applications investigated. Due to the variability between animals at each of the two collection sites examined, and between sites, the expression of AhR2 does not bode well for routine use as a biomarker in field studies. While the expression level of CYP1A is the most common biomarker for

exposure to AhR2 ligands, the nature of the ligand(s) is usually unknown in field studies. It may be possible to use mAb 5B6 to “trap” AhR2 in wild-caught mummichogs, and then to determine the actual ligand(s) bound in the tissues, thus allowing for a means to compared AhR2 ligands present at the time of sampling.

LITERATURE CITED

- Abraham, B.J. 1985. Species profile: Life Histories and Environmental requirements of Coastal fishes and Invertebrates: Mummichog and Striped Killifish. *Biological Reports*. 82:1-14.
- Abnet CC, Tanguay RL, Hahn ME, Heideman W, and Peterson RE (1999a) Two forms of aryl hydrocarbon receptor type 2 in rainbow trout (*Oncorhynchus mykiss*). Evidence for differential expression and enhancer specificity. *J Biol Chem* **274**:15159–15166.
- Andreasen, E.A., Spitsbergen, J.M., Tanguay, R.L., Stegeman, W.H., Peterson, R.E. 2002. Tissue-specific expression of AHR2, ARNT2, and CYP1A in zebrafish embryos and larvae: effects of developmental stage and 2, 3, 7, 8-Tetrachlorodibenzo-p-dioxin exposure. *Toxicological Sciences* 68: 403-419.
- Andreasen, E.A., Hahn, M.E., Heideman, W., Peterson, R.E., Tanguay, R.L. 2002. The zebrafish (*Danio rerio*) aryl hydrocarbon receptor type 1 is a novel vertebrate receptor. *Mol. Pharmacol* 62:234-239.
- Armstrong, P.B., Child, J.S. 1965. Stages in the normal development of *Fundulus heteroclitus*. *Biol. Bull.* 128:143-168.
- Baird, S. F., Girard C. 1853. Description of new species of fishes, collected by Captains R. B. Marcy, and Geo. B. M’Clellum, in Arkansas. *Proc. Acad. Nat. Sci. Phil.* 6(7):387-390.
- Belair, C.D., Peterson, R.E., Heideman, W. 2001. Disruption of erythropoiesis by dioxin in the zebrafish. *Dev. Dyn.* 222: 581-594.
- Bigelow H.B., Schroeder W.C. 1953. Fishes of the Gulf of Maine. *Fisheries Bulletin*, US 53:1-577.
- Boelsterli, U.A. 2007. 2nd Ed. Mechanistic toxicology: the molecular basis of how chemicals disrupt biological targets. *Cytochrome P450 (CYP)*. New York. 65-68. CRC Press.
- Brunnberg S, Andersson P, Lindstam M, Paulson I, Peollinger L, and Hanberg A. 2006 The constitutively active Ah receptor (CA-AhR) mouse as a potential model for dioxin exposure—Effects in vital organs. *Toxicology*. 224:191-201.
- Chapman, L. J., Chapman, C. A., and Chandler, M. (1996). Wetland ecotones as refugia for endangered fishes. *Biological Conservation*, 78, 263-270.

- Cherng SH, Lin ST, Lee H. 1996. Modulatory effects of polycyclic aromatic hydrocarbons on the mutagenicity of 1-nitropyrene: a structure-activity relationship study. *Mut Res* 367(4):177–85.
- Chen, H-S. and G. H. Perdew. 1994. Subunit composition of the heteromeric cytosolic aryl hydrocarbon receptor complex. *J Biol Chem* 269:27554–27558.
- Clark BW, Matson CW, Jung D, Di Giulio RT. 2010. AHR2 mediates cardiac teratogenesis of polycyclic aromatic hydrocarbons and PCB-126 in Atlantic killifish (*Fundulus heteroclitus*). *Aquatic Toxicology*, 99:232-240.
- Cochran RC, Zabludoff SD, Paynter KT, DiMichele L, Palmer RE. 1988. Serum hormone levels associated with spawning activity in the mummichog, *Fundulus heteroclitus*. *Gen Comp Endocrinol* 70(2):345-354.
- Cox, MB, and Miller CA. 2004. Cooperation of heat shock protein 90 and p23 in aryl hydrocarbon receptor signaling. *Cell Stress Chaperones*. 9:4-20.
- Denison, M. S., Pandini, A., Nagy, S. R., Baldwin, E.P., Bonati, L. (2002). Ligand binding and activation of the Ah receptor. *Chem. Biol. Interact.* 141: 3-24.
- Denison, M.S., Nagy, S.R. 2003. Activation of the aryl hydrocarbon receptor by structurally diverse exogenous and endogenous chemicals. *Annu. Rev. Pharmacol. Toxicol.* 43: 309-334.
- Duvernell, D.D., Lindmeier, J.B., Faust, K.E., Whitehead, A., 2008. Relative influences of historical and contemporary forces shaping the distribution of genetic variation in the Atlantic killifish, *Fundulus heteroclitus*. *Molecular Ecology* 17, 1344–1360.
- Elferink CJ. 2003. Aryl hydrocarbon receptor-mediated cell cycle control. *Progress in Cell Cycle Research*, 5:260-267.
- Erickson, M.D. 1997. Analytical chemistry of PCBs. 2-14. 2ND Ed. CRC Press LLC.
- Frederick, L.A., P.A. Van Veld, and C.D. Rice. 2007. Bioindicators of immune function in creosote-adapted estuarine killifish, *Fundulus heteroclitus*. *Journal of Toxicology and Environmental Health Part A* 70(17): 1433–1442.
- Griffith, R. W. 1974. Environmental and salinity tolerance in the genus *Fundulus*. *Copeia* 1974:319- 331.

- Gu, Y., Hogenesch, J. B., Bradfield, C. A. (2000). The PAS superfamily: Sensors of environmental and developmental signals. *Annu. Rev. Pharmacol. Toxicol.* 40: 519-561.
- Gunter, G. 1950. Distributions and abundance of fishes on the Aransas National Wildlife Refuge, with life history noted. *Publ. Inst. Mar. Sci., Univ. Tex.* 1(2):89-101.
- Hahn ME, Karchner SI, Shapiro MA, and Perera SA (1997) Molecular evolution of two vertebrate aryl hydrocarbon (dioxin) receptors (AHR1 and AHR2) and the PAS family. *Proc Natl Acad Sci USA* **94**:13743–13748.
- Hahn, M.E. (1998). The aryl hydrocarbon receptor: A comparative perspective. *Comp. Biochem. Physiol. Part C.* 121: 23-53.
- Hahn, M.E. 2002. Aryl hydrocarbon receptors: diversity and evolution. *Chemico-Biological Interactions.* 141 (1-2): 131-160.
- Hahn, M. E., Merson, R.R., Karchner, S.I., 2005. Xenobiotic receptors in fish: Structural and functional diversity. In: Mommsen, T.P., Moon, T.W. (Eds.), *Environmental Toxicology*, 6: 191-228.
- Hahn, M.E., Karchner, S.I., Evans, B.R., Franks, D.G., Merson, R.R., Lapsertis, J.M.. 2006. Unexpected diversity of aryl hydrocarbon receptors in non-mammalian vertebrates: insights from comparative genomics. *Journal of Exp. Zoo.* 305A: 693-706.
- Hardy, J. D., Jr. 1978. Development of fishes of the mid- Atlantic Bight, Vol. II. *Anguillidae through Syngnathidae*. U.S. Fish and Wildlife Service Publication FWS/OBS-78/12.
- Holsapple MP, Karras JG, Ledbetter JA, Schieven GL, Burchiel SW, Davila DR, Schatz AR, Kaminski NE. 1996. Molecular mechanisms of toxicant-induced immunosuppression: role of second messengers. *Annu Rev Pharmacol Toxicol.* 36:131-59
- Hsiao, S.-M., Meier, A.H. 1988. Semilunar ovarian activity of the gulf killifish, *Fundulus grandis*, under controlled laboratory conditions. *Copeia* 1: 188-195.
- Hsiao, S-M., Meier, A.H. 1989. Comparison of semilunar cycles of spawning activity in *Fundulus grandis* and *F. heteroclitus* held under constant laboratory conditions. *Exp. Zool.* 252: 2 13-2 18.

- Karchner, S.I., Franks, D.G., Powell, W.H., Hahn, M.E., 2002. Regulatory interactions among three members of the vertebrate aryl hydrocarbon receptor family: AHR repressor, AHR1, and AHR2. *J. Biol. Chem.* 277, 6949–6959.
- Karchner, S.I., Powell, W.H., Hahn, M.E. 1999. Identification and functional characterization of two highly divergent aryl hydrocarbon receptors (AHR1 and AHR2) in the teleost *Fundulus heteroclitus*. *The Journal of Biological Chemistry.* 274 (47): 33814-33824.
- Kewley, R. J., Whitelaw, M. L., Chapman-Smith, A. (2004). The mammalian basic helix-loop-helix/PAS family of transcriptional regulators. *Int. J. Biochem. Cell Biol.* 36: 189-204.
- Kneib, R. T. and A. E. Stiven. 1978. Growth, reproduction, and feeding of *Fundulus heteroclitus* (L.) on a North Carolina salt marsh. *J. Exp. Mar. Biol. Ecol.* 31:121-140.
- Kneib, R. T. 1986. The role of *Fundulus heteroclitus* in salt marsh trophic dynamics. *American Zoologist* 26:259-269.
- MacGregor, R., III, Greeley, M.S., Trimble, W.C, Tatum, W.M. 1983. Seasonal variation of reproduction and fattening in gulf killifish from brackish mariculture ponds. *Northeast Gulf Sci.* 6: 23-32.
- Merson, R. R., Franks, D. G., Karchner, S. I., Hahn, M. E. 2006. Development and characterization of polyclonal antibodies against the aryl hydrocarbon receptor protein family (AHR1, AHR2, and AHR repressor) of atlantic killifish, *Fundulus heteroclitus*. *Comp. Biochem. and Physiology Part C* 142:85-94.
- Mimura, J., Ema, M., Sogawa, K., Fujii-Kuriyama, Y. 1999. Identification of a novel Mechanism of regulation of Ah (dioxin) receptor function. *Gene Dev.* 13: 20-25.
- Mitchell, K.A, Elferink, C.J. 2008. Timing is everything: consequences of transient and sustained AhR activity. *Biochemical Pharmacology.* 77 (6): 947-956.
- Moennikes O, Loeppen S, Buchmann A, Anderson P, Ittrich C, Poellinger L, and Schwarz M. 2004. A constitutively active dioxin/aryl hydrocarbon receptor promotes hepatogenesis in mice. *Cancer Research,* 64:4707-4710.

- Munns, W. R., Black, D. E., Gleason, Salomon, K., Bengtson, D., Gutjahr-Gobell, R. 1997. Evaluation of the effects of dioxin and pcbs on *Fundulus heteroclitus* populations using a modeling approach. *Environmental Toxicology and Chemistry* 16 (5): 1074-1081.
- Nacci D.E., Champlin D., Jayaraman S. 2010. Adaptation of the estuarine fish *Fundulus heteroclitus* (atlantic killifish) to polychlorinated biphenyls (PCBs). *Estuaries and Coasts* 33: 853-864.
- Nebert D, Roe A, Dieter M, Solis W, Yang Y, Dalton T. 2000. Role of aromatic hydrocarbon receptor and Ah gene battery in the oxidative stress response, cell cycle control and apoptosis. *Biochem Pharmacol* 59:65-85.
- Nevid, N. J. and A. H. Meier (1993). "A day-night rhythm of immune activity during scale allograft rejection in the gulf killifish, *Fundulus grandis*." *Dev Comp Immunol* 17(3): 221-8.
- Nevid, N. J. and A. H. Meier (1994). "Nonphotic stimuli alter a day-night rhythm of allograft rejection in gulf killifish." *Dev Comp Immunol* 18(6): 495-509.
- Overstreet, R.M, Barnes, S.S., Manning, C.S., Hawkins, W.E. 2000. Facilities and husbandry (small fish models). *The handbook of experimental animals: The laboratory fish*. Ostrander, G.K., 45. Academic Press.
- Powell, W.H., Bright, R., Bello, S.M., Hahn, M.E. 2000. Developmental and tissue-specific expression of AHR1, AHR2, and ARNT2 in dioxin-sensitive and – resistant populations of the marine fish *Fundulus heteroclitus*. *Toxicological Sciences* 57: 229-239.
- Prasch, A.L., Teraoka, H., Carney, S.A., Dong, W., Hiraga, T., Stegeman, J.J., Heideman, W., Peterson, R.E. 2003. Aryl hydrocarbon receptor 2 mediates 2,3, 7, 8-Tetrachlorodibenzo-p-dioxin developmental toxicity in zebrafish. *Toxicological Sciences* 76: 138-150.
- Quattrochi, L.C., Vu, T., Tukey, R. H. 1994. The human CYP1A2 gene and induction by 3-methylcholanthrene: a region of DNA that supports ah-receptor binding and promoter-specific induction. *The Journal of Biological Chemistry* 126 (9): 6949-6954.
- Rice CD, Schlenk D, Ainsworth J, Goksoyr A. 1998. Cross-reactivity of monoclonal antibodies against peptide 277-294 of rainbow trout CYP1A1 with hepatic CYP1A among fish. *Marine Environmental Research* 46(1-5):87-91.

- Rice, C. D. and Y. Xiang (2000). "Immune function, hepatic CYP1A, and the reproductive biomarker response in the gulf killifish, *Fundulus grandis*, during dietary exposures to endocrine disrupters." *Marine Environmental Research* 50: 163-168.
- Roszell, L. R. and Rice, C.D. (1998). "Innate cellular immune function of anterior kidney leucocytes in the gulf killifish, *Fundulus grandis*." *Fish & Shellfish Immunology* 8(2):129-142.
- Roy NK and Wirgin I (1997) Characterization of the aromatic hydrocarbon receptor gene and its expression in Atlantic tomcod. *Arch Biochem Biophys* **344**:373–386
- Simpson D. G., Gunter G., 1956. Notes on habitats, systematic characters and life histories of Texas salt water cyprinodontes. *Tulane Stud. Zool.*, 4. 115-134.
- Solangi, M. A. and R. M. Overstreet (1980). "Biology and pathogenesis of the coccidium *Eimeria funduli* infesting killifishes." *J Parasitol* 66(3): 513-526.
- Tanguay RL, Abnet CC, Heideman W, and Peterson RE (1999) Cloning and characterization of the zebrafish (*Danio rerio*) aryl hydrocarbon receptor. *Biochim Biophys Acta* **1444**:35–48.
- Tanguay RL, Andreasen E, Heideman W, and Peterson RE (2000) Identification and expression of alternatively spliced aryl hydrocarbon nuclear translocator 2 (ARNT2) cDNAs from zebrafish with distinct functions. *Biochim Biophys Acta* 1494:117–128.
- Taylor, M. H., Dimichele, L. 1980. Ovarian changes during the lunar spawning cycle of *Fundulus heteroclitus*. *Copeia* 1980:118-125.
- Teo, S. L. H., Able, K.W. 2003. Growth and production of the mummichog (*Fundulus heteroclitus*) in a restored salt marsh. *Estuaries and Coasts* 26(1):51-63.
- Teraoka, H., Dong, W., Ogawa, S., Tsukiyama, S., Okuhara, Y., Niiyama, M., Ueno, N., Peterson, R.E, Hiraga, T. 2002. 2, 3, 7, 8-Tetrachlorodibenzo-p-dioxin toxicity in the zebrafish embryo: altered regional blood flow and impaired lower jaw development. *Toxicol. Sci.* 65: 192-199.
- Vogelbein W.K., Fournie J.W., Van Veld P. A., Huggett R.J. 1990. Hepatic neoplasms in the mummichog *Fundulus heteroclitus* from a creosote-contaminated site. *Cancer Research* 50: 5978-5986.

- Wassenberg DM, Swails EE, Di Giulio RT. 2002. Effects of single and combined exposures to benzo(a)pyrene and 3,3',4,4'-pentachlorobiphenyl on EROD activity and development in *Fundulus heteroclitus*. *Marine Environmental Research* 54(3-5): 279-283.
- Weis, J.S. 2002. Tolerance to environmental contaminants in the mummichog, *Fundulus heteroclitus*. *Human and Ecological Risk Assessment* 8 (5) 933-953.
- Whitlock, J.P. (1999). Induction of cytochrome P4501A1. *Annu. Rev. Pharmacol. Toxicol.* 39:103-125.
- ZeRuth, G., Pollenz, R. S. 2007. Functional analysis of cis-regulatory regions within the dioxin-inducible CYP1A promoter/enhancer region from zebrafish (*Danio rerio*). *Chemico-Biological Interactions* 170 (2): 100-113.