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MECHANISMS OF RESISTANCE TO HALOGENATED AND NON-HALOGENATED AHR LIGANDS IN CHRONICALLY CONTAMINATED KILLIFISH POPULATIONS

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ABSTRACT IOF DISSERTATION

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The Graduate School

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ABSTRACT OF DISSERTATION

A dissertation submitted in partial fulfillment of the requirements for the degree in Doctor of Philosophy in the Graduate School at the University of Kentucky

By

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ABSTRACT OF DISSERTATION

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Abstract:

Chronically contaminated killifish from Newark Bay (NB) NJ, and New Bedford Harbor (NBH) MA, have developed resistance to halogenated aromatic hydrocarbons that bind to and activate the aryl hydrocarbon receptor (AHR). To study the mechanisms of resistance, adult killifish were exposed to halogenated and non-halogenated AHR ligands and enzymatic and toxicological endpoints were measured in adult and embryonic fish.

The chlorinated and non-chlorinated AHR ligands 3,3'4,4'-tetrachlorobiphenyl (PCB77) and benzo-a-pyrene (B[a]P) induced cytochrome P450 1A (CYP1A) in reference site, but not in NB killifish. Expression of CYP3A (not part of the AHR gene battery) was inducible only in Flax Pond killifish. Basal expression of the phase II enzyme glutathione-s-transferase (GST) was higher in NB killifish. These results suggest that NB killifish are resistant to CYP1A induction by chlorinated and non-chlorinated AHR ligands. Higher basal GST activity observed in NB killifish could be protective against toxicity caused by contaminants found in this site.

Activation of AHR and induction of CYP1A, by AHR ligands has been associated with the toxic effects caused by these chemicals. To determine the association between resistance to CYP1A induction and the toxicity caused by AHR ligands, CYP1A activity, developmental deformities and reactive oxygen species (ROS) production were measured in reference site and contaminated (NB and NBH) killifish embryos exposed to AHR ligands. 3,3'4,4'5-pentachlorobiphenyl (PCB126) and 3-methylcholantherene (3-MC) induced CYP1A, and ROS production in reference site embryos. NB and NBH embryos were resistant to PCB126 induction of CYP1A, but responded to 3-MC. Killifish embryos from NB and NBH were resistant to PCB126 induced deformities. PCB126 and 3-MC did not increase ROS production in NB or NBH killifish embryos. Alpha-naphthoflavone (ANF) (an AHR/CYP1A inhibitor) blocked PCB126 mediated deformities and CYP1A induction in reference site embryos, but increased ROS

production. The P450 inhibitor, piperonyl butoxide (PBO) was able to block PCB126 mediated induction of CYP1A activity and ROS production. These results suggest that PCB126 induced deformities are dependent on activation of AHR and CYP1A induction.

In chronically contaminated killifish populations, loss of sensitivity to coplanar PCBs and PAHs could be through reduced expression of AHR, or altered DNA sequence or methylation status of the CYP1A gene promoter. Hepatic AHR expression, measured by photoaffinity labeling, was lower in NB killifish than reference site animals, suggesting that NB killifish express less AHR protein. DNA sequence analysis did not reveal considerable differences between contaminated and reference site populations, however additional DNA fragments were observed in some promoters but not in others. The methylation of the CYP1A promoters was studied using methylation sensitive restriction enzymes and no differences were detected between reference site and NB killifish. Treatment with the DNA methyltransferase inhibitor AzaC did not restore CYP1A induction by PCB126 in NB killifish.

These studies suggest that resistance to activation of AHR and induction of xenobiotic activating enzymes (CYP1A and CYP3A) in combination with increased expression of conjugating enzymes (GST) protects chronically contaminated killifish against these chemicals.

Keywords: AHR, CYP1A killifish, polychlorinated biphenyls, polycyclic aromatic hydrocarbons, resistance.

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DISSERTATION

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For Javier Arzuaga

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Chapter One

Introduction:

Pollution problems are frequent in many parts of the American continent. In the north Atlantic coast of the United States, as in other parts of the word, these problems are associated with industrial, and urban development. Environmentally relevant toxic chemicals have a negative impact in exposed ecosystems. Fish populations that inhabit contaminated sites have higher levels of DNA adducts and increased frequency of liver and skin tumors (Baumann, 1998). For example killifish (*Fundulus heteroclitus*) and brown bullhead (*Ameiurus nebulosus*) collected from contaminated portions of the Elizabeth River VA, and the Anacostia River (Washington, DC) have higher incidence of DNA adducts (Pinkney et al., 2004; Rose et al., 2000) and liver tumors (Pinkney et al., 2001; Vogelbein et al., 1990) than clean site animals.

The pollution present in impacted aquatic systems consists of hundreds environmental contaminants. Sediment samples from Newark Bay, NJ and the Hudson River estuary NY are contain complex mixtures that contain heavy metals, raw sewage, pesticides, etc. Co-planar Halogenated Aromatic Hydrocarbons (HAHs) and Polycyclic Aromatic Hydrocarbons (PAHs) can be found among the contaminants in these sites (O'Connor, 1988). Exposure to these chemicals negatively affects animals. Laboratory studies using sediment extracts from contaminated sites demonstrate that exposure to these chemicals can increase teratogenesis (developmental deformities), oxidative stress, cancer and altered behavior (Baumann, 1998; Weis et al., 2003). Most of the toxicological effects caused by HAHs and PAHs of co-planar aromatic hydrocarbons are mediated through the aryl hydrocarbon receptor (AHR). The AHR is a cytosolic receptor that functions in a similar fashion to hormone receptors. Upon binding of HAH or PAH ligands, the AHR is transformed in to its active form and initiates transcription of responsive genes. One of the most studied responses to AHR activation is induction of the monooxygenase cytochrome P450 1A (CYP1A). Under laboratory conditions, exposure to AHR ligands present in complex mixtures can induce CYP1A expression in different fish models (Levine, 1999; Wassenberg et al., 2002a).

Some species as well as individuals within a population may be more vulnerable than others to environmental stressors (Carvalho et al., 2004; Willett et al., 2000). If the

selective pressures are strong enough then certain individuals, possessing and expressing genotype that permits survival under the new pressure, can have greater chance to reproduce than others. Recent studies have discovered fish populations that inhabit contaminated sites and have developed resistance to the immediate toxic effects caused by these pollutants (Wirgin and Waldman, 2004). It is possible that contaminated fish populations have adapted to the conditions of their environments through a selection of the more toxicant-resistant individuals. This process could potentially reduce the gene pool of the population and affect its ability to adapt to new stressors.

This thesis examines two killifish populations from the contaminated Newark Bay NJ, and New Bedford Harbor MA. Both killifish populations have been chronically exposed to environmental contaminants. Although the sources of pollution are different for the two sites, the contaminants present in this environment include different levels of xenobiotic AHR ligands such as halogenated aromatic hydrocarbons (HAHs) and polycyclic aromatic hydrocarbons (PAHs). Recent studies have demonstrated that killifish from Newark Bay and New Bedford Harbor have developed resistance to immediate toxic effects caused by HAHs and PAHs. The purpose of the studies described herein was to determine the biochemical and molecular mechanistic adaptations that allow these animals to survive in these contaminated environments.

Study organism: The Killifish (*Fundulus heteroclitus*)

Killifish live in shallow waters in estuarine areas. Their range is from the Gulf of St Lawrence, to northeastern Florida. They usually measure from 2 to 4 inches in length, but some can be 6 inches long. Their life expectancy is from 3 to 4 years (Armstrong and Child, 1965b). Killifish are a non-migratory species and their home range is approximately 36 meters during the winter months (Lotrich, 1975). Killifish form an important part of the trophic structure of the ecosystem and are consumed by other species of commercial relevance such as the blue crab (*Callinectes sapidus*), and stripped bass (*Morone Saxatilis*) (Weis, 2002). They are omnivores and their diet includes sea grass fragments, crustaceans, diatoms, amphipods mollusks, small fishes, and fish eggs (including their own). Killifish spawn during the summer months and most of the spawning coincides with new and full moons. The sexually mature fish show strong

dimorphisms during the spawning season. Killifish eggs are about 2mm wide, the eggs are colorless or pale yellowish, and very sticky, so they mass up together or stick to the material where they rest upon. The incubation time takes from 9 to 18 days. They also display a large degree of genetic variability (Brown, 1991). The AHR pathway is functional in this species of fish. AHR ligand dependent CYP1A induction can be observed in a variety of tissues and developmental stages (Elskus et al., 1999; Van Veld et al., 1997; Wassenberg et al., 2002b). Killifish are frequently used as research models for various advantages. They are easy to collect and maintain in the lab, unless they come to the lab with some kind of infection, and the chorionic membrane covering egg is transparent so researchers can easily view developmental stages.

Background material:

Studies on pollution resistance were conducted on killifish from Newark Bay and New Bedford Harbor. These are two superfund sites located on the north east coast of the United States. Both sites are contaminated with a variety of chemicals. The sources for pollution are different in these two sites. A brief description of the types of contamination in two these sites is presented bellow.

Newark Bay:

The Newark Bay (NJ) is probably one of the most highly populated and industrially active areas in the United States. Since the 1800s the New Jersey estuary has been exposed to pollution originated from industrial development and domestic sources. This area includes the Hackensack River, Passaic River, Kill Van Kull, and Arthur Kill (Figure 1). The contamination of Newark Bay includes heavy metals, pesticides, polychlorinated biphenyls (PCBs), polycyclic aromatic hydrocarbons (PAHs), dioxins and conventional pollutants (e.g. organic matter, ammonia, and bacterial and viral pathogens) (Crawford et al., 1995; Wenning, 1994). Polychlorinated dibenzo-p-dioxins (PCDDs) including 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD), are among the most potent carcinogens. The source of these chemicals in Newark Bay was the production of herbicide components of Agent Orange by an industrial facility located in the bank of the Passaic River. Concentrations of 21,000 parts per trillion (ppt) have been reported for

Newark Bay sediment samples collected near the production site. It has been estimated that the total deposition of TCDD in this site has been 4 to 8 kg since the 1940s (Bopp, 1991). TCDD has been found in lobsters (*Homarus americanus*), blue crabs and striped bass collected from this area (O'Connor, 1988; Wenning et al., 1992). The PCBs and PAHs in this area originated from, petroleum and chemical storage, refinery facilities, chemical manufacturing, and industrial discharges in to the area (Huntley et al., 1993; Wenning, 1994). Killifish residing in Newark Bay have elevated tissue levels of PCBs (Elskus et al., 1999; Monosson et al., 2003).

New Bedford Harbor:

New Bedford Harbor is also contaminated with PCBs, PAHs, metals and dioxins, but most if the contamination is mostly composed of PCBs (Pruell, 1990). Body burdens of PCBs in killifish collected from this area range from 1370 to 655 ppm (Lake, 1995). These are among the highest PCB concentrations reported. The main source of PCB contamination in New Bedford Harbor was an industrial facility that used PCB mixtures for manufacturing capacitors. This facility was open from the 1940s to the 1970s. Additional sources for contamination in this site are industrial and municipal discharges (Bello, 1999; Pruell, 1990). Organisms that inhabit these areas suffer from developmental abnormalities, altered behavior, immune function, and enzyme expression and activity (O'Connor, 1988).

The AHR pathway:

Much of the toxic effects caused by co-planar halogenated and non-halogenated aromatic hydrocarbons occur through activation of the aryl hydrocarbon receptor (AHR). The AHR forms part of the basic helix-loop-helix/Per-ARNT-Sim (bHLH-PAS) protein family. It remains inactive in the cytosol forming a stable complex with two heat chock proteins (HSP 90), the HSP90 co-chaperone p23 and the X-associated protein (or Ara9) (Carlson and Perdew, 2002; Safe, 2001). Upon activation by co-planar aromatic hydrocarbons, the AHR translocates to the nucleus and forms a complex the AHR nuclear translocator (ARNT) (Figure 2). This complex will recognize specific DNA sequences known as xenobiotic response elements (XREs) which are found in the promoter regions

of responsive genes (Swanson, 2002; Whitlock, 1999). Activation of the AHR leads to altered expression of hundreds of genes (Puga et al., 2000), including phase I and phase II xenobiotic metabolizing enzymes. This group responsive genes is known as the AHR gene battery. Because of their importance in xenobiotic metabolism the following phase I and phase II enzymes were studied in this thesis: cytochrome P450 1A (CYP1A), Dt-diaphorase, glutathione-s-transferase (GST), and uridine diphosphate-glucuronyl transferase (UDPGT).

The AHR and most of the genes involved in this pathway have been identified, cloned and sequenced, in fish. The AHR functional domains are conserved between different vertebrate models(Abnet et al., 1999; Hahn, 1998a; Hahn et al., 1994). Interestingly, some fish species, including killifish, have two AHR genes: AHR1 and AHR2. Ligands like TCDD bind to the two AHRs with almost equal efficiency, but the expression profile of the two killifish AHRs are different (Hahn, 1998a). AHR1 mRNA is expressed in killifish heart, brain, ovary, and testis. It also expressed in liver, kidney and gills, but at much lower levels. The expression of AHR2 mRNA is uniform in the organs mentioned above (Karchner et al., 1999).

Phase I and phase II xenobiotic metabolizing enzymes:

CYP1A:

Phase I xenobiotic metabolizing enzymes transform AHR ligands in to more water-soluble forms through hydrolysis, oxidation or reduction reactions. Cytochrome P450 1A (CYP1A) and DT-diaphorase are phase I xenobiotic metabolizing enzymes. CYP1A is a heme containing protein whose function is to add an oxygen atom to the xenobiotic substrate, and thus increases its hydrophylicity. CYP1A is one of the most studied enzyme within the AHR gene battery since it is strongly induced by AHR ligands (Lemaire, 1996; Zhang, 1990). Unfortunately, not all of the catalytic reactions performed by CYP1A result in excretion of xenobiotics. In some cases CYP1A can convert it's substrate in to an unstable form that can bind to DNA (Eberhart et al., 1992; Nebert et al., 2004) or generate reactive oxygen species through oxidation reduction cycles. Halogenated AHR ligands, such as dioxins and PCBs, can also uncouple the CYP1A

catalytic cycle which results in release of ROS from the enzyme's active site (Schlezinger et al., 2000; Shertzer et al., 2004).

In addition to CYP1A, cytochrome P450 1B1 (CYP1B1) can also be induced by AHR ligands in vertebrate and cell culture models. Like CYP1A, CYP1B1 can also metabolize PAHs in to unstable metabolites that can bind to other macromolecules and cause toxic damage (Nebert et al., 2004; Shimada et al., 2001).

DT diaphorase:

Xenobiotics that bind to and activate AHR can be converted in to quinones by CYP1A. These metabolites are considered toxic because they go through oxidation-reduction reactions that generate ROS (Di Giulio et al., 1995; Joseph et al., 1998). DT-diaphorase is a flavoprotein that catalyzes the two-electron reduction of quinoid compounds in to more stable hydroquinones, thereby preventing redox cycling and superoxide generation (Burdick et al., 2003; Joseph and Jaiswal, 1994; Ross et al., 2000).

Although this enzyme is considered to be part of the AHR gene battery (Ross et al., 2000), studies using fish models have yielded conflicting results. For example: (Lemaire, 1996) found that DTD activity was inducible by the AHR ligand β-naphthoflavone in rainbow trout (*Oncorhynchus mykiss*), but not in sea bass (*Dicentrarchus labrax*) and dab (*Limanda limanda*).

GST:

Phase II enzymes primarily act on functional groups added to xenobiotics during phase I reactions. Phase II metabolism of xenobiotics results in a large increase in hydrophilicity. Two of the more commonly studied phase II enzymes that for part of the AHR gene battery are: GST, and uridine diphosphate-glucuronyl transferase UDPGT.

Most of the GST enzymes are soluble and present in the cell cytosol. They are composed of two units and conjugate a variety of compounds with the endogenous tripeptide glutathione (GSH). The mammalian GSTs are divided in to four different families: alpha (α), pi (π), mu (μ), and theta (θ) (Sheweita, 2000). GSTs can convert toxic PAH metabolites generated from phase I metabolism in to a less harmful form. Whole animal, and in-vitro studies have found that GSTs can conjugate (±)-anti-

benzo[a]pyrene-trans-7,8-dohydrodiol-9,10-epoxide (BPDE) (Gallagher, 1996; Xia et al., 1998). BPDE is a benzo-a-pyrene (B[a]P) metabolite generated by CYP1A and other P450 enzymes. It is highly unstable, mutagenic and carcinogenic. These findings suggest that high GST enzyme activity potentially protects against formation of PAH-DNA adducts. In addition to metabolism of toxic xenobiotics, GST is also associated with detoxication of lipid peroxides and protection against oxidative tress (Yang et al., 2004; Zimniak et al., 1997).

Studies using fish models have generated conflicting results on GST induction after treatment with AHR ligands (Lemaire, 1996). Basal expression and AHR dependent induction of this enzyme varies from one fish model to another, suggesting possible differences in the mechanism for induction, and susceptibility to contaminants. For example: studies using the comparing two species of catfish show that channel catfish have higher basal expression of GST, than brown bullheads. Channel catfish are also less vulnerable to DNA adduct formation and toxic effects caused by B[a]P (Ploch et al., 1998).

UDPGT:

Conjugation with uridine diphosphate-glucuronic acid (UDP-glucuronic acid) acid is an important mechanism in xenobiotic metabolism and elimination. This reaction is carried out by UDPGT, is a membrane-associated protein located in the endoplasmic reticulum (Sheweita, 2000). In most cases, glucurinic metabolites are more water soluble and easily excreted, but conjugation with glucuronic acid can also generated toxic metabolites. Induction of UDPGT has also been associated with depletion of thyroid stimulating hormone (Schuur et al., 1997). Studies using various fish models have demonstrated that AHR ligands (PAHs and PCBs) can induce UDPGT activity in liver tissue (Nacci et al., 2002; Novi et al., 1998; Zhang, 1990).

Toxic effects cause by PAHs and HAHs in fish

Exposure to AHR ligands causes a variety of toxic effects in exposed fish. For example; under laboratory conditions PCBs and PAHs have been shown to cause developmental deformities (Henry et al., 1997), suppression of the immune system

(Carlson et al., 2002; Duffy et al., 2003), altered expression of pro and anti-oxidant enzymes (Otto and Moon, 1995; Schlezinger and Stegeman, 2001), and increased cell proliferation and cancer (Baumann, 1998; Grinwis et al., 2000). These studies described in this thesis were aimed at studying AHR ligand dependent induction of developmental deformities and oxidative stress in contaminated and reference site killifish.

Development

Embryonic exposure to AHR ligands increases CYP1A induction, and teratogenesis during early developmental stages of fish and other vertebrates (Hoffman et al., 1996; Nacci et al., 1998). Frequently observed developmental effects caused by AHR ligand exposure include edema, cardiovascular dysfunction, hemorrage, craniofacial malformations and increased mortality (Henry et al., 1997; Meyer and Di Giulio, 2002).

There is still conflicting data on the role of AHR and CYP1A in mediating xenobiotic induced deformities. For example; studies using morpholio nucleotides, that bind to target mRNA and block transcription, have shown that AHR, but not CYP1A mediates TCDD induced deformities in zebrafish embryos (Carney et al., 2004; Prasch et al., 2003). Other studies using P450 inhibitors suggest that CYP1A is involved in these toxic effects in medaka embryo development (Cantrell et al., 1996). Another interesting observation is that exposure to AHR and CYP1A inhibitors in combination with a PAH increases deformities (Hawkins et al., 2002; Wassenberg and Di Giulio, 2004). But when AHR and CYP1A inhibitors are given with PCB126 the co-treatment reduced PCB126 induced deformities (Wassenberg, in press). It is possible that PAHs and PCBs increase developmental toxicities through different mechanisms.

Oxidative stress

Exposure to AHR ligands has been shown to cause oxidative stress by increasing lipid peroxidation, DNA strand breaks, expression of stress proteins, alteration of oxidized vs. reduced glutathione levels, as well as enzymes that regulate oxidative status (Dalton et al., 2002; Nebert et al., 2000). Oxidative stress is a cellular response to increased levels of oxygen radicals (e.g., superoxide, and hydrogen peroxide and

hydroxyl radicals). Increased production of reactive oxygen species (ROS) can affect cellular health and function (de Vries, 1996; Di Giulio et al., 1995).

Exposure to coplanar polychlorinated biphenyls (co-PCBs) and polychlorinated dibenzo-p-dioxins (e.g. 2,3,7,8-tetrachlorodibenzo-p-dioxin, TCDD), strongly induces oxidative stress in vertebrate research models such as rodents (Fadhel et al., 2002; Hassoun et al., 2002), human hepatoma cells (Park et al., 1996), and fish (Palace et al., 1996; Schlezinger and Stegeman, 2001). Oxidative stress caused by TCDD and PCBs is associated with AHR activation (Alsharif et al., 1994; Park et al., 1996). It has been hypothesized that AHR ligand induced ROS production could result from a variety of mechanisms, including AHR mediated induction of P450 enzymes (e.g. CYP1A1/2), and antioxidant and pro-oxidant enzymes (Dalton et al., 2002; Nebert et al., 2004).

Resistance

Various groups have documented that some fish populations from contaminated sites show extraordinary resilience to immediate toxic effects caused by AHR ligands and other toxicants(Wirgin and Waldman, 2004). For example; Atlantic tomcod (*Microgadus tomcod*) from the Hudson River are resistant to co-planar PCB mediated induction of CYP1A activity and developmental deformities (Courtenay et al., 1999; Wirgin and Waldman, 2004).

There are three identified killifish populations in contaminated sites of the United States east coast that are resistant to the immediate toxic effects caused by PAHs and PCBs. They are located in Newark Bay (NJ), New Bedford Harbor (MA), and a PAH contaminated portion of the Elizabeth River (VA). Bellow is a brief description of killifish from these sites.

Newark Bay killifish:

As mentioned above, Newark Bay is contaminated with a variety of pollutants from very different origins, and killifish as well as other organisms that inhabit this aquatic environment are exposed to this complex mixture. Although killifish from Newark Bay have higher tissue concentrations of PCBs than reference site animals (Elskus et al., 1999; Monosson et al., 2003), laboratory studies have revealed that they

have become resistant to exposure to AHR ligands. For example; waterborne or dermal exposure increases EROD in adult killifish from reference site animals, but not in Newark Bay fish (Prince and Cooper, 1995b). Reference site animals were also more sensitive to TCDD induced hemorrhaging, edema, and mortality than Newark Bay killifish (Prince and Cooper, 1995a). Exposures to environmentally relevant mixtures of PCBs have revealed that Newark Bay first generation (F1) killifish larvae are resistant to PCB mediated EROD induction, suggesting that resistance to CYP1A induction is heritable in this population (Elskus et al., 1999).

New Bedford Harbor killifish:

Despite the fact that New Bedford Harbor killifish inhabit a site that is heavily contaminated with PCBs, these animals have also developed resistance to AHR ligand effects. Adult NBH killifish are resistant to B[a]P, and 2,3,7,8-tetrachlorodibenzofuran (TCDF) mediated induction of CYP1A mRNA, protein and enzymatic activity (Bello et al., 2001; Nacci et al., 2002). Lab raised, F1, and second generation (F2) killifish embryos from NBH are resistant to co-planar PCB induced EROD and developmental deformities (Nacci et al., 1999). This suggests that the mechanism of resistance to CYP1A induction is genetic.

Elizabeth River killifish:

A killifish population from the southern branch of the Elizabeth River (VA), has also developed resistance to AHR ligands. The sediments from this area are contaminated with PAHs generated from a wood treatment facility (Atlantic Wood, Industries) that is no longer operating. Although killifish from this site have a higher incidence of hepatic tumors, and DNA adducts than reference site animals (Vogelbein et al., 1990), studies by various groups have revealed that these killifish have developed resistance to PCBs and PAHs. Adult killifish from this site are resistant to PAH mediated induction of CYP1A (Van Veld and Westbrook, 1995) and F1 generation embryos are resistant to PCB induction of EROD and deformities (Meyer and Di Giulio, 2002). Also, hepatic basal expression of GST in Elizabeth River killifish is six fold higher than reference site fish (Armknecht et al., 1998). Since GST is associated with conjugation

and elimination of unstable xenobiotic metabolites (Sundberg et al., 2002), higher basal GST expression is could be protective against PAH toxicity (Weis, 2002).

The mechanism for resistance to CYP1A induction in this population is apparently different from Newark Bay and New Bedford Harbor killifish. Lab raised, F1 generation killifish from the Elizabeth River are resistant to CYP1A induction, but F2 killifish embryos are responsive to CYP1A induction by AHR ligands (Meyer et al., 2002). This suggests that resistance to CYP1A induction in Elizabeth River killifish is caused by a non-genetic mechanism. It has been proposed that these animals gain resistance by an epigenetic mechanism such as DNA methylation of the CYP1A gene.

Specific aims

Differential sensitivities to halogenated and non-halogenated AHR ligands has been observed in different strains, species, populations and sexes in experimental animals. As mentioned above, *Fundulus heteroclitus* populations exposed to anthropogenically introduced AHR ligands over multiple generations have developed resistance to their acute toxic effects. In Newark Bay killifish, resistance is characterized by a lack of CYP1A induction after exposure to HAHs, includingTCDD and co-planar PCBs (Elskus et al., 1999; Prince and Cooper, 1995a). The main hypothesis of this thesis is that in chronically contaminated killifish, recalcitrance AHR activation and CYP1A induction is protective against AHR ligands. We also hypothesized that altered AHR expression or CYP1A promoter sequence grants resistance to AHR ligands in Newark Bay and New Bedford Harbor killifish. To prove these hypothesis I addressed the following questions:

1. Are phase I and phase II enzymes associated with AHR ligand metabolism and excretion equally regulated in contaminated site killifish and reference site killifish?
2. Are Newark Bay killifish resistant to CYP1A induction by co-planar PCBs, and non chlorinated AHR ligands during different developmental stages?
3. Is resistance to CYP1A induction associated with resistance to AHR ligand induced teratogenesis, and ROS production?
4. Is altered AHR expression or CYP1A promoter sequence associated with resistance in killifish?

Figure 1-1: Map of collection site (*): Newark Bay, NJ.

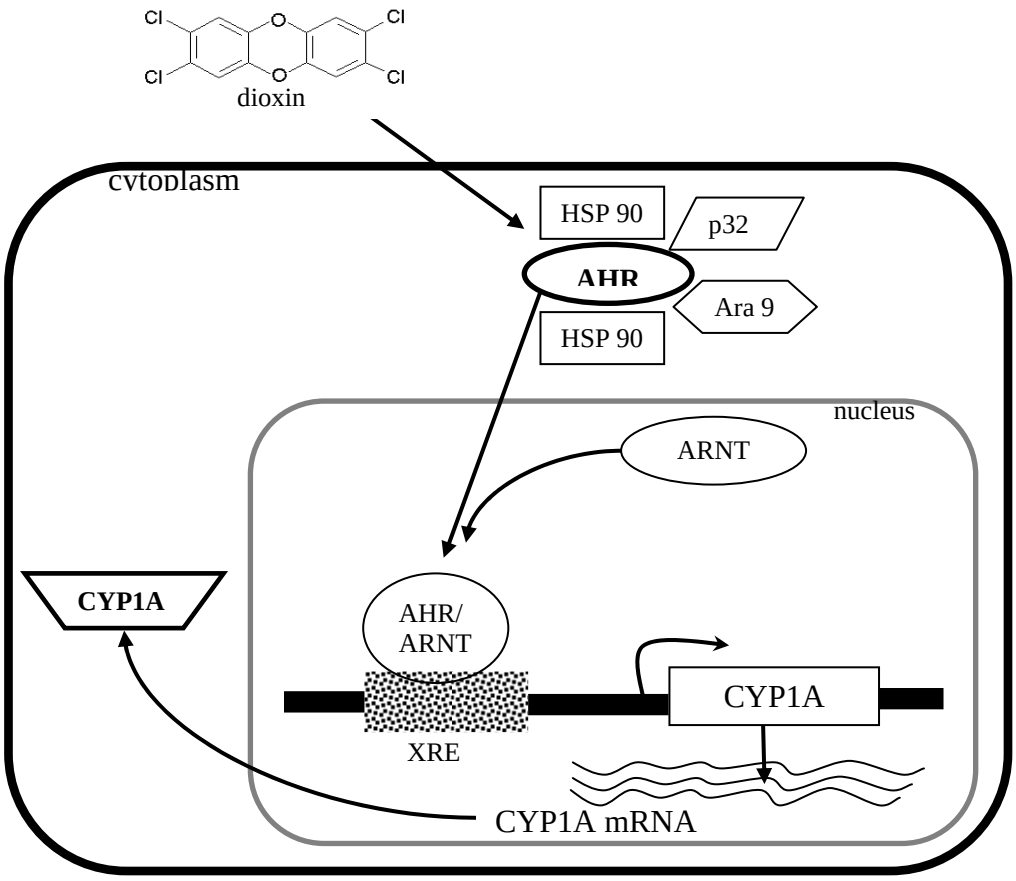


Figure 1-2: The AHR pathway. AHR ligand (dioxin) diffuses in to the cell and activates the AHR, which then translocated to the nucleus, where in combination with ARNT, binds to xenobiotic response elements (XREs) and initiates transcription of responsive genes. Modified from (Hahn, 1998b; Safe, 2001)

Chapter Two

Evidence for resistance to B[a]P and PCB-77 in a chronically polluted *Fundulus heteroclitus* population

Abstract

Halogenated aromatic hydrocarbons (HAHs) and polycyclic aromatic hydrocarbons (PAHs) are major environmental contaminants. Fish species that are chronically exposed can develop resistance to their toxic effects. In all fish species studied to date, toxicant resistance has been accompanied by decreased inducibility of the xenobiotic metabolizing enzyme, cytochrome P450 1A (CYP1A). CYP1A induction is mediated through the Aryl Hydrocarbon Receptor (AHR). Although these compounds mediate their effects through this pathway, there have been resistant populations in which one chemical class cannot induce CYP1A expression (HAHs) while the other (PAHs) can. Cross-resistance to HAHs and PAHs was examined in a HAH-resistant population of *F. heteroclitus* (Newark Bay, NJ). Fish were injected intraperitoneally with the HAH 3,4, 3',4'-tetrachlorobiphenyl (PCB77), benzo[a]pyrene (B[a]P, a PAH) or vehicle and sacrificed after two (B[a]P) or five days (PCB77, vehicle). Our data showed no significant increase in CYP1A mRNA levels in resistant Newark Bay *F. heteroclitus* treated with either B[a]P or PCB, while there was a 3.9 fold (PCB) and 4.2 fold (B[a]P) increase in CYP1A mRNA in Flax fish. AHR labeling studies revealed significantly lower levels of AHR ($P<0.05$) in Newark fish (1770 ± 1693.2 DPM) than in Flax fish (6082.5 ± 1709.9 DPM). Overall, these data suggest resistance to both PAHs and HAHs in Newark *F. heteroclitus* at the level of CYP1A mRNA, which might be mediated, in part, though lower expression of AHR.

Introduction

Exposure to HAHs causes transcriptional activation of genes involved in cell growth and differentiation, wasting syndrome and tumor promotion. Harmful effects of HAHs have also been observed in fish (Guiney et al., 1997; Henry et al., 1997). Much of

the biological activity of these compounds is mediated through the AHR (Mimura and Fujii-Kuriyama, 2003; Nebert et al., 2004)

In fish, resistance to HAHs has been observed in populations chronically exposed to these compounds and is characterized, in part, by lack of inducibility of the xenobiotic metabolizing enzyme CYP1A (Hahn, 1998b; Wirgin and Waldman, 2004). Although resistant fish have a functional AHR, it is highly resistant to activation by xenobiotic AHR ligands (Bello et al., 2001). For example: killifish from New Bedford Harbor MA, a PCB contaminated site are resistant to CYP1A induction when treated with TCDF (one of the most potent AHR ligands). This lower sensitivity to inducers suggests that resistance is likely mediated by changes in the AHR pathway (e.g. altered receptor affinity, alterations in the CYP1A promoter). Further evidence that inducibility is not completely lost in resistant fish is demonstrated by the finding that some resistant populations show selective responses to different chemical classes. (Courtenay et al., 1999) showed that Hudson River tomcod, which are resistant to HAHs, respond to treatment with PAHs with increased expression of CYP1A mRNA and enzyme activity. Since Newark Bay *F. heteroclitus* are chronically exposed to both these chemical classes in their environment, we hypothesized that resistance to both types of CYP1A inducers may have developed in the Newark population.

Materials and methods

Killifish collection and maintenance

Male killifish were collected from a reference site (Flax Pond, NY) and a site contaminated with organic and inorganic pollutants (Newark Bay, NJ). Fish ranged from 6.7 ± 0.3g (Flax Pond) to 2.3 ± 0.2g (Newark Bay) bw; and were reproductively mature (Flax 1.6 ± 0.4% GSI; Newark 4.5 ± 1.2% GSI). Fish were injected intraperitoneally with B[a]P 50mg/kg bw, 1mg PCB77/kg, or corn oil (vehicle) and held in recirculating SW systems ($35^{0}/_{00}$) at 20°C under a 14/10 L/D cycle and fed daily. Fish were sampled after two (B[a]P) or five days (PCB, vehicle), times when teleost CYP1A mRNA is maximally induced (Hahn and Stegeman, 1994; Kloepper-Sams and Stegeman, 1989).

Northern blot analysis

Northern blot analysis of CYP1A mRNA was performed using hepatic RNA extracted from *F. heteroclitus*. Total RNA was prepared using Trizol (Invitrogen, Carlsbad, CA) as recommended by the manufacturer (Gibco BRL). RNA (10 µg) was electrophoresed (1% agarose gel), transferred to a nylon membrane, and probed with a digoxigenin (DIG) labeled CYP1A *F. heteroclitus* RNA probe. Riboprobe was made from a *F. heteroclitus* CYP1A partial cDNA construct by using DIG-11-UTP as recommended by the manufacturer (Roche Molecular Biochemicals).

Microsome preparation and analysis

Liver microsomes were isolated as described (Elskus et al., 1999). Hepatic microsomal CYP1A catalytic activity was measured fluorometrically as ethoxyresorufin-O-deethylase (EROD) in 48 well plates in triplicate. CYP1A protein was measured by immunoblotting using monoclonal antibody MAb 1-12-3 which recognizes CYP1A from *F. heteroclitus* (Kloepper-Sams et al., 1987) as described (Elskus et al., 1999). For quantification of AHR, liver cytosols from untreated Flax and Newark fish were prepared and quantified for total AHR by photoaffinity labeling with [^{125}I] iodo-7,8-dibromodibenzo-p-dioxin as described (Hahn et al., 1994).

Statistical analysis

Statistical analysis was performed using SYSTAT version 10 (Systat software Inc., Point Richmond CA). Square root transformation of the data was performed before statistical analysis. Two-way ANOVA followed by Fishers Least Significant Difference (LSD) was used to detect significant differences between treatment groups and populations. Level of significance was 0.05.

Results and Discussion

Northern blots revealed a significant increase ($P<0.05$) in hepatic CYP1A mRNA in both PCB and B[a]P treated Flax Pond, but not Newark Bay, *F. heteroclitus* (Figure 1). This suggests that the Newark Bay killifish population is resistant to CYP1A induction by both compounds. CYP1A catalytic activity and protein levels were highly variable

within treatment groups (Figures 2, and 3). This may be due to short depuration time (2 months). Freshly caught Flax *F. heteroclitus* have high EROD activity (J. Zielinski, personal communication), while livers of freshly caught Newark *F. heteroclitus* contain considerable tissue concentrations of PCBs (~1600ppb) (Monosson et al., 2003). As the half-life of PCBs in fish tissue is approximately four months (Niimi, 1983), Newark Bay killifish livers may have contained enough PCB to competitively inhibit CYP1A activity (Gooch et al., 1989; White et al., 1997). The high number of animals per pool (5-10) may also be a source of variability, with high or low responders making outsized contributions. High variability in resistant Newark *F. heteroclitus* has also been noted by others (Elskus et al., 1999; Prince and Cooper, 1995a).

AHR levels in hepatic cytosols of Newark Bay *F. heteroclitus*, (1770.0±1693.17 DPM) were significantly lower than levels in Flax fish (6082.5 ± 1709.9 DPM), a 3.4 fold difference. Lower AHR levels could be part of a mechanism by which Newark Bay killifish gain resistance to CYP1A induction. For example, absence of AHR is characterized by lack of inducibility of CYP1A1/2 in mammalian AHR knockout models (Shimizu et al., 2000). Lower AHR levels, resulting in fewer AHR-DNA complexes, accompanied by lower sensitivity of this pathway in resistant fish (Bello et al., 2001), may contribute to the resilience to CYP1A induction observed in the Newark Bay killifish population.

Spare AHR has been observed in various models. One of them is PLHC-1 cells obtained from the teleost *Poeciliposis lucida* (Hesterman et al., 2000). According to the model proposed by this group, a reduction of up to 85% of AHR levels can still allow approximate maximal induction of CYP1A levels, given that the EC50 value would increase by 7-fold (Hesterman et al., in Press). It is not yet known if the remaining AHR in polluted site killifish could still be activated.

Experiments using a higher number of fish that were allowed to depurate for four months are described in chapter three. Studies aimed at determining if resistance to CYP1A induction is due to alterations in the DNA sequence or methylation status of the CYP1A promoter are described in chapter eight.

Figure legends

Figure 2-1 (a): CYP1A mRNA from corn oil, PCB77 and B[a]P treated killifish from Flax Pond (reference site) and Newark Bay (resistant population). Each treatment group consists of three pools of 5 (Flax) to 10 (Newark) livers (n=3). (b) CYP1A protein, expressed as relative absorbance; (c) EROD activity (pmol/min/mg microsomal protein)

Figure 2-2: Liver cytosolic AHR levels ± SD in Flax and Newark *Fundulus heteroclitus* measured by photoaffinity labeling .

Figure 2-1a. CYP1A mRNA from corn oil, PCB77 and B[a]P treated killifish

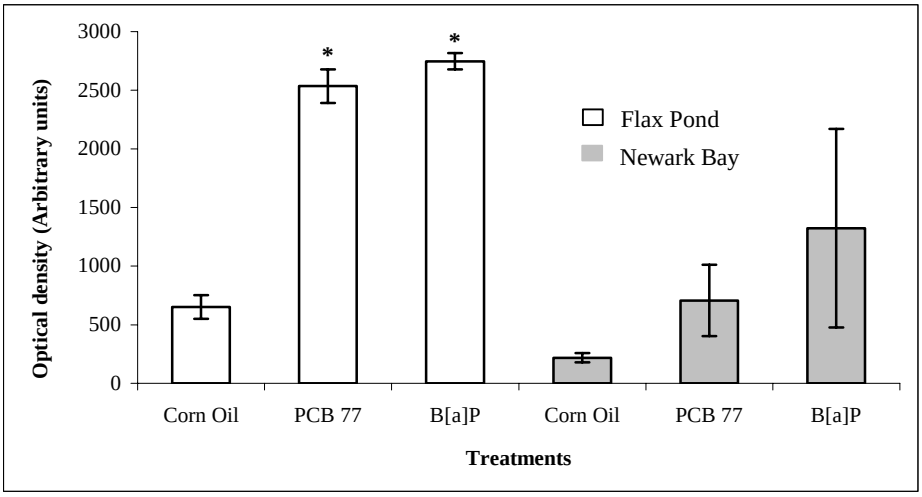


Figure 2-1b. CYP1A protein from corn oil, PCB77 and B[a]P treated killifish

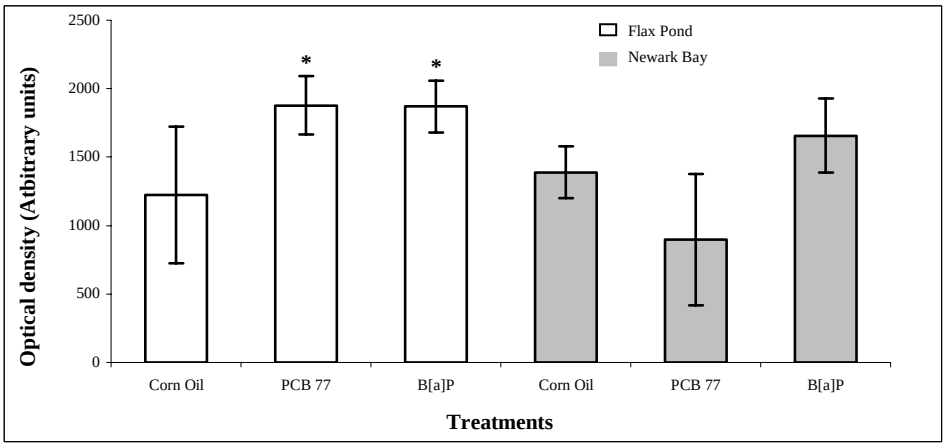


Figure 2-1c. CYP1A activity from corn oil, PCB77 and B[a]P treated killifish

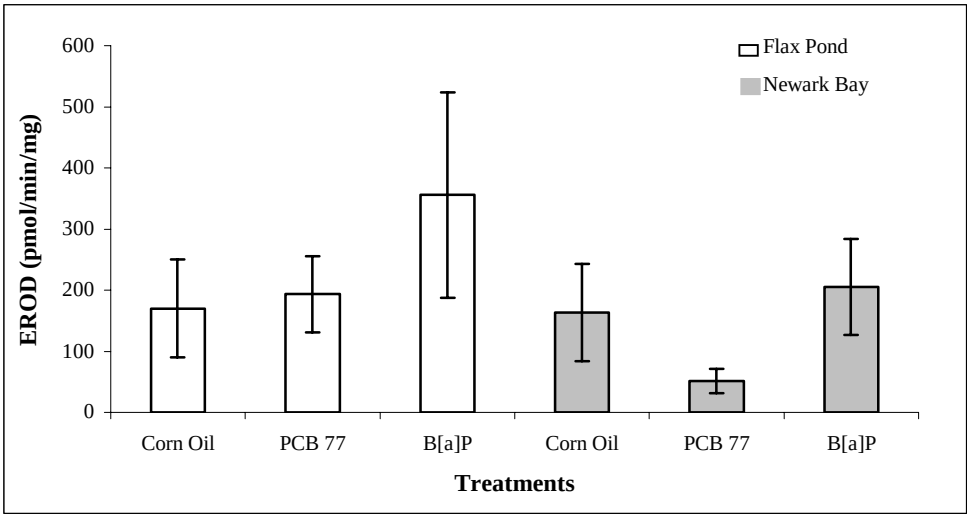
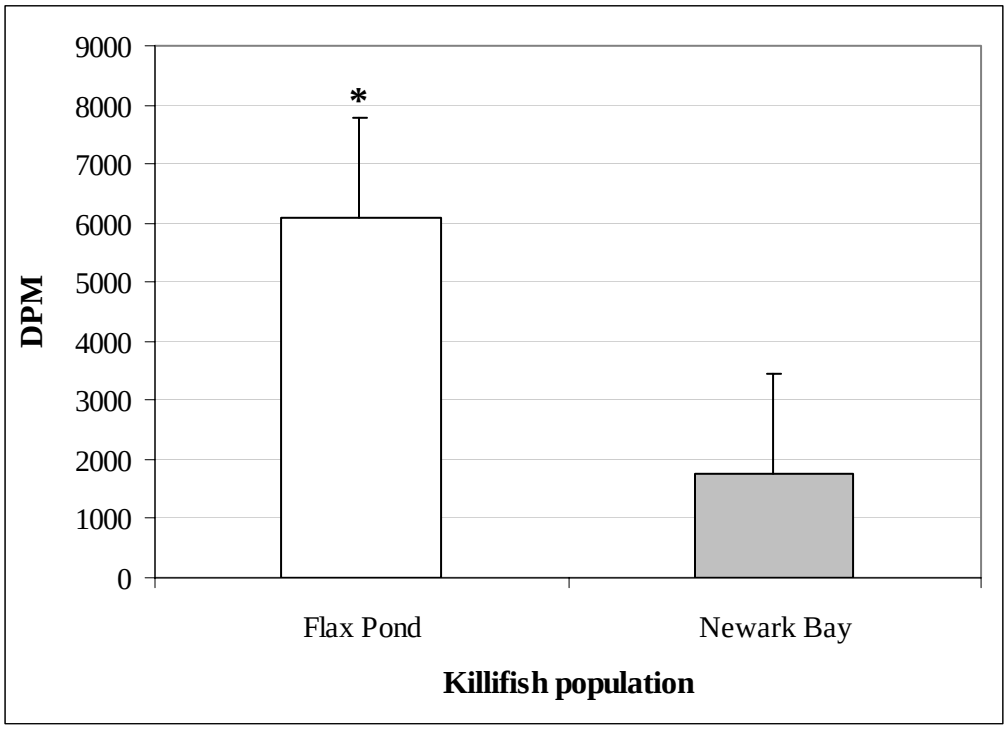


Figure 2-2. Liver cytosolic AHR levels ± SD in Flax Pond and Newark Bay killifish



Chapter Three

Differential regulation of the AHR gene-battery in resistant and responsive killifish (*Fundulus heteroclitus*) populations.

Abstract

Exposure to aryl hydrocarbon receptor ligands leads to induction of a variety of genes collectively known as the AHR gene battery. Among the genes that respond to this molecular signal there are important enzymes involved in the metabolism of these chemicals. This study focuses on chemical regulation of aryl hydrocarbon receptor (AHR) battery genes (cytochrome P450 1A (CYP1A), glutathione-S-transferase (GST), uridine diphosphate glucuronyl transferase (UDPGT), and DT-diaphorase) and a monooxygenase not regulated via the AHR, P450 3A (CYP3A), in two populations of killifish with different tolerance to toxicants. Resistant (Newark Bay, NJ) and responsive (Flax Pond, NY) killifish were treated by intraperitoneal (ip) injection with vehicle (corn oil), 3,3',4,4'-tetrachlorobiphenyl (PCB77, 1 mg/kg) or benzo-a-pyrene (B[a]P, 50 mg/kg). Fish were killed after forty eight hours (B[a]P) or 5 days (vehicle, PCB77) and liver tissue prepared for measurement of microsomal CYP1A protein content and catalytic activity (ethoxyresorufin-o-deethylase, EROD), microsomal CYP3A protein content, activity of 4-nitrophenol UDPGT, and cytosolic 1-chloro-2,4-dinitrobenzene (CDNB) GST, and dichlorophenol-indophenol (DCPIP) DT-diaphorase. Treatment with PCB77 or B[a]P significantly induced both CYP1A and CYP3A in responsive killifish, producing a 3.3 and 4.1 fold induction in CYP1A activity (EROD), a 7.4 and 10.1 fold induction in CYP1A protein, and a 2.8 and 3.6 fold induction in CYP3A protein, respectively, relative to controls. In contrast, PCB77 and B[a]P treatment had no effect on these enzymes in resistant fish. UDPGT and GST activities in resistant and responsive fish failed to respond to either treatment. However, basal GST activity was higher (2.2 fold) in resistant compared to responsive fish. DT-diaphorase activity was not detected in killifish, although our method did detect this activity in other fish species. Together, our results suggest that the chronically polluted Newark Bay killifish population has developed resistance to CYP1A induction by multiple AHR ligands, that

resistance extends to other inducible P450 forms involved in toxicant metabolism, and that despite regulation through a common r eceptor, expression of AHR battery genes is not coordinately regulated. We speculate that resistance to chemical toxicity in PCB tolerant fish may be conferred, at least in part, by a combination of suppressed toxicant activation accompanied by elevated detoxication (via conjugation) activity.

Introduction

Environmental pollutants that activate the aryl hydrocarbon receptor (AHR) can cause a wide variety of toxic effects, including immune system suppression, developmental abnormalities, tumor promotion and carcinogenesis (Carlson et al., 2002; Meyer and Di Giulio, 2002; Safe, 2001; Teraoka et al., 2002). Binding of xenobiotics, such as halogenated aromatic hydrocarbons, HAHs (e.g. tetrachlorodibenzo-p-dioxins and co-planar polychlorinated biphenyl congeners), and polycyclic aromatic hydrocarbons (PAHs, e.g. benzo[a]pyrene), to the AHR results in altered expression of a group of genes that are collectively referred to as the AHR gene battery (Nebert et al., 2000). Activities of some of the enzymes that respond to AHR activation are associated with the toxicity caused by such pollutants. For example, cytochrome P450 1A (CYP1A) has been shown to convert xenobiotics in to toxic intermediates that can bind to macromolecules, and increase reactive oxygen species production (Guengerich and Shimada, 1998; Schlezinger and Stegeman, 2001). Yet, other genes that form part of this response are involved with metabolism and excretion of the same xenobiotics. For example, glutathione-s-trasferase (GST), uridine diphosphate glucuronyl transferase (UDPGT) and DT-dipahorase are involved with detoxification xenobiotic PAH metabolites (Joseph and Jaiswal, 1994; Sheweita, 2000). The expression pattern that results from such exposures could lead to metabolism of the parent compound in to toxic intermediates, or result in detoxification and excretion of these xenobiotics.

Exposure to HAHs can result in altered expression of AHR battery genes for prolonged periods of time. For example; rainbow trout (Oncorhynchus mykiss) exposed to a polychlorinated biphenyl (PCB) mixture over a period of twenty weeks become resistant to CYP1A induction by PCBs and the PAH 3-methylcholanthrene (Celander and Forlin, 1995), and have increased activity of the xenobiotic metabolic enzymes: GST, Dt-

dipahorase, UDPGT and glutathione reductase (GR) (Forlin et al., 1996). Also, MCF-7 cells exposed to benzo-a-pyrene over a six month period develop resistance to dimethylbenz[a]anthracene (DMBA) induced apoptosis, DNA adduct formation, and 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) activation of CYP1A1 transcription (Ciolino et al., 2002). This suggests that altered expression of toxicant metabolizing enzymes could be essential in adapting to environmental xenobiotics (Farber, 1990).

Exposure to environmental contaminants containing AHR ligands has deleterious effects on populations of aquatic organisms. Despite chronic exposure to potent toxicants, some populations continue to thrive (Wirgin and Waldman, 2004). The biochemical mechanisms underlying such chemical resistance are not well understood, however there is growing evidence that resistance to toxicants that activate AHR may be mediated, at least in part, through altered expression of AHR regulated genes (Weis, 2002). In some fish populations, chronic exposure to these chemicals lead to the development of resistance to their toxic effects, as observed in killifish (Fundulus heteroclitus) exposed to PCBs in New Bedford Harbor MA, (Bello et al., 2001; Nacci et al., 1999), PAHs, in the Elizabeth River VA, (Van Veld and Westbrook, 1995), and mixtures of both these chemical classes with metals and pesticides in Newark Bay NJ, (Elskus et al., 1999; Prince and Cooper, 1995b).

The Newark Bay (NJ) environment has been exposed to pollution since the 1800's. Contamination in the New Jersey estuary originated from industrial development and domestic sources. High levels of dioxins (such as TCDD), PCBs, PAHs, heavy metals, pesticides and other types of contaminates can be found in sediments (Crawford et al., 1995). Killifish residing in Newark Bay are resistant to the toxic effects of chemicals at this site (Prince and Cooper, 1995b), despite elevated levels of PCBs in their tissues (Elskus et al., 1999; Monosson et al., 2003). It is possible that these animals achieve tolerance to their toxic effects by altering the expression of AHR gene battery enzymes.

The purpose of this study is to compare the expression and activity of genes from the AHR gene battery between the Newark Bay killifish population and a reference site population (Flax Pond, NY). We studied expression and metabolic activity of CYP1A, GST, UDPGT, and DT-diaphorase, after treatment with the AHR ligands PCB77 or

benzo-a-pyrene (B[a]P). These enzymes were chosen because of their potential role in toxic metabolic activation (Shimada and Fujii-Kuriyama, 2004) or detoxification (Miller and Ramos, 2001; Sundberg et al., 2002) of AHR ligands. We also studied expression of cytocrome P450 3A (CYP3A) protein. This enzyme does not form part of the AHR gene battery, but because of its involvement in xenobiotic metabolism (Guengerich and Shimada, 1998; Shimada et al., 1989), we hypothesized that its expression is also altered in Newark Bay killifish. As demonstrated in chapter five, the expression and activity of these enzymes is associated with survival of highly contaminated killifish.

Materials and Methods

Materials:

PCB77 was a generous gift from Dr. Hans-Joachim Lehmler (University of Iowa). Benzo[a]pyrene and 4 nitrophenol were purchased from Sigma (St. Louis, MO). Resorufin and 7-ethoxyresorufin, dicoumarol, and 1-cloro-2, 4-dinitrobenzene (CDNB), were purchased from Molecular Probes (Eugene, OR), CalBiochem (La Jolla, CA), and Mid West Scientific (St. Louis, MO) respectively. Polyacrylamide gradient gels (4 to 12%) as well as gel running and transfer apparatus were from Invitrogen (Carlsbad, CA). Nitrocellulose membrane (0.45 μm) for western blots was purchased from Schleisher and Schull (Keene, NH). All other biochemicals were from Sigma.

Killifish collection and maitenance:

Killifish were collected from Flax Pond, NY and the Roanoke Yacht Club in Newark Bay, NJ using minnow traps, and were depurated for four months in static, recirculating systems with artificial seawater. Temperature was maintained between 18 and 20°C, and salinity at 30 ppt. Approximately 90% of the water volume in the tanks was replaced every month. Killifish were kept on a daily diet of Tetramin. Animals were distributed into different tanks according to the treatments they received. There were a total of 27 males and 11 females from Newark Bay, and 30 males and 9 females from Flax Pond. The experiment was performed in January 2001 when the fish were gonadally regressed.

Fish treatments:

Killifish from Flax Pond (6 ± 2g) and Newark Bay (6 ± 3g) were injected intraperitoneally with vehicle (corn oil), 3, 3', 4, 4' tetrachlorobiphenyl (PCB77) (Ballschmiter and Zell, 1980) at 1mg/kg body weight, or B[a]P at 50mg/kg body weight. Animals injected with B[a]P were sampled 2 days after treatment, and those receiving PCB77 or vehicle, after 5 days. Treatment regimes were based on dose response and kinetic experiments performed by other groups (Gooch et al., 1989; Hahn and Stegeman, 1994; Kloepper-Sams and Stegeman, 1989). Fish were killed by cervical transsection, the liver removed, weighed, taking care not to rupture the gall bladder, and immediately frozen in liquid nitrogen for later biochemical analysis within two months.

Tissue preparations:

Hepatic microsomes and cytosols were prepared as previously described (Stegeman et al., 1979). Briefly, livers were pooled (≥ 0.2g/pool; 2 to 5 livers per pool). Liver tissue was homogenized in ice cold 50 mM Tris (pH 7.5) using a teflon homogenizer. Homogenates were spun at 1,500 x g (10 min.) and 10,000 x g (10 min.) to pellet the nucleus, cell membranes and mitochondria. The supernatant was spun at 100,000 x g for one hour and the resultant microsomal pellet homogenized in re-suspension buffer (50mM Tris, 1mM EDTA, 1mM DTT and 20% glycerol) and stored in liquid nitrogen. Glycerol was added to the cytosolic fraction (20% v:v) and stored in liquid nitrogen.

Determinations of protein concentration and enzyme assays:

Microsomal and cytosolic protein concentrations were determined fluorometrically as described (Lorenzen and Kennedy, 1993) using bovine serum albumin (BSA) as the standard. All samples were run at least in duplicate.

CYP1A catalytic activity ethoxyresorufin-O-deethylase (EROD) activity was measured in 48 well plates (Costar) as previously described (Hahn et al., 1996). Reaction mixtures contained liver microsomes (10 μl; 30 to 130 ug of protein), 7-ethoxyresorufin (2.0 μM, final concentration), in 50mM Tris, 0.1 M NaCl. The reaction was initiated by

addition of NADPH (1.34mM, final concentration). The final reaction volume was 150µl. Enzymatic activity (CYP1A conversion of 7-ER to resorufin) was measured fluorometrically using a Cytofluor 4000 (PE Biosystems, Foster City, CA) plate reader. All EROD assays were performed at room temperature,. The reaction was followed for 10 minutes and the linear portion of the curve used to determine reaction rate. All samples were run in triplicate.

Cytosolic GST activity was measured using the nonspecific substrate 1-chloro-2,4-dinitrobenzene (CDNB) as described in (Armknecht et al., 1998). Reactions were carried out in 1 ml quartz cuvettes containing 0.1M potassium phosphate buffer, pH 6.5, 1mM glutathione, 1mM CDNB and 15 µl (41.4-140 µg) of cytosolic protein in a final reaction volume of 1 mL. The formation of the CDNB conjugate was monitored on a dual-beam uv-vis spectrophotomoeter (Shimadzu UV-1601, Norcross, GA), at a wavelength of 340 nm for eighty seconds. All reactions were performed at room temperature in triplicate.

Uridine diphosphate glucuronyl transferase (UDPGT) activity was measured as described (Nacci et al., 2002), with 4-nitrophenol as the substrate. Liver microsomes (15 µl; 50 to 190µg) were added to a reaction buffer containing 250mM Tris-HCl (pH 7.4), 5mM MgCl and 0.5 mM 4-nitrophenol. Reactions were initiated by addition of UDP-glucuronic acid (UDPGA) (final concentration 5mM) and followed for 30 minutes at 25 °C. Reactions were carried out in two duplicate sets: one set containing UDPGA and the other deionized H_2O (dI H_2O) to monitor the reduction in color resulting from the formation of 4-nitrophenol β-glucuronide. The final reaction volume was 200µl. After 30 minutes, reactions were stopped by adding 2 volumes of ice cold 0.5M trichloroacetic acid (TCA), and then neutralized by adding 1 volume of 2M sodium hydroxide (NaOH). Finally, the reaction was diluted in 3 volumes of dI H_2O and absorbance measured at 405 nm. Enzyme activity was calculated using a 4-nitrophenol extinction coefficient of 18.1 cm^2/mol (Burchell and Weatherill, 1981). All samples assays were performed in duplicate.

DT-diaphorase (NAD(P)H oxidoreductase) activity was measured as described (Hasspieler and Di Giulio, 1992). Briefly, the following reagents were mixed in a 1 ml quartz cuvette: 0.3-1.0 µg of cytosolic protein, assay buffer (50 mM Tris-HCl, pH 7.5),

0.1% bovine serum albumin (BSA) and 1.0 mM NADH, and 0.05 mM dichlorophenol-indophenol dissolved in assay buffer. The final reaction volume was 1 ml. Blanks contained all the reagents mentioned above plus 10 μM dicoumarol (DC). DC is a powerful DT-diaphorase inhibitor. The reaction was initiated by addition of the enzyme substrate (dichlorophenolindiphenol, DCPIP) to the sample and reference cuvettes. The disappearance of DCPIP was monitored by measuring changes in absorbance (600 nm) over 1 minute. Enzyme activity was calculated using the DCPIP extinction coefficient ($21 mM^{-1}\ cm^{-1}$, (Lind et al., 1990)). All samples were run at least in duplicate.

Detection of CYP1A and CYP3A protein:

Microsomal CYP1A protein was measured by immunoblot as described (Elskus et al., 1999). Briefly, 10 μg of microsomal protein was resolved on 4 to 12 % polyacrylamide gradient gels, transferred to nitrocellulose sheets (0.45 μm, pore size) and subjected to immunoblotting using the monoclonal antibody (MAb) 1-12-3 against scup (Stenotomus chrysops) CYP1A1. This antibody shows high specificity for Fundulus heteroclitus CYP1A as well as other vertebrate species (Kloepper-Sams et al., 1987) and was a generous gift from Dr. John Stegeman (Woods Hole Oceanographic Institution, MA). An HP conjugated goat anti-mouse IgG was used as secondary antibody and enhanced chemiluminescence (ECL+, Amersham) was used for detection. Microsomal CYP3A-like proteins were detected using a rabbit polyclonal antibody against scup P450 A (Celander et al., 1996). This antibody was a generous gift from Dr. Malin Celander (Göteborg University, Sweden). Chemiluminescent signal was detected using the Storm 860 phosphoimager (Molecular Dynamics). Intensity of the CYP1A and CYP3A bands were measured using Scion Image (NIH Image software). Microsomes from (benzo-a-pyrene)-induced killifish (1, 5, 10 ug microsomal protein) were loaded onto every gel to determine linearity of signal. To facilitate comparisons between blots, sample CYP1A band densities were normalized to the CYP1A signal from a single pool of B[a]P treated Flax Pond killifish run on every gel.

Statistical analysis:

Statistical analysis was performed using Statistica (StatSoft Inc. Tulsa, OK). Homogeneity of variance was tested using Levene's test. If the data were not normally distributed, a square root transformation was performed. Two-way ANOVA was used to detect significant differences between treatment groups and populations. Three-way ANOVA was used to detect sex, treatment and population effects at $P<0.05$. Tukey's test was used for post hoc comparisons. Level of significance was 0.05.

Results

Gonadal somatic index (GSI) and Hepatic somatic index (HSI)

GSI values correspond to sexually immature animals, except for female Newark Bay killifish treated with PCB77, where GSI values approached those reported for sexually mature fish (Bradford and Taylor, 1987). Three-way ANOVA detected significant treatment effects. Post Hoc analysis (Tukey's test) revealed that GSI values for PCB-treated Flax Pond male and Newark Bay female killifish were significantly higher than respective controls (Table 3-1).

Hepatic somatic indices differed with site and sex, demonstrating population ($p<0.002$) and population-sex ($p<0.05$) effects, but no effects of treatment (Table 3-1). HSI values were significantly higher in Newark Bay than Flax Pond killifish. In Flax Pond killifish, HSI values were significantly higher in females than males, but in Newark Bay fish HSI values were significantly higher in males compared to females.

CYP1A enzyme activity and protein

Flax Pond and Newark Bay killifish responded differently to CYP1A inducers (Figure 3-1). CYP1A activity (EROD) was significantly elevated in Flax Pond fish treated with PCB77 (3.3 fold) or B[a]P (4.1 fold), relative to controls. In contrast, CYP1A activity was not significantly altered in the resistant (Newark Bay) killifish treated with either CYP1A inducer. High variability in the BaP-treated Newark Bay fish is due to much higher activity in two of the six microsomal pools (261.5 and 286.5

pmol/min/mg compared to an average of 84.1 ± 34 pmol/min/mg for the remaining four pools).

CYP1A protein expression reflected CYP1A activity. Flax animals displayed a 7.4 and 10.1 fold induction of CYP1A protein when treated with PCB-77 or B[a]P, respectively, while no significant induction was observed in Newark Bay fish (Figure 3-2).

CYP3A protein in killifish

Immunoblot analysis revealed that Flax Pond killifish exposed to either PCB77 or B[a]P had small, but significantly higher (2.8 fold and 3.6 fold respectively) expression levels of hepatic CYP3A protein compared to vehicle treated animals (Fig. 3-3a). Newark Bay killifish did not show CYP3A induction upon exposure to either compound. The CYP3A antibody used in these experiments revealed two different bands on the immunoblotts, which could potentially correspond to two different CYP3A proteins (Fig. 3-3b). The intensity of the top band was quantified in these experiments.

Glutathione-s-transferase (GST)

Cytosolic GST activity was unaffected by treatment with either PCB-77 or B[a]P. However, 2-way ANOVA revealed a significant population effect. GST activity in Newark Bay killifish was consistently 2.2 fold higher than levels in Flax Pond killifish, regardless of treatment (Figure 3-4).

Uridine diphosphate glucuronyl transferase (UDPGT)

UDPGT enzyme activity towards 4-nitrophenol was not altered by treatment with PCB77, or B[a]P in either killifish population (Figure 3-5). Unlike GST activity, there were no differences in enzyme activity between populations. One pool from the Flax Pond vehicle treatment group displayed UDPGT enzyme activity more than one standard deviation higher than the mean. Conducting 2-way ANOVA with and without this sample did not alter significance.

DT-diaphorase

DT-diaphorase activity was measured in Flax Pond and Newark Bay killifish, but no enzyme activity was detectable using the method described above. Using the same method on rainbow trout and channel catfish (*Ictalurus punctatus*) liver cytosol we detected enzyme activities of 27.53 and 55.8 nmol/min/mg respectively. These values are comparable to rainbow trout and channel catfish DT-diaphorase enzyme activities reported by others: 29.09 nmol/min/mg, (Parker et al., 1993) and 39 nmol/min/mg (Hasspieler and Di Giulio, 1992) respectively. This indicates that the assay protocol we used in these studies was working properly.

Discussion

The results of this study suggest that chronic exposure to AHR ligands has resulted in altered expression of AHR battery genes in Newark Bay killifish. We also discovered that in addition to resistance to PCB induction of CYP1A protein and activity, Newark Bay killifish are also tolerant to B[a]P, and that CYP3A protein can be induced by AHR ligands in the killifish model. Altered expression of these, and possibly additional genes, could be related to tolerance to some of the toxic effects provoked by these compounds.

This study, in conjunction with previous work (Chapter two), demonstrates that resistance to CYP1A induction in Newark Bay killifish extends to PAHs and occurs at the level of mRNA and protein expression and catalytic activity. Previous studies had determined that these animals are resistant to induction of CYP1A protein and catalytic activity by coplanar PCBs and TCDD (Elskus et al., 1999; Prince and Cooper, 1995a). Our results are also in agreement with studies of other killifish populations chronically exposed to AHR ligands. (Bello et al., 2001) observed that New Bedford Harbor killifish are tolerant to HAH (tetrachlorodibenzofuran, TCDF) mediated induction of hepatic CYP1A mRNA, protein, and catalytic activity, while (Nacci et al., 2002) found that this same population is also resistant to induction of CYP1A by B[a]P. Creosote (PAH) contaminated Atlantic Wood (Elizabeth River, VA) killifish populations are resistant to both PAHs and PCBs (Meyer and Di Giulio, 2002; Meyer et al., 2002; Van Veld and Westbrook, 1995). However, studies using Atlantic tomcod (*Microgadus tomcod*) from

the Hudson River demonstrated that these animals have developed resistance to PCB induction of CYP1A mRNA expression, but they do respond to PAHs, suggesting possible differences in induction mechanisms of CYP1A activation by different kinds of AHR ligands (Courtenay et al., 1999).

Although the experiments performed in this study had a higher number of replicates that the ones presented in Chapter two, a high degree of variability among treatment groups was notizable. It is possible that this variability could have arisen from a pooling effect, as explained in Chapter two, or that the microsomes used were obtained from pooled male and female fish. Estrogens secreted during spawning events (Bradford and Taylor, 1987) can block CYP1A induction by AHR ligands in female fish (Elskus, 2004; Jana et al., 2000). This would result in different responses in male and female fish. However, the GSI data coolected at the time of liver sampling suggests that the killifish used in these experiments were not actively spawning, therefore hormone dependent alterations of PCB and PAH activation of AHR and CYP1A induction should have been minimal.

In other research models, resistance to activation of the AHR pathway is related to a reduction in chemical toxicity. Reduced AHR activation can lead to a decrease in AHR ligand mediated induction of oxidative stress (Alsharif et al., 1994; Hassoun and Stohs, 1996; Jin et al., 2001), liver toxicity (Uno et al., 2001), cellular death, DNA damage (Ciolino et al., 2002) and carcinogenesis (Shimizu et al., 2000). Such findings suggest that suppression of the AHR pathway plays an important role in reducing xenobiotic toxicity. These theories are tested in chapter five, where PCB induced deformities in fish embryos were demonstrated to be dependent on AHR activation and CYP1A induction and chronically contaminated killifish were shown to be resistant to these toxic effects.

Although CYP3A is not considered to be part of the AHR gene battery, treatment with PCB77 or B[a]P induced CYP3A protein expression in reference site killifish. Newark Bay killifish were resistant to CYP3A induction by these chemicals. CYP3A is highly expressed in mammalian (Gibson et al., 2002) and fish liver tissue (Hegelund and Celander, 2003; Kullman et al., 2000; Lee et al., 1998). CYP3A is inducible by steroid hormones such as glucocorticoids and antiglucocorticoids as well as xenobiotics like phenobarbital (PB) and phenobarbital (PB)-like compounds (Quattrochi and Guzelian,

2001). Additional experiments using AHR inhibitors could help determine if CYP3A induction in killifish is AHR dependent or not. CYP3A can metabolize various xenobiotics, including the model procarcinogen, B[a]P (Maurel, 1996; Shimada et al., 1989; Yun et al., 1992). Therefore, resistance to CYP3A induction in Newark Bay fish could also be beneficial if it results in reduced formation of toxic metabolites.

CYP3A induction by AHR ligands has been observed in few species, and it is usually modest. Previous studies by others have shown that CYP3A was not inducible by B[a]P in Arctic charr (*Salvelinus alpinus*) (Jorgensen et al., 2001), the PCB mixture Clophen A in rainbow trout (Celander et al., 1996), PCB126 in scup (*Stenotomus chrysops*) (Schlezinger and Stegeman, 2001) or by B[a]P in Atlantic cod (*Gadus morhua*) (Husoy et al., 1994). However, mild increases in CYP3A expression have been documented in scup exposed to PCB77 (White et al., 1997). Also, AHR ligands (Arochlor 1254, 3-methylcholanthrene and beta-naphthoflavone) have been shown to provoke a moderate increase in CYP3A2 and 3A4 in mammalian models (Cooper et al., 1993; Hansen et al., 2000). Species differences in CYP3A regulation may reflect differences in the promoter region of the gene and/or elements of the cellular environment, such as transcription factors, that are important for enzyme induction (Gibson et al., 2002; LeCluyse, 2001).

Recent studies have shown that oxidative stress can induce CYP3A expression in cultured erythroleukemia cells (Nagai et al., 2004). Since activation of AHR is known to cause oxidative stress (Palace et al., 1996; Shertzer et al., 1998), it is possible that AHR dependent increases reactive oxygen species (ROS) production activate expression of CYP3A in killifish.

The higher expression levels of GST in resistant fish could be associated with elevated conjugation activity, and resistance to xenobiotics. Although PCB77 or B[a]P did not increase either of the phase II enzymes we measured (GST and UDPGT), basal GST activity was 2.2 fold higher in Newark Bay killifish than in Flax Pond killifish. This is similar to previous reports by others on the PAH resistant Atlantic Wood killifish population, which had 4.4 fold higher GST activity when compared to reference site fish (Armknecht et al., 1998). Elevated GST activity has also been documented in two fish species with different susceptibilities to PAH-induced liver cancer: the relatively PAH

tolerant channel catfish (*Ictalurus punctatus*) and the more vulnerable brown bullheads (*Ameiurus nebulosus*). Cancer resistant channel catfish had 1.2 fold higher basal GST activity than cancer-prone brown bullheads. It is possible that higher GST activity could accelerate the elimination of PAH metabolites that would otherwise cause cell and tissue damage (Willett et al., 2000). Also, cell culture studies have shown that over expression of cytosolic GSTs can confer resistance to prooxidant chemicals (Mari and Cederbaum, 2001; Zimniak et al., 1997). Increased hepatic GST expression in the PCB resistant Newark Bay killifish is consistent with the biochemical changes observed in cancer cells: a decrease in phase I and increased phase II xenobiotic metabolizing enzyme expression (Farber, 1990; Farber and Rubin, 1991). Elevated GST activity would increase excretion rates of potentially toxic metabolites in comparison to reference site animals.

Induction of CYP1A but not UDPGT by PCB77 or B[a]P could reflect differences in the lag time between chemical treatment and enzyme expression between these AHR-regulated genes. Our results are in accordance to previous studies using resistant killifish from New Bedford Harbor and reference site killifish, where seven days after treatment with TCDF (0.0, 0.06, .31, 1.5, and 7.6 nmol TCDF/kg) killifish UDPGT activity remained unaltered (Bello et al., 2001). Other studies have generated conflicting data with the results we report in this study. Hepatic cytosol of reference site killifish treated with B[a]P (50mg/kg) and sampled after ten days had 2.8 fold higher UDPGT activity than vehicle treated animals (Nacci et al., 2002). In a caging study, EROD activity was significantly induced in carp (*Cyprinus carpio*) kept caged at a polluted site for two weeks, and UDPGT activity was significantly induced in animals kept caged for four weeks (Van der Oost et al., 1998). In our study, killifish were sampled after two days (B[a]P treatment) or five days (controls and PCB77 treatment). It is possible that induction of UDPGT by B[a]P or PCB77 is slower than induction of CYP1A and requires more time to reach it's peak, suggesting that expression of these enzymes is not coordinately regulated by the AHR pathway. Additional studies are needed to fully understand the dynamics of UDPGT expression and induction in this fish model.

Our inability to detect DT-diaphorase activity in either killifish population may reflect species differences in expression, type of methodology used, or both. Based on its role in xenobiotic metabolism (Joseph and Jaiswal, 1994), and it's regulation by AHR

ligands (Ross et al., 2000), we expected DT-diaphorase to respond to CYP1A inducers in killifish. However, there are clear species differences in regulation of this enzyme. For example, European flounder (*Platichthys flesus*) primary hepatocytes treated with B[a]P (100 μM) had significantly higher DT-dipahorase activity than vehicle treated animals (Winzer et al., 2002), yet studies using the AHR ligand β-naphthoflavone failed to detect significant DT-diaphorase induction in sea brass (*Dicentrarchus labrax*) and gilthead seabream (*Sparus aurata*) (Novi et al., 1998; Pretti et al., 2001). One study reported activity levels varying as much as 100-fold among 12 fish species (Förlin et al., 1995). We have used the same method for measuring DT-diaphorase activity in channel catfish and rainbow trout liver cytosol and obtained enzyme activity values similar to those reported by others (Hasspieler and Di Giulio, 1992; Parker et al., 1993). Together, these studies suggest that our inability to detect DT-diaphorase activity in killifish is more likely related to species differences than to methodology concerns.

Our data on CYP1A protein and catalytic activity, and GST, UDPGT and DT-diaphorase catalytic activity, demonstrate that these AHR battery genes are not coordinately regulated in killifish. Despite having significantly lower AHR protein levels (Arzuaga and Elskus, 2002), resistant killifish had constitutively higher activity of the AHR battery enzyme GST relative to responsive fish, while basal activity of other AHR battery enzymes (CYP1A, UDPGT) were similar between the responsive and resistant population. Moreover, treatment with AHR ligands significantly induced one AHR battery gene, CYP1A, in responsive fish, but had no effect on others (GST, DT-diaphorase, UDPGT). Together, these data provide evidence for lack of coordinated regulation of genes in the killifish AHR gene battery.

Resistance to CYP1A induction could be an important adaptation for animals living in an environment contaminated with AHR ligands. Induction of CYP1A accelerates the metabolism and excretion of xenobiotics, but it can also increase cellular and tissue damage by transforming parent toxicants, such as B[a]P, into reactive intermediates that can bind to macromolecules such as DNA, protein and lipids (Schrenk, 1998; Shimada and Fujii-Kuriyama, 2004), or increasing oxidative stress (Schlezinger and Stegeman, 2001). Studies in fish demonstrate that induction of CYP1A is related to microsomal production of oxygen radicals in the presence of coplanar PCBs (Schlezinger

et al., 2000; Schlezinger and Stegeman, 2001); developmental abnormalities (Henry et al., 1997) and altered immune function (Carlson et al., 2002). A possible benefit of resistance to CYP1A induction could be that resistant killifish, in comparison to reference site animals, produce fewer reactive metabolites and reactive oxygen species after exposure to PAHs and/or PCBs (see chapter six). In fact, PCB resistant killifish from New Bedford Harbor produce fewer DNA adducts than reference site fish after exposure to B[a]P (Nacci et al., 2002). Such an adaptation in combination with increased phase II metabolism would make resistant animals more fit to survive in polluted environments.

In summary we conclude that Newark Bay killifish, which are chronically exposed to mixtures of AHR ligands, have become resistant to their CYP1A inducing effect, that AHR ligands induce CYP3A in killifish and resistance extends to suppressed induction of this P450 enzyme, and that resistance to toxicants results from altered expression of both phase I and phase II xenobiotic metabolizing enzymes in resistant fish populations.

Figure Legends:

Figure 3-1: Hepatic CYP1A activity (EROD) in killifish from Flax Pond (☐ open bars) and Newark Bay (■ shaded bars), treated with vehicle (corn oil), PCB77 (1 mg/kg) or B[a]P (50 mg/kg). Bars represent the mean ± S.D. n = 4 to 5 pools (2 to 5 fish per pool). *Significantly different from respective controls ($p<0.05$).

Figure 3-2: Hepatic CYP1A protein expression in Flax Pond (☐ open bars) and Newark Bay (■ shaded bars) killifish treated with vehicle (corn oil), PCB77 (1 mg/kg) or B[a]P (50 mg/kg). Bars represent the mean ± S.D. n = 4 to 5 pools (2 to 5 fish per pool) . *Significantly different from respective controls ($P<0.05$).

Figure 3-3 (a and b): a) Hepatic CYP3A protein expression detected in Flax Pond (☐ open bars) and Newark Bay (■ shaded bars) killifish treated with vehicle (corn oil), PCB77 (1 mg/kg) or B[a]P (50 mg/kg). Bars represent the mean ± S.D. n = 4 to 5 pools (of 2 to 5 fish) per treatment. *Significantly different from respective controls ($P<0.05$). b) Representative immuno blot of CYP3A detection. Lanes 1, 2, and 3 correspond to detected CYP3A protein in Flax Pond killifish treated with vehicle (corn oil), PCB77 (1 mg/kg) or B[a]P (50 mg/kg) respectively, and lanes 4, 5, and 6 correspond to Newark Bay killfish treated with vehicle (corn oil), PCB77 (1 mg/kg) or B[a]P (50 mg/kg) respectively.

Figure 3-4: Hepatic glutathione-S- transferase activity in killifish from Flax Pond (☐ open bars) and Newark Bay (■ shaded bars) treated with vehicle (corn oil), PCB77 (1mg/kg) and B[a]P (50 mg/kg). Bars represent the mean ± S.D. n = 5 to 7 pools (2 to 5 fish per pool) # Significantly different from corresponding Flax Pond (responsive population) treatment groups.

Figure 3-5: Hepatic UDPGT activity in Flax Pond (☐ open bars) and Newark Bay (■ shaded bars) killifish treated with vehicle (corn oil), PCB77 (1mg/kg) and B[a]P (50 mg/kg). Bars represent the mean ± S.D. = 5 to 7 pools (2 to 5 fish per pool).

Table 3-1. Mean gonad somatic index (GSI) and hepatic somatic index (HSI) of control, PCB77 (1 mg/kg) and B[a]P (50 mg/kg) treated killifish..

Population:	**Average GSI (%) (± SD):**			**Average HSI (%) (± SD):**		
Flax Pond:	Vehicle	PCB77	B[a]P	Vehicle	PCB77	B[a]P
Females	1.25 ± 0.62	3.90 ± 2.26	1.34 ± .78	3.67 ± 1.60	4.04 ±0.52	4.96 ± 0.99
	(n=3)	(n=2)	(n=4)	(n=3)	(n=2)	(n=4)
Males	1.67 ± 1.02	3.16 ± 1.35*	1.27 ± 0.76	3.14 ± 1.27	3.67 ±0.78	3.86 ± 1.56
	(n=8)	(n=11)	(n=11)	(n=8)	(n=11)	(n=11)
Newark Bay:						
Females	2.10 ± 0.17	8.0 ± 4.37*	1.65 ± 0.74	5.00 ± 1.04	3.77 ± 0.52	5.45 ± 0.46
	(n=4)	(n=4)	(n=3)	(n=4)	(n=4)	(n=3)
Males	1.79 ± 0.74	2.06 ± 2.06	1.50 ± 1.20	6.77 ± 2.07	4.72 ± 1.44	5.84 ± 1.2
	(n=7)	(n=12)	(n=8)	(n=7)	(n=12)	(n=8)

Significantly different from corresponding control group at $p < 0.05$.

Figure 3-1. Hepatic CYP1A activity in Flax Pond and Newark Bay killifidh treated with corn oil, PCB77 or B[a]P.

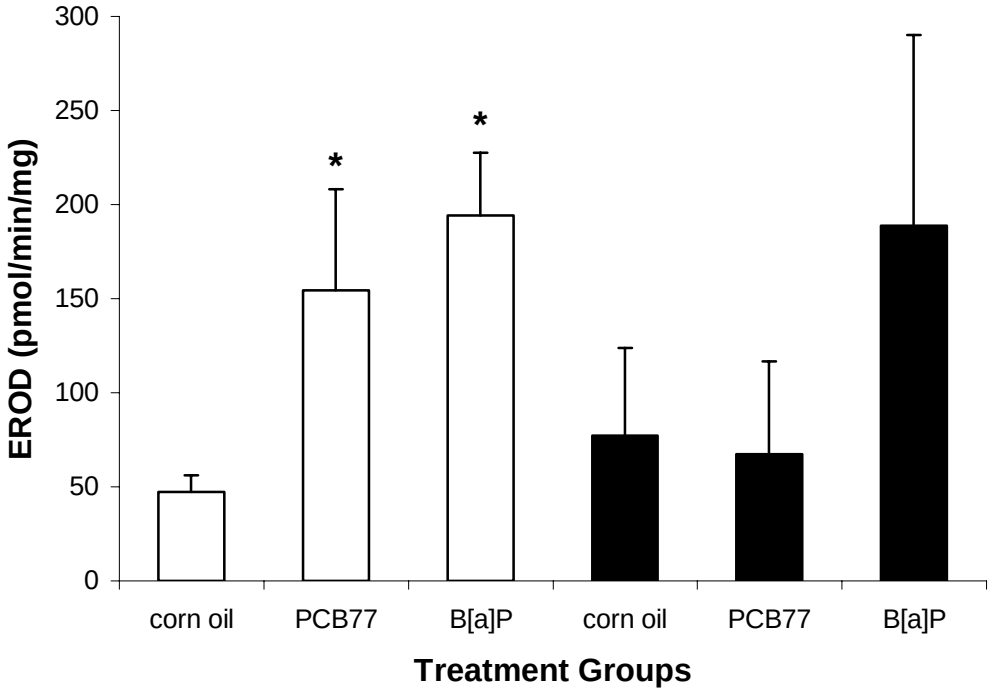


Figure 3-2. Hepatic CYP1A protein in Flax Pond and Newark Bay killifidh treated with corn oil, PCB77 or B[a]P.

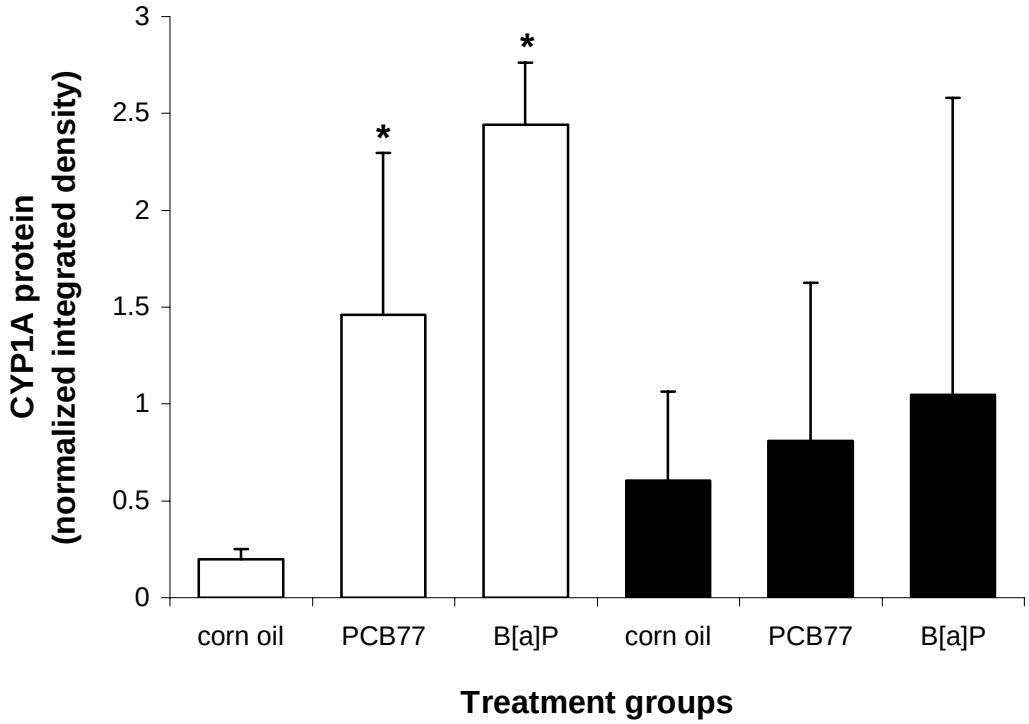


Figure 3-3a. Hepatic CYP3A protein in Flax Pond and Newark Bay killifidh treated with corn oil, PCB77 or B[a]P.

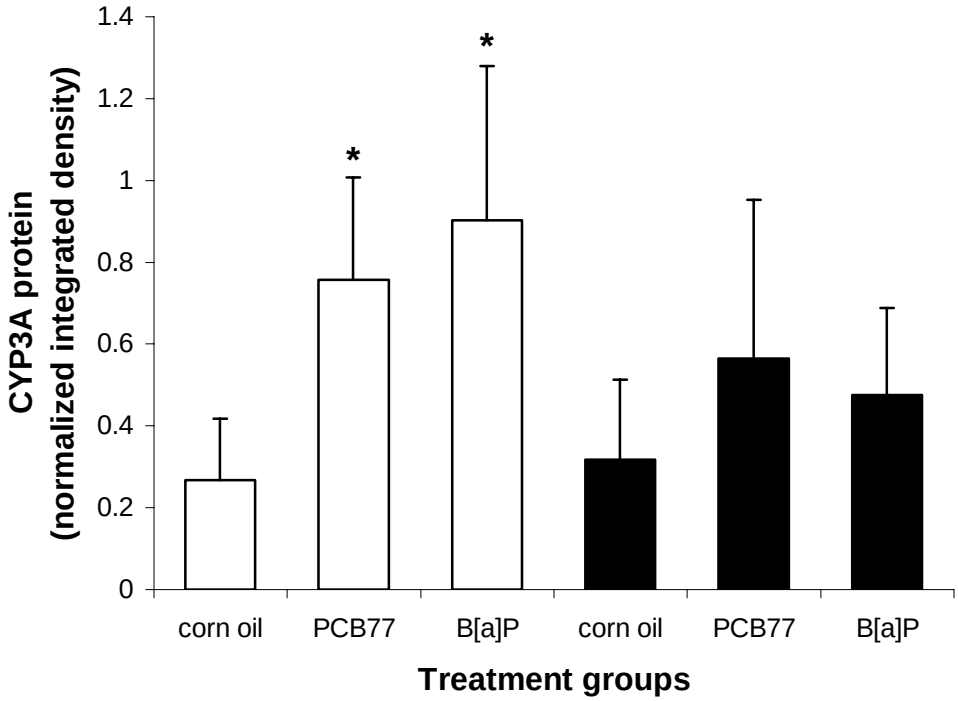


Figure 3-3b. Immunoblott for killifish CYP3A detection.

Lanes: 1 2 3 4 5 6

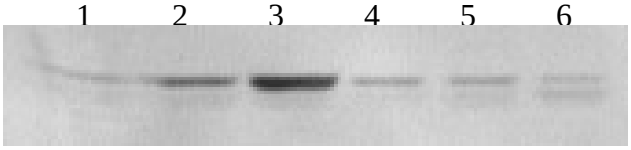


Figure 3-4. Hepatic GST activity in Flax Pond and Newark Bay killifidh treated with corn oil, PCB77 or B[a]P.

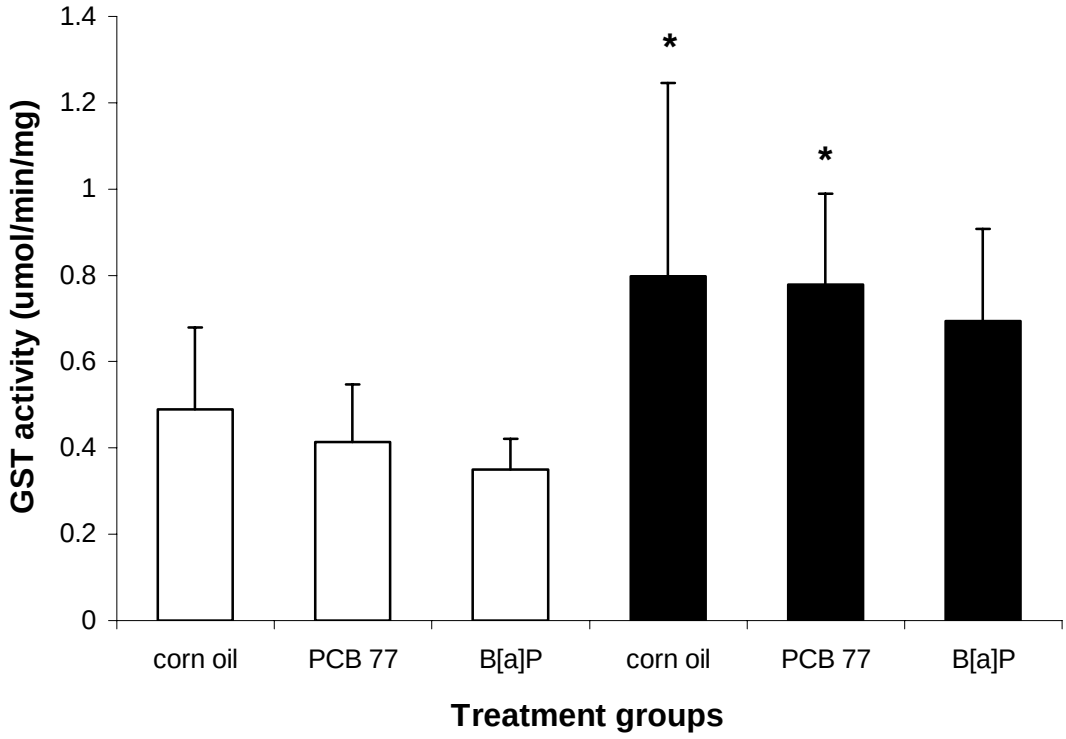
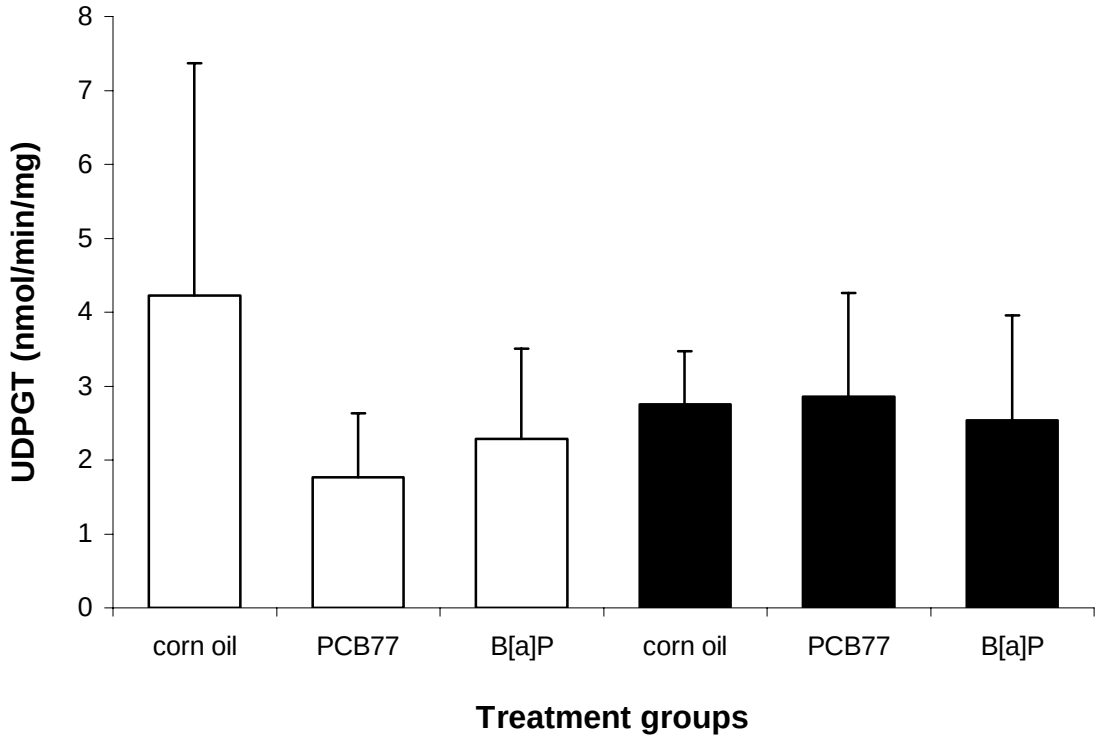


Figure 3-5. Hepatic UDPGT activity in Flax Pond and Newark Bay killifidh treated with corn oil, PCB77 or B[a]P.



Chapter Four

Evidence for AHR ligand induction of reactive oxygen species (ROS) during early embryonic development in killifish (*Fundulus heteroclitus*).

Abstract

Exposure to dioxin-like chemicals that activate the aryl hydrocarbon receptor (AHR) can result in increased cellular and tissue production of reactive oxygen species (ROS). Little is known of these effects during early fish development. We used the fish model, *Fundulus heteroclitus,* to determine if AHR ligands increase ROS production during killifish development, if this increase is associated with AHR activation (measured as induction of CYP1A activity), and to test a novel method for measuring ROS non-invasively in a living organism. The superoxide sensitive fluorescent dye, dihydroethidium (DHE), was used to detect ROS production microscopically in developing killifish exposed to the AHR agonist and potent CYP1A inducer, 3,4,5, 3',4'-tetrachlorobiphenyl (PCB126). Both *in ovo* CYP1A activity and ROS were induced by PCB126. CYP1A activity (ethoxyresorufin-o-deethylase, EROD) was maximally induced in embryos after a 5 day exposure (2 to 7 days post-fertilization, dpf) to 0.3nM PCB126. ROS production was detectable in embryonic liver as early as 5 dpf (0.3 nM PCB126) and was inducible by PCB126 concentrations as low as 0.003 nM. To determine if non-chlorinated AHR ligands induce ROS production, and if ROS production is associated with CYP1A induction potency, killifish embryos were exposed to vehicle, PCB126 (0.3 nM), or a 100-fold higher dose of the less potent, non-chlorinated polycyclic aromatic hydrocarbon (PAH) 3-methylcholanthrene (3-MC, 33.5 nM). PCB126 and 3-MC increased EROD by 17 and 12 fold, and ROS production by 7.6 and 2.2 fold, respectively. Our data demonstrate that AHR ligands can increase ROS production in killifish embryos, that ROS production occurs even in early life stages, that potency as a CYP1A inducer reflects potency to induce ROS, and that PCBs are stronger oxidants than PAHs. The superoxide detection assay (SoDA) described in this chapter provides a sensitive, easily measured, early indicator of altered ROS production that can be used in conjunction with simultaneous *in ovo* measurements of CYP1A activity to

explore functional relationships among biochemical, physiological and developmental responses to AHR ligands.

Introduction

Oxidative stress is a cellular response to increased levels of oxygen radicals (e.g., superoxide, and hydrogen peroxide and hydroxyl radicals). Increased production of reactive oxygen species (ROS) can affect cellular health and function (de Vries, 1996; Di Giulio et al., 1995). Exposure to chlorinated environmental contaminants such as coplanar polychlorinated biphenyls (co-PCBs) and polychlorinated dibenzo-p-dioxins (e.g. 2,3,7,8-tetrachlorodibenzo-p-dioxin, TCDD), strongly induces oxidative stress in vertebrates, including rodents (Fadhel et al., 2002; Hassoun et al., 2002; Mohammadpour et al., 1988; Senft et al., 2002), human hepatoma cells (Park et al., 1996), and lake trout (Palace et al., 1996).

Oxidative stress caused by TCDD and PCBs is associated with activation of the aryl hydrocarbon receptor (AHR) (Alsharif et al., 1994; Park et al., 1996) Activation of AHR is followed by increased expression and activity of cytochrome P450 1A (CYP1A) and other phase I and phase II metabolic enzymes (Safe, 2001). ROS production could result from a variety of AHR associated mechanisms, including altered expression and activity of P450 enzymes (e.g. CYP1A1/2), and antioxidant and pro-oxidant enzymes (Dalton et al., 2002).

Measuring ROS typically involves laborious assays performed on excised tissues or in isolated cells. Some of the more commonly used techniques to study ROS production are measurement of lipid peroxides by the thiobarbituric acid reacting substances (TBARS) test (Fadhel et al., 2002; Uchiyama, 1978), oxidative DNA damage (Park et al., 1996), protein carbonyl content (Leitao et al., 2003; Reznick and Packer, 1994), and use of superoxide-sensitive fluorescent dyes like dihydroethidium (DHE) and diclorodihydrofluorescin diacetate (DCFH-DA) (Schlezinger et al., 1999). DHE has been used extensively in *in vivo* and *in vitro* experimental models to study ROS production. Examples of DHE-based ROS assays include microsomal superoxide production in scup liver microsomes after treatment with PCB126 (Schlezinger and Stegeman, 2001; Schlezinger et al., 1999), ROS production after ischemia-reperfusion in an embryonic

chick cardiomyocyte model (Shao et al., 1999), cyclosporin A induced ROS production in primary rat hepatocytes (Andres et al., 2000), and increased mitochondrial production of superoxide after hypoxia in human umbilical vein endothelial cells (Pearlstein et al., 2002). Here we use a novel method employing DHE to measure ROS production non-invasively in a living vertebrate, the developing killifish.

The objectives of this study were to determine if AHR ligands can increase ROS production in killifish embryos, how early in development ROS production can be detected, and to test a novel method for non-invasively measuring superoxide production in a living organism.

Materials and methods

Materials

7-Ethoxyresorufin and DHE were purchased from Molecular Probes (Eugene, OR). PCB126 was purchased from AccuStandard (New Haven, CT), salt (Bio-crystals) for preparing 30ppt sea water was purchased from Marine Enterprises International (Baltimore, MD).

Killifish collection and maitenance

Spawning adult killifish (*Fundulus heteroclitus*) were collected from Beaufort NC in August (2003) and maintained in 20-gallon recirculating tanks equipped with charcoal Whisper^{TM} filters, in 30 ppt artificial seawater at 23-25°C on a 16/8 L/D cycle. Fifty percent of the tank water was replaced every other week.

Killifish are batch spawners that release eggs on the new and full moons (Bradford and Taylor, 1987). Killifish embryos were collected during the weeks of new and full moon from August to December 2003. In the laboratory, female and male killifish spawn against plastic mesh surfaces placed in their tanks. However, if not prevented from doing so, the adults will consume the embryos. To optimize collection of embryos, we constructed spawning baskets based on a design provided by Dr. Diane Nacci (US EPA, ERLN, Narragansett, RI). Spawning baskets consisted of two concentric PVC circles. Nylon filtration screen (300 microns, Aquatic Ecosystems, Apopka, FL) is

affixed to the bottom of the larger circle (25 cm diameter x 5 cm deep) using aquarium sealant. Wide gauge plastic netting (mesh size: 1/4", Aquatic Ecosystems, Apopka, FL) is affixed to the top of the smaller circle (20 cm diameter x 2.5 cm deep) using plastic ties and this circle placed inside the larger circle. Fish spawn against the wide gauge screen and the embryos fall through and are retained on the fine mesh bottom. During evening hours, baskets were placed in the bottom of the tank housing the parental fish (twelve females and five males). The following day (day 1), spawned eggs were collected and placed individually in 20 mL scintillation vials containing 3 ml of sea water (30ppt). On day two, eggs were examined microscopically for evidence of fertilization and assigned to treatment groups. Developmental stage was determined as described by (Armstrong and Child, 1965b).

In ovo superoxide detection assay (SoDA)

To measure superoxide using our superoxide detection assay (SoDA), killifish embryos were exposed to known ROS inducers and evaluated microscopically for superoxide production. Killifish embryos were aqueously exposed to test compounds dissolved in acetone diluted in 30 ppt seawater. Treatments consisted of vehicle (0.001% acetone) or one of two AHR ligands, 3,4,3',4'-tetrachlorobiphenyl (PCB126) or 3-methylcholanthrene (3-MC). See figures for exposure details. Embryos were exposed from two to seven days post fertilization (dpf) to 10 mL of exposure media in 20 mL scintillation vials (one embryo per vial) and held at 25°C on a 12/12 L/D cycle. Observations and measurements were made on days three, five and seven pf. Two hours prior to ROS measurement, the exposure media was replaced with 2 mL of fresh media (vehicle or ligand) containing DHE dissolved in seawater. DHE reacts with ROS to form ethidium, which intercalates with DNA and amplifies its fluorescence (Bindokas et al., 1996). DMSO (0.01%) was used as vehicle for DHE, and the DHE sea water solution was sonicated for 20 minutes prior to embryo incubation to dissolve the DHE entirely. Embryos were kept in the dark on a plate shaker (~70 rpm) from 0.5 to 1.5 hours, after which the DHE solution was replaced with 10ml of clean artificial seawater. DHE staining of embryo tissues was visualized on a Nikon E800 fluorescence microscope (Melville, NY) equipped with a Nikon, 100W mercury lamp and digital camera (spot RT

Diagnostic Instruments, Sterling Heights, MI). Images were captured using Spot RT software interfaced with Adobe Photoshop (San Jose, CA). Image exposure times were determined separately for each experiment, and were based on the amount of time needed to detect a signal in the vehicle controls. Exposure times in our experiments ranged from 0.8 to 1.5 seconds for the ethidium signal (a measurement of superoxide) using a Texas Red filter (EX-560/55 EM-645/75 DM-595). Images were analyzed using Scion Image (Frederick, MD).

In ovo CYP1A activity

In ovo CYP1A activity (ethoxyresorufin-o-deethylase, or EROD) was conducted as described by (Nacci et al., 1998; Nacci, in press). Briefly, at 2 dpf embryos were treated with vehicle alone (0.001% acetone final concentration), PCB126 or 3-MC. The seawater contained 7-ethoxyresorufin (7-ER) dissolved in DMSO (0.001%), at a final concentration of 21ug/L. At 7 dpf exposure media was replaced with clean seawater. *In ovo* EROD was measured at 9 dpf using a fluorescence microscope with Texas Red filters (as described above for SoDA assay). CYP1A induction was evaluated based on the accumulation of the metabolite resorufin (RR) in the animal's bladder relative to vehicle controls and is presented as the intensity of the RR signal (Texas Red), as described (Nacci et al., 1998). Exposure times were 70 milliseconds (ms) for the RR image.

Histology, hematoxylin and eosin (HE) staining

Seven day post-fertilization embryos were preserved in 10% formalin until analysis. Prior to sectioning, embryos were washed with 20 ml of deionized water (dI H_2O) for one hour, followed by overnight incubation at 4 °C in phosphate buffered saline (PBS) buffer containing 30% sucrose. Embryos were embedded in Optimal Cutting Temperature compound (Tissue-Tek, Torrance, CA) and cryosectioned to 12 μm sections using a Cryotome Cryocut 1800 (Reichert-Jung, Germany). Tissue slides were stained with hematoxylin and eosin (Carson, 1990), and slides were visualized on a Nikon E800 microscope (Melville, NY) equipped with a digital camera (spot RT Diagnostic Instruments, Sterling Heights, MI).

Statistical analysis

For analysis of in ovo EROD data, and ROS production by PCB126 and 3-MC, one way ANOVA followed by Tukey's test was performed on the data using SAS software (Cary, NC). A student t-test was used for determining significant differences in ROS production between PCB treated animals and respective controls.

Results

0.3 nM PCB126 induces maximal EROD response in developing killifish embryos.

Since ROS is associated with activation of the AHR pathway and CYP1A induction by dioxin-like compounds in vertebrates (Dalton et al., 2002) we first determined the maximal CYP1A inducing dose of our test chemical, PCB126, to use when optimizing the SoDA assay. On day two-post fertilization, killifish embryos were exposed to PCB126 at 0.03, 0.3 and 1.5 nM (0.01, 0.1 and 0.5 μg/L). *In ovo* EROD was measured on day nine. Maximal EROD induction was observed at the 0.3 nM PCB126 concentration, while the highest dose used (1.5 nM PCB126) was toxic (increased frequency of developmental abnormalities) and induced only sub-maximal EROD activity (Figure 4-1). For these reasons, the 0.3 nM PCB126 dose was the dose used for all subsequent experiments.

ROS production detected by DHE staining is significantly higher in PCB treated animals.

To determine if the DHE-based superoxide assays used in cell culture and isolated tissues would also detect superoxide in our fish embryo model, and to determine which tissues produced the strongest ROS signal, we exposed killifish embryos to vehicle or 0.3 nM PCB126 for 5 days (from 2 to 7 dpf), and incubated the embryos with DHE (50 μM, for 1.5 hours) before ROS detection at 7 dpf. After staining, one organ in the PCB treated animals presented a very strong fluorescence signal. This organ was located to the left of the body axis, when viewed from above (Figure 4-2a), corresponding to the location of the embryonic liver (Armstrong and Child, 1965b). Histological analysis of cryosections of the embryo by Dr. Michael Moore (Woods Hole Oceanographic Institution, WHOI) confirmed the location of the embryonic liver (Figure 4-2b), and

comparison of histological slides to *in ovo* images (Figure 4-2a) confirmed that the ethidium stained (ROS producing) tissue was the embryonic liver.

50 μM is the optimal DHE concentration for staining killifish embryos.

In cell culture experiments DHE is commonly used at concentrations ranging from 5 to 10 μM. According to (Horobin and Rashid, 1990), the transport of a biological probe in the cell interior depends on the solubility of the probe through the cell membrane and in different cell compartments, which can be thought of as different layers. Thus, more layers results in less probe reaching the desired target. They referred to this as the "Chinese box model". In a killifish embryo, DHE would have to cross through the chorion, in addition to cell membranes. To account for this, we chose a DHE concentration (50μM) 5 to 10 fold higher than concentrations typically used in cell culture in addition to a 10μM DHE.

While the 10uM DHE concentration detected a significant increase in ROS between vehicle and 0.3 nM PCB126-treated embryos (2.0 fold), an even greater difference (4.4 fold) was measured in these embryos when the higher DHE concentration, 50 μM, was used (Figure 4-3). This suggests that either insufficient DHE for maximal response reached the target site (embryonic liver) at the 10 μM dose, or that the 10 μM DHE concentration was saturated by the superoxide produced in the embryo liver. Based on these results, 50 μM DHE was used in all subsequent experiments for ROS detection.

1.5 hours is the optimal DHE incubation time for ROS detection.

To determine how long embryos need to be exposed to DHE for the superoxide reaction to go to completion, we treated embryos with vehicle or PCB126 (0.3 nM) for 5 days (2 to 7 dpf) and at 7 dpf incubated them in 50uM DHE for 0.5, 1, and 1.5 hours. In all 3 incubation periods DHE staining in PCB treated groups was significantly higher than vehicle treated embryos (Figure 4-4). After 0.5, 1.0 and 1.5 hours of DHE incubation the ROS production in PCB treated embryos was 7.4, 5.5 and 5.8 fold higher, respectively, than vehicle treated controls measured at the same time points. Because the 1.5h DHE incubation allowed easier identification of the embryo liver during image

analysis and produced the strongest fluorescence staining, we used this staining time for all future experiments.

ROS can be detected as early as 5 dpf

To determine how early in development PCB-induced ROS generation can be detected, embryos were exposed to vehicle or PCB126 (0.3nM) beginning at 2 dpf and stained with 50 μM DHE on days 3, 5, 7, and 9 dpf. Insufficient liver development in 3 dpf embryos made positive identification of this tissue difficult and precluded us from making ROS measurements at this stage of development. Significant differences in ROS production were detected between PCB and vehicle treated embryos measured on 5, 7 and 9 dpf (Figure 4-5), with fold inductions over controls of 7.2, 3.6, and 2.2, respectively. The low signal in the 9 dpf embryos (2.2. fold) is likely due to increased tissue pigmentation at this developmental stage, reducing transmission of fluorescence through the embryo. Although the strongest signal was found in the 5dpf embryos (7.2 fold), liver tissue identification was most reliable at 7dpf, and thus 7dpf embryos were used for all subsequent ROS assays in this study.

In ovo EROD is more sensitive than SoDA for detecting a biochemical response to PCB treatment.

To determine which detection technique (SoDA or EROD) is the more sensitive biochemical response to PCB exposures in our model, embryos were exposed to vehicle or a low PCB126 dose (0.003 nM) for 5 days (2 - 7 dpf). ROS production was measured at 7 dpf and in-ovo EROD at 9 dpf. A student t-test revealed significant induction of both *in ovo* EROD and ROS, however induction relative to vehicle controls was higher for EROD (2.2 fold) than for ROS (1.5 fold) (Figure 4-6).

3-MC increases ROS production in killifish embryos.

Recent *in vitro* studies using liver microsomes (Shertzer et al., 2004) suggest that the degree of halogenation of AHR ligands is associated directly with microsomal ROS production. To determine if, in our *in vivo* model, a halogenated AHR ligand is a more potent inducer of ROS than a non-halogenated AHR ligand, killifish embryos were

exposed from 2 - 7 dpf to vehicle, 3-MC (33.5nM), which is non-halogenated, or PCB126 (0.3nM), which has 5 halogen substitutions. ROS production and *in ovo* EROD were quantified at 7 dpf and 9 dpf, respectively. As expected, 0.3 nM PCB126 significantly induced both CYP1A (25 fold, Figure 4-7a) and ROS (7.6 fold, Figure 4-7b) relative to vehicle controls. In contrast, a 111-fold higher molar dose of the non-halogenated ligand, 3-MC, provoked a similar induction of EROD (12.8 fold), but only a weak induction of ROS (2.2 fold), relative to vehicle controls (Figure 4-7b). These data suggest that, 3-MC is a less potent ROS inducer in comparison to PCB126.

Discussion

The objectives of this study were to determine if AHR ligands can increase ROS production in killifish embryos, how early in development ROS production can be detected, and to test a novel method for non-invasively measuring superoxide production in a living organism. Our data demonstrate that the coplanar PCB126 is capable of inducing ROS production during early fish development, that the primary site for ROS production in early life stages is the liver, that *in ovo* EROD is more sensitive than *in ovo* ROS as a measure of biochemical response to PCB exposure, and that PCB126 is a more powerful oxidant than the non halogenated AHR ligand 3-MC.

As reported in studies with isolated tissues and cell cultures, AHR ligands induced superoxide production in our *in ovo* system, demonstrating the competency of early life stages to produce reactive oxygen in response to prooxidants. Significant differences in ROS production were detected between PCB126 and vehicle treated embryos as early as 5 dpf, earlier detection (3 dpf) being precluded by insufficient development of the embryonic liver. Both halogenated and non-halogenated AHR ligands were able to induce ROS in embryos, although the potency of induction varied tremendously, which may reflect differences in the pathways of ROS generation by these chemical classes (discussed below).

Potency as a CYP1A inducer reflected relative potency to induce ROS production. The potent CYP1A inducer, PCB126, and the weaker inducer, 3MC both significantly elevated both EROD and ROS relative to vehicle controls, however a 111 fold higher dose of 3MC was needed to achieve similar induction levels. This suggests

there may be a mechanistic link between CYP1A induction and ROS production. Studies using CYP1A inhibitors to investigate mechanistic linkages are described in chapter five.

Although PCB126 and 3-MC both induce CYP1A in killifish embryos (Nacci et al., 1998), it is likely these chemicals work through different pathways to increase in ovo ROS production. PAHs, such as 3 MC and the model toxicant, benzo[a]pyrene, are metabolized by CYP1A to quinones, reactive intermediates that can generate ROS (Burdick et al., 2003; Kantoniemi et al., 1996; Kerzee and Ramos, 2000). Studies using cell culture systems have demonstrated that PAHs increase ROS production, but the observed increase is rather small, yet significant and reproducible (Fabiani et al., 1999; Fabiani et al., 1998). In contrast, halogenated aromatic chemicals, such as PCBs and TCDD, are not readily metabolized but instead generate ROS via uncoupling of CYP450 enzymes (Schlezinger et al., 1999; Shertzer et al., 2004). Since PAHs are metabolized and excreted faster than PCBs, it is possible that by 7 dpf (when ROS was measured), PCB126 levels remained high, causing continuous production of ROS while levels of 3-MC (and quinones) were low due to metabolism and clearance. This would also explain, in part, our finding that PCB126 was a stronger oxidant than 3MC, as even at a molar dose 111 times higher, 3MC still provoked weaker ROS production (2.2 fold) than PCB126 (7.6 fold). It is also possible that ROS production by PAHs could also arise though mechanisms additional to CYP1A mediated metabolism (Dalton et al., 2002).

Staining killifish embryos with DHE proved to be a simple, sensitive, non-invasive method for measuring AHR ligand induced ROS production *in vivo*. Many of the methods used for detection of ROS production are vulnerable to artifacts and require the disruption of cells and/or excision of tissues (Moore and Roberts, 1998). Dihydroethidium (DHE) is used extensively to detect *in vitro* microsomal production of superoxide in PCB treated vertebrate models (Schlezinger et al., 2000; Schlezinger and Stegeman, 2001; Schlezinger et al., 1999). Recent studies have shown that the reaction between superoxide and DHE generates both ethidium, the product measured in DHE-based superoxide assays, and a second, novel and as yet unidentified product with a different fluorescence spectrum (Zhao et al., 2003). Our filter detects the classic ethidium product.

To optimize detection of superoxide in our assay, embryos required longer incubations in higher concentrations of DHE than are typically used for cell culture. Maximal ROS detection in embryonic liver required staining embryos with 50μM DHE for a period of 1.5 hours (Figure 4). Cell culture (hepatocyte) studies use considerably lower DHE concentrations (5 to 10 μM) and shorter incubation times (30 to 45 minutes) (Andres et al., 2000; Shishido et al., 2003). This difference may reflect more rapid penetration of DHE through hepatocyte cell membranes than through fish chorion membranes. The "Chinese box model" concept of biological probe penetration states that the more layers a probe needs to penetrate, the less probe will reach the target tissue (Horobin and Rashid, 1990). The chorion of *F. heteroclitus* embryos (10-12 microns,) (Armstrong and Child, 1965b) is many times thicker than rat hepatocyte cell membranes (9nm) (Lehninger, 1975), suggesting it is likely that longer incubation times at higher concentrations could are needed for sufficient DHE to reach the embryo than are needed to penetrate hepatocytes in cell culture.

The decline in ethidium signal strength observed from 5 dpf to 9 dpf may result from decreased superoxide production, increased superoxide removal, or decreased signal detection as the embryo grows. Activity of the antioxidant, glutathione peroxidase (Otto and Moon, 1995), may increase during development, leading to decreased superoxide levels at later life stages. Alternatively, superoxide levels may be decreased by ROS-dependent downregulation of the AHR pathway (Barouki and Morel, 2001; Dalton et al., 2002). It is also possible that superoxide levels are not diminished, but rather our ability to detect the ethidium signal declines with increasing pigmentation and thickening of the tissues as the embryo develops. Although signal detection was maximal at 5dpf, the liver was more easily identified in 7dpf embryos. Where optimal sensitivity is required, we recommend using 5 dpf killifish embryos.

In our model embryo system, *in ovo* CYP1A activity (EROD) was more sensitive than *in ovo* ROS for detecting biological response to the toxic pollutant, PCB126. Our lowest test dose of PCB126, 0.003 nM, provoked a 2.2 fold increase in *in ovo* EROD, but only a 1.5 fold increase in ROS. Even when ROS was measured in 5 dpf embryos, the life stage showing the strongest ROS signal, and at higher PCB doses (0.3 nM), similar

results were obtained. *In ovo* EROD in 9 dpf embryos was induced 17.4 fold while ROS in 5 dpf embryos was elevated only 7.2 fold over vehicle controls.

It should be noted that although embryos used for ROS and EROD analyses were exposed to PCB126 for the same number of days prior to quantification of signal (5 day exposures), we did not measure ROS and EROD at the same stage of development. Rather, signals were quantified on the day when the signal was maximal (9 dpf, EROD, (Nacci et al., 1998) or the liver was most easily identified (7 dpf, ROS). To determine which assay, *in ovo* EROD or *in ovo* ROS, provides the strongest signal at the earliest stage of development, future experiments should examine which assay, ROS or EROD, exhibits the strongest induction over controls at the same life stage (e.g. 5 dpf embryos).

As mentioned above, DHE is converted to the fluorescent ethidium bromide in the liver, which can bind to DNA and cause mutations. Killifish stained with DHE were still alive after 24 and 48 hours, suggesting that DHE is not immediately toxic to killifish embryos. Future experiments should study long-term effects of DHE exposure.

Overall, results from this study suggest that PCBs and PAHs both enhance ROS generation during early development stages in fish, that the liver is the primary site of ROS production in these exposures, that PCB126 is a stronger prooxidant than 3-MC, and that DHE can be effectively used to detect changes in ROS production in living embryos. PCB induced ROS production was highest at 5 dpf and detection declined thereafter, possibly though induction of an antioxidant mechanism, inactivation of the AHR pathway or increased embryo size and pigmentation. The PAH model 3-methylcholantherene was also capable of inducing increased ROS production, although much higher doses (> 100 fold higher) were needed relative to PCB126, suggesting differences in the mechanism for ROS production between PAHs and PCBs. In combination with *in ovo* EROD and *in ovo* detection of developmental deformities, the *in ovo* superoxide detection assay (SoDA) described in this paper provides an additional tool for studying the effects of AHR ligands during early embryological development in vertebrates. Studies defining the role of AHR and CYP1A in ROS production, and determining if chronically contaminated killifish populations that are resistant to CYP1A induction are also resistant to these prooxidants are described in chapter five.

Figure legends

Figure 4-1. Induction of *in ovo* EROD in killifish embryos treated with vehicle or PCB126. Two day post-fertilization (2 dpf) embryos were treated with 0.03, 0.3, or 1.5nM PCB126 (0.01, 0.1, and 0.5 ug/L) or vehicle for 5 days (2- 7 dpf) and bladder fluorescence measured at 9 dpf. Bars represent average percent of vehicle EROD ± standard error for n = 17 - 24 individuals per treatment. Image exposure times were 70 ms (Texas Red). Different letters represent statistically significant differences at $p < 0.05$.

Figure 4-2a. PCB126 treated embryos display stronger HE fluorescence (ROS production) than vehicle treated animals. Two day post-fertilization (2 dpf) embryos were treated with 0.3 nM PCB126 for 5 days (2 to 7 dpf) and stained (50 uM HE, 1.5 h) for ROS at 7 dpf. Digital images taken under bright light (left) and Texas red (right).

Figure 4-2b. H and E stained cryosections of a 7 dpf killifish embryo. 20 micron section, scale bar represents 100 microns.

Figure 4-3. Incubation trials to determine DHE concentration for optimal detection of ROS in embryonic liver. Embryos were treated with vehicle or PCB126 (0.3nM) at 2 dpf and ROS production measured at 7 dpf after a 1.5 h incubation with either 10 or 50 μM DHE. Bars represent average percent of vehicle ROS ± standard error for n= 19 to 21 individuals per treatment for vehicle (white) and PCB126-treated (black) embryos. Image exposure time was 0.8 sec (Texas Red). *Significantly different from respective vehicle control at $p < 0.05$.

Figure 4-4. Incubation trials to determine DHE incubation time for optimal detection of ROS in embryonic liver. Embryos were treated with vehicle or PCB126 (0.3nM) for five days (2 - 7 dpf) and ROS production measured at 7 dpf. Bars represent average percent of vehicle ROS ± standard error for n=13 to 15 individual embryos per treatment for

vehicle (white) and PCB126-treated (black) embryos. Image exposure time was 0.8 sec (Texas Red). *Significantly different from vehicle control at $p < 0.05$

Figure 4-5. ROS production induced by PCB126 during killifish early embryonic development. Embryos were treated with vehicle or PCB126 (0.3nM) for five days (2 to 7 dpf) and ROS production measured at 5, 7 and 9 dpf. Bars represent average percent of vehicle ROS ± standard error for n = 18 to 21 individuals per treatment for vehicle (white) and PCB126-treated (black) embryos. Image exposure time was 1.5 sec (Texas Red). *Significantly different from vehicle control at $p < 0.05$.

Figure 4-6 (a and b). Relative sensitivity of embryo CYP1A and ROS to induction by PCB126. Embryos were treated with vehicle or PCB126 (0.003nM) for 5 days (2 to 7 dpf). *In-ovo* EROD was measured at 9 dpf and ROS production at 7 dpf. a) *In ovo* EROD. Bars represent average percent of vehicle EROD ± standard error for n = 23 - 24 individuals per treatment. Image exposure time was 70 ms (Texas Red). b) *in ovo* ROS. Bars represent average percent of vehicle ROS ± standard deviation for n = 19 – 20 individuals per treatment. Image exposure time was 800 ms (Texas Red). * Significantly different from vehicle control at $P < 0.05$.

Figure 4-7 (a and b). 3-MC induction of *in ovo* EROD and ROS production in developing killifish embryos. Embryos were treated with vehicle, 3-MC (33.5nM) or PCB126 (0.3nM) for 5 days (2 to 7dpf) and measured for EROD (9 dpf) or ROS (7 dpf). a) *In ovo* EROD. Bars represent average percent of vehicle EROD ± standard error for n = 19 - 22 individuals per treatment. Different letters represent statistically significant differences at $P < 0.05$. Image exposure time was 70 ms (Texas Red). b) *In ovo* ROS. Bars represent average percent of vehicle ROS ± standard deviation for n = 20 individuals per treatment. Image exposure was 700 ms (Texas Red). *Significantly different from vehicle control at $P < 0.05$.

Figure 4-1. *In ovo* EROD in killifish embryos treated with vehicle or PCB126.

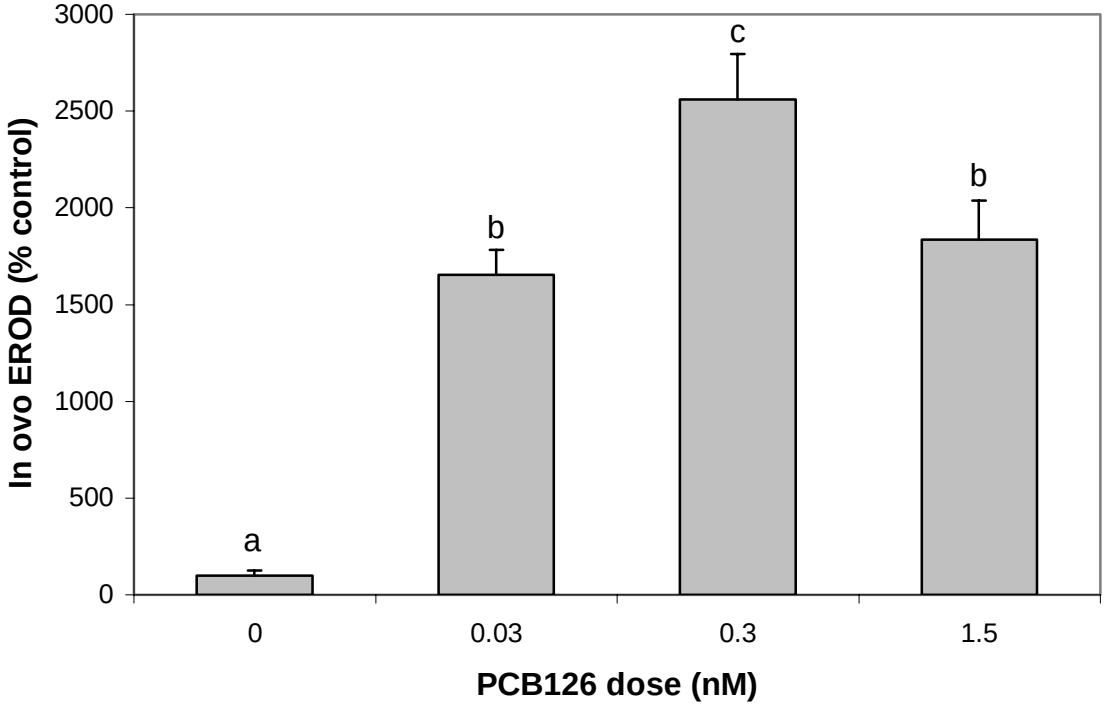


Figure 4-2a: HE fluorescence (ROS production) in vehicle and PCB126 treated embryos.

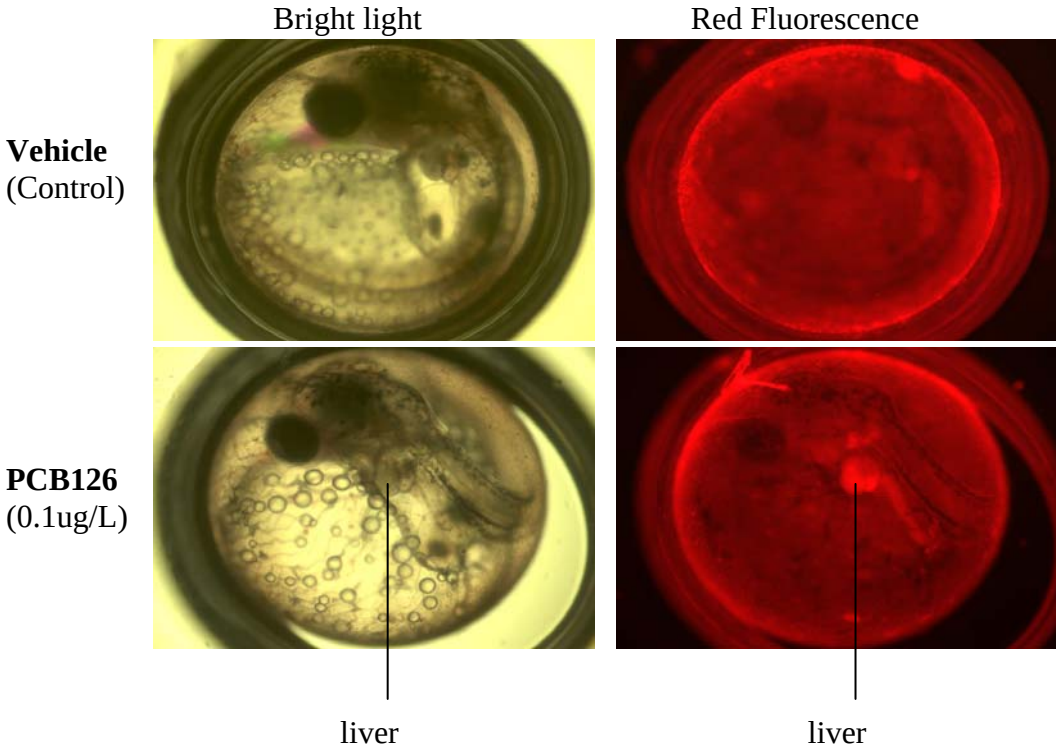


Figure 4-2b. H and E stained cryosections of a 7 dpf killifish embryo.

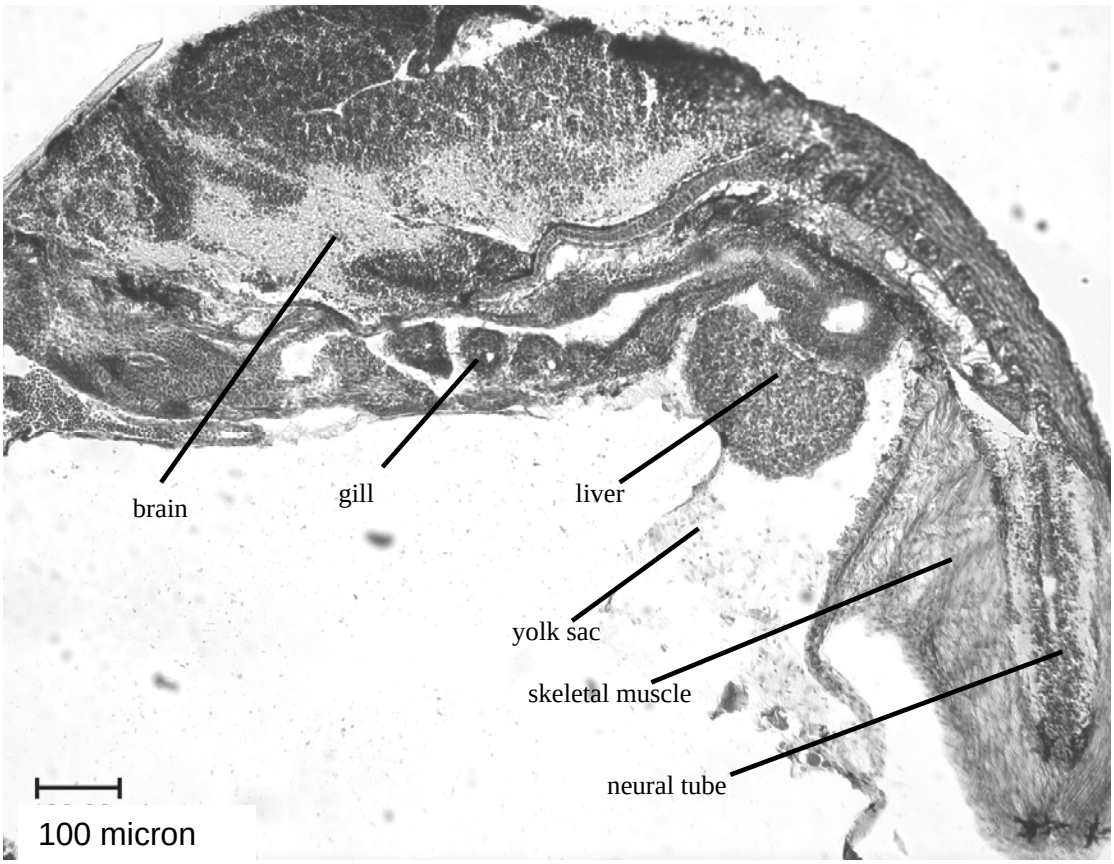


Figure 4-3. DHE concentration for optimal detection of ROS in embryonic liver

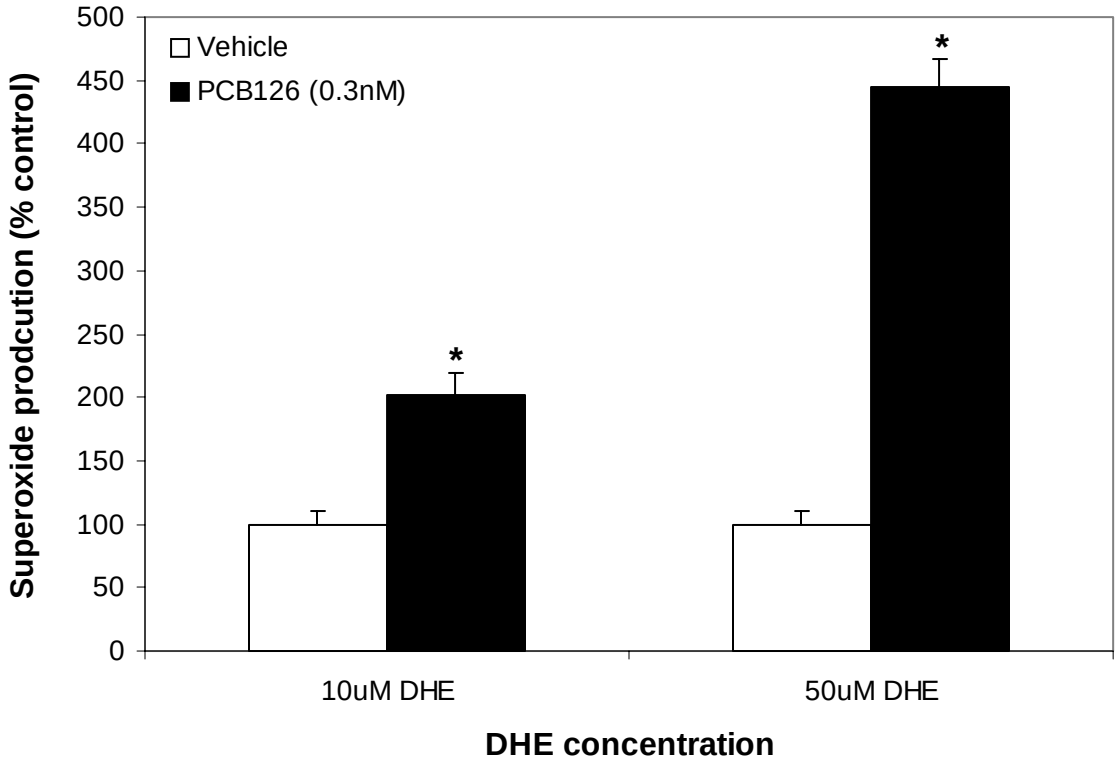


Figure 4-4. DHE incubation time for optimal detection of ROS in embryonic liver

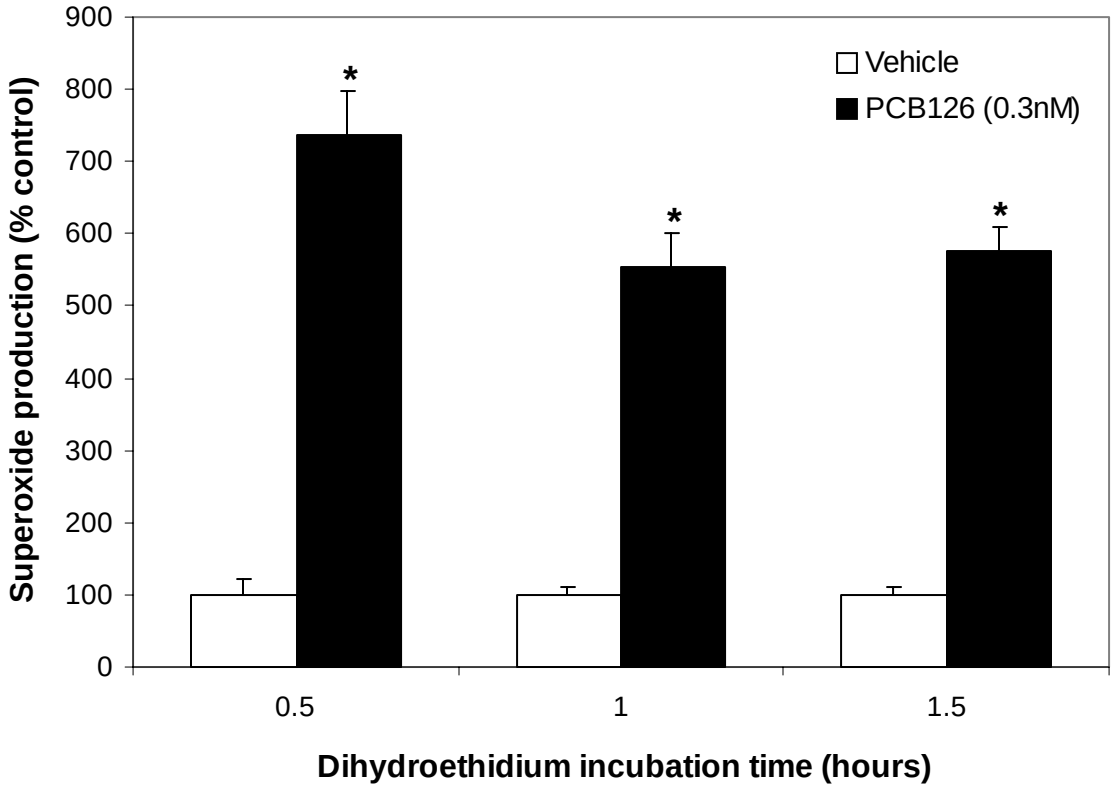


Figure 4-5. ROS production induced by PCB126 during killifish early embryonic development.

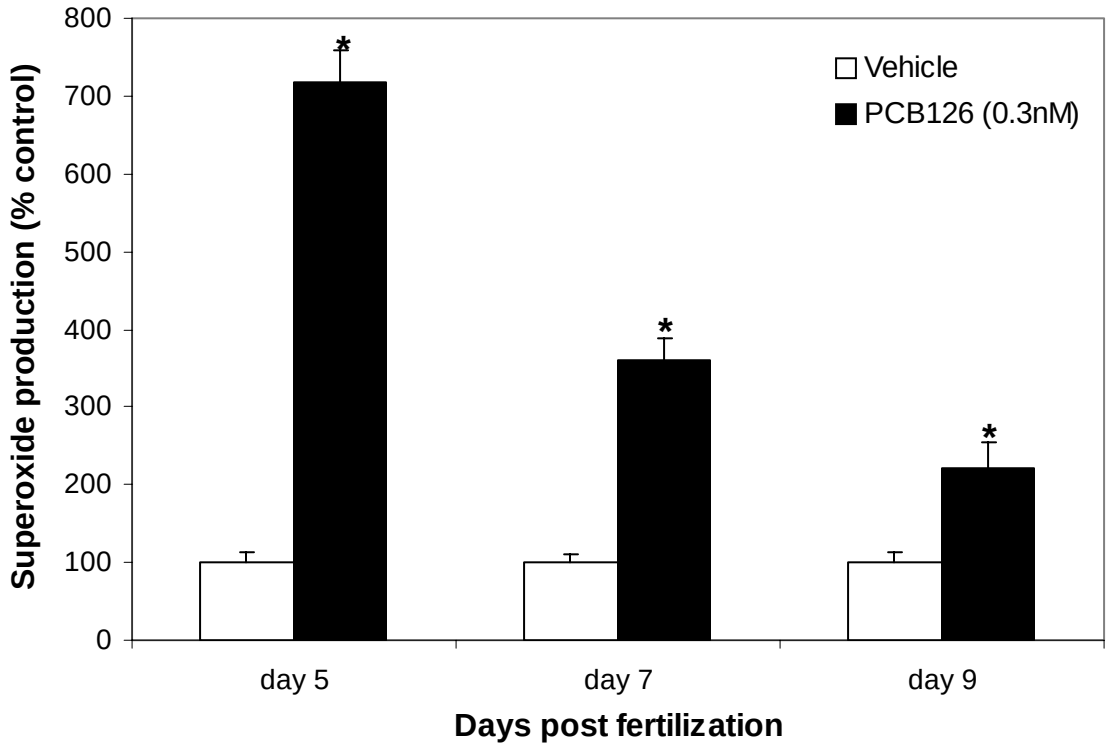


Figure 4-6a. Sensitivity of embryo CYP1A to induction by 0.003nM PCB126.

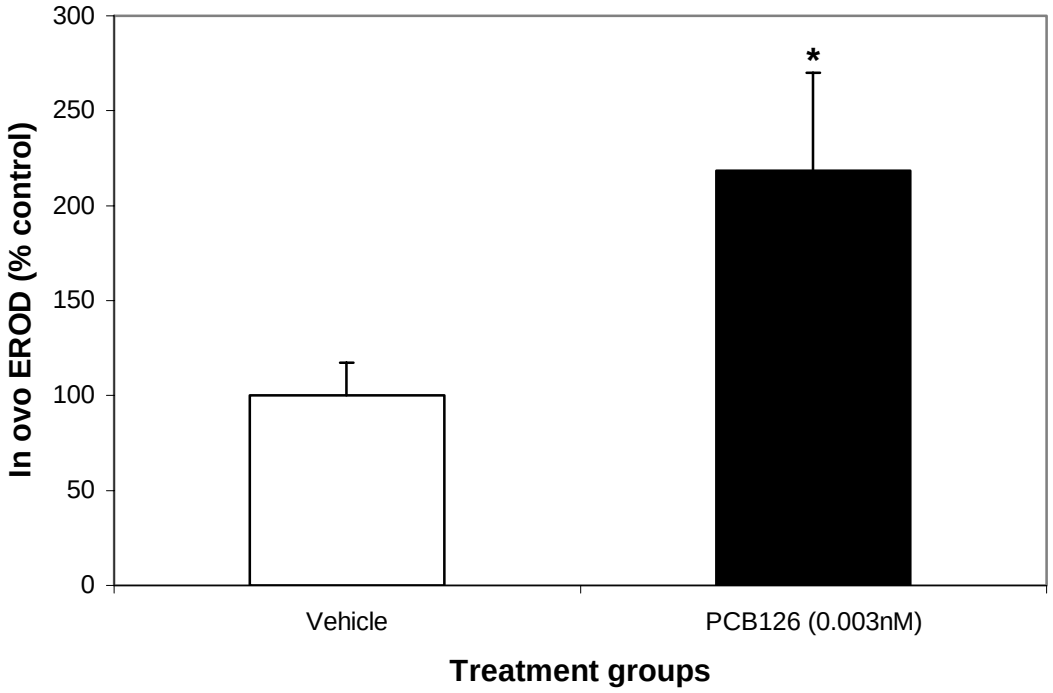


Figure 4-6b. Sensitivity of embryo ROS production to induction by 0.003nM PCB126.

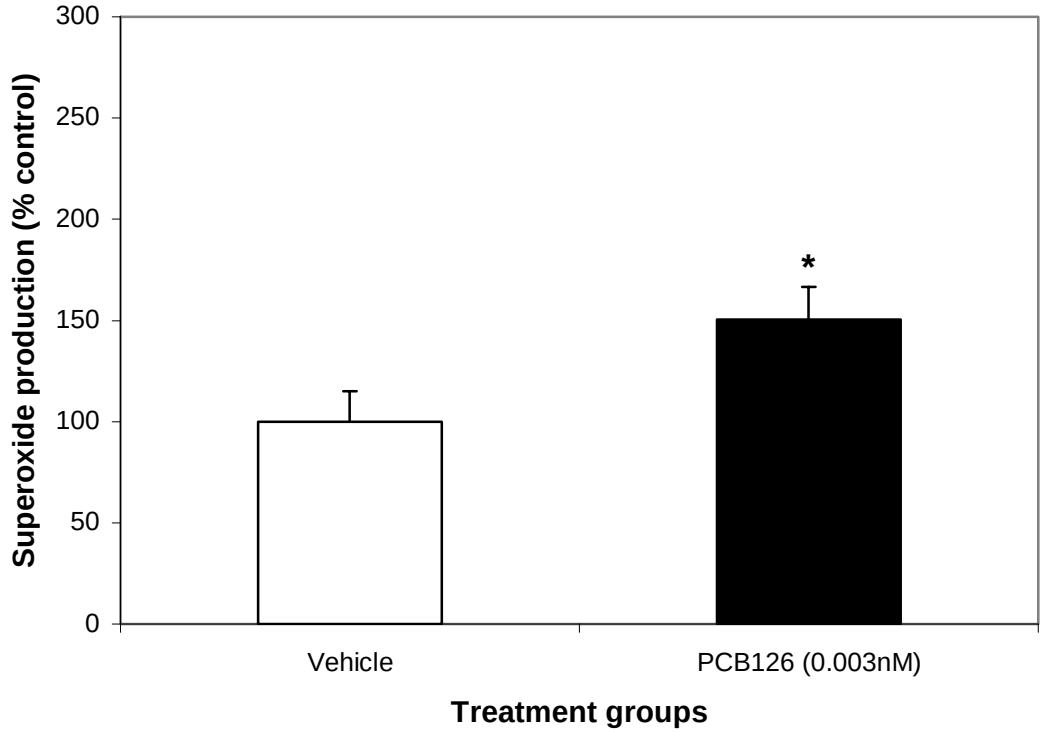


Figure 4-7a. 3-MC induced in ovo EROD in developing killifish embryos.

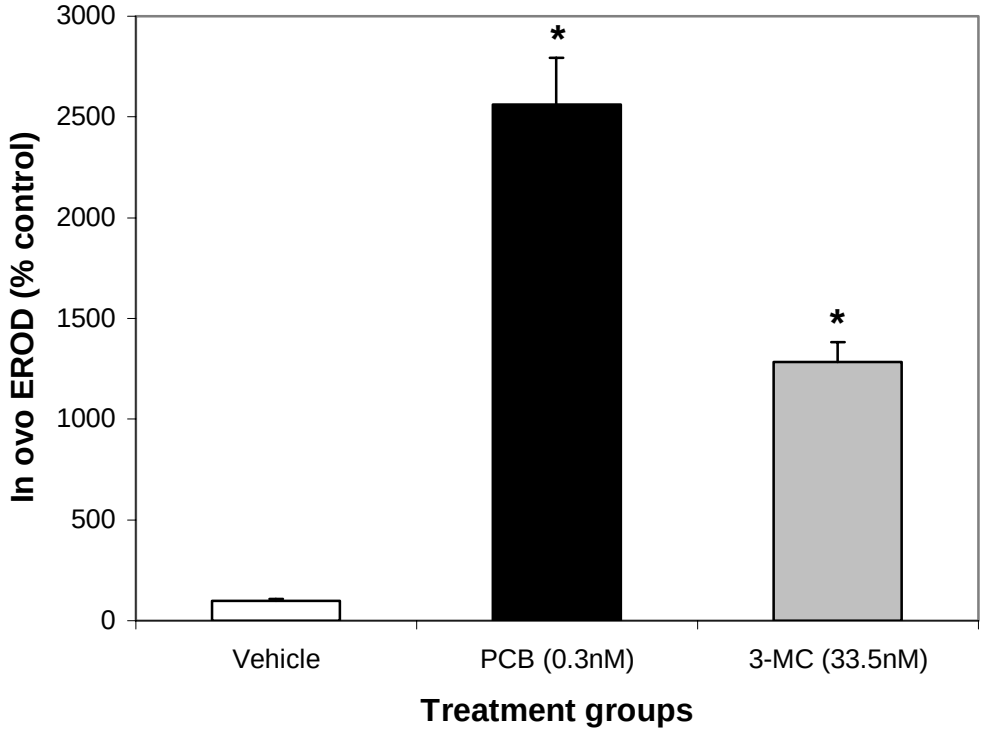
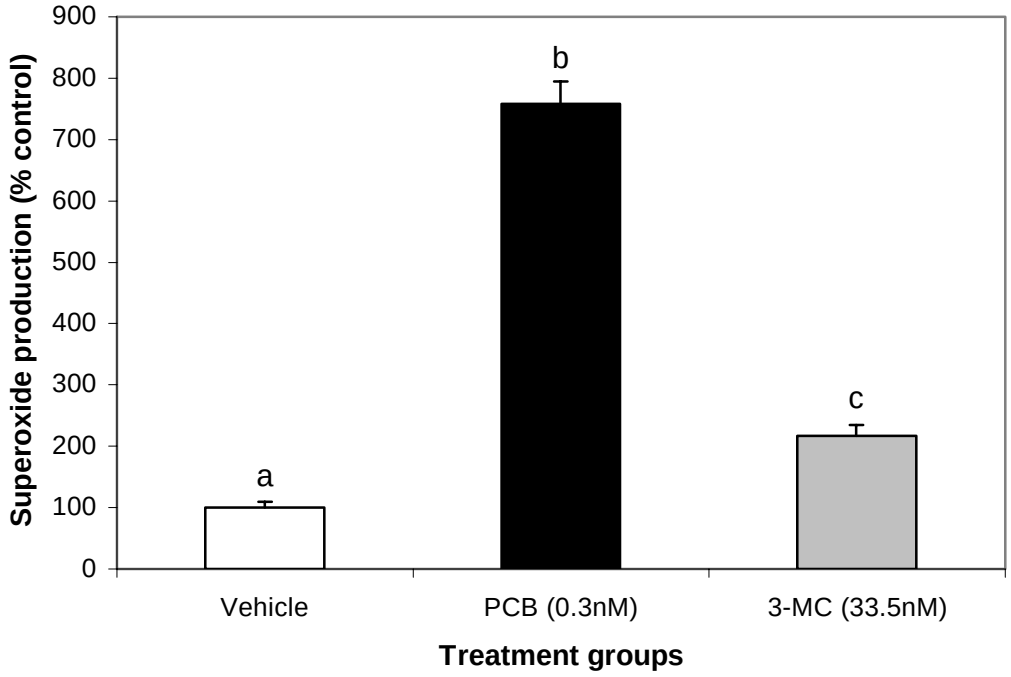


Figure 4-7b. 3-MC induced in ROS produciton in developing killifish embryos.



Chapter Five

Chronically contaminated killifish (*Fundulus heteroclitus*) embryos from polluted sites are resistant to AHR ligand mediated induction of CYP1A activity, ROS production and developmental deformities.

Abstract

Newark Bay NJ, and New Bedford Harbor MA, are contaminated with a myriad of pollutants, including polychlorinated biphenyls (PCBs) and polycyclic aromatic hydrocarbons (PAHs). Exposure to coplanar PCBs and PAHs can affect embryonic development, induce expression of cytochrome P450 1A (CYP1A), and increase reactive oxygen species (ROS) production (see chapter four). These effects are though to be mediated by the aryl hydrocarbon receptor (AHR). Killifish (*Fundulus heteroclitus*) from New Bedford Harbor (NBH) and Newark Bay (NB) have been chronically exposed to xenobiotic AHR ligands, and have developed resistance to PCB mediated induction of CYP1A (see chapters two and three), and developmental deformities. We hypothesized that NB and NBH killifish have also developed resistance to PCB and PAH mediated induction of ROS production. Killifish embryos from the polluted and reference sites were exposed to the AHR ligands: 3,3'4,4'5-pentachlorobiphenyl (PCB126) or 3-methylcholanthrene (3-MC). ROS production was quantified on day seven post fertilization (pf), in ovo EROD on day nine pf and deformities on day ten pf. Reference site animals were responsive to CYP1A induction, developmental deformities and ROS production caused by exposure to PCB126 and 3-MC. Killifish embryos from NB and NBH were resistant to PCB126 induced deformities and PCB126 and 3-MC induced ROS production. While treatment with PCB126 and 3-MC significantly induced in CYP1A activity in reference site animals, NB and NBH killifish embryos only responded to treatment with 3-MC. These differences in responsiveness to PCB126 and 3-MC suggest that additional molecular mechanisms could be controlling CYP1A induction in killifish. Recent studies have shown that fish collected from contaminated environments have higher levels of antioxidants. To determine if the resistance to AHR/CYP1A activation or higher antioxidant levels is protective against PCB126 induced oxidative

stress and deformities, reference site animals were treated with PCB126 in combinations with 1) the AHR/CYP1A inhibitors: alpha-naphthoflavone (ANF), or quercetin (QE); 2) the P450 inhibitor, piperonyl butoxide (PBO) or 3) the antioxidant: n-acetyl-cysteine (NAC). Our results suggest that CYP1A induction and developmental deformities are AHR dependent and that co-planar PCB induced P450 activity is a source for ROS. We conclude that resistance to AHR/CYP1A activation by co-planar PCBs protects contaminated site killifish against the toxic effects caused by these contaminants.

Introduction

Environmental contaminants like coplanar polychlorinated biphenyls (PCBs) and polycyclic aromatic hydrocarbons (PAHs) are commonly found in polluted aquatic environments (O'Connor, 1988; Wenning, 1994). These chemicals have been shown to cause a wide variety of toxic effects in exposed organisms (O'Connor, 1988), yet previous studies have identified pollution exposed fish populations that are resistant to the immediate toxic effects caused by exposure to PAHs and PCBs (Weis, 2002; Wirgin and Waldman, 2004). These adaptations have been documented for killifish populations from New Bedford Harbor, MA (Bello et al., 2001; Nacci et al., 1999), Newark Bay, NJ (Prince and Cooper, 1995a; Prince and Cooper, 1995b), and a portion if the Elizabeth River, VA (Meyer et al., 2002; Van Veld and Westbrook, 1995). Although all three populations are contaminated with PAHs and PCBs, the composition of these chemicals is very different from one site to another. New Bedford Harbor is a site that is heavily contaminated with PCBs (Lake, 1995), the Elizabeth River is polluted with PAHs (O'Connor, 1988) and in Newark Bay PAHs, PCBs and dioxins can be found (Wenning, 1994; Wenning et al., 1992). Killifish from these sites are resistant to PCB and PAH induced mortality, developmental deformities, and have altered expression of phase I and phase II xenobiotic metabolizing enzymes (Weis, 2002).

Many of the toxic effects caused by exposure to PCBs and PAHs are mediated by the aryl hydrocarbon receptor (AHR) (Mimura and Fujii-Kuriyama, 2003; Nebert et al., 2004). The AHR is a cytosolic receptor whose activation initiates transcription of hundreds of genes including phase I and phase II xenobiotic metabolizing enzymes (Puga et al., 2000; Schrenk, 1998). One of the most commonly studied responses to AHR

activation is induction of the monooxygenase cytochrome P450 1A or CYP1A (Whitlock, 1999). This enzyme metabolizes xenobiotic substrates, but its activity has also been shown to be detrimental. CYP1A can convert PAHs in to unstable metabolites that can bind to DNA (Jonsson et al., 2004; Leadon et al., 1988) or increase reactive oxygen species (ROS) production (Nebert et al., 2004; Telli-Karakoc et al., 2002). Halogenated aromatic hydrocarbons (HAHs) such as PCBs and dioxins can also bind to the enzyme and uncouple its catalytic cycle, which leads to ROS production (Schlezinger and Stegeman, 2001; Shertzer et al., 2004).

Early developmental stages of fish and other vertebrates are particularly sensitive to the toxic effects caused by these chemicals. Exposure to PCBs and/or PAHs during early development can induce CYP1A expression, increase ROS production and teratogenesis (Henry et al., 1997; Hoffman et al., 1996; Wassenberg et al., 2002b). Studies using different vertebrate models suggest that these effects are mediated though activation of AHR and induction of CYP1A (Dong et al., 2001; Dong et al., 2002; Hassoun and Stohs, 1996; Jin et al., 2001).

Chronically contaminated killifish populations from Newark Bay (NB), and New Bedford Harbor (NBH), have altered AHR/CYP1A response to these chemicals and exposure to AHR ligands does not induce CYP1A expression in embryonic (Arzuaga et al., 2004; Nacci et al., 1999), larval (Elskus et al., 1999) or adult killifish (Bello et al., 2001; Elskus et al., 1999; Nacci et al., 2002). We hypothesized that by acquiring resistance to AHR activation and CYP1A induction Newark Bay and New Bedford Harbor killifish are less sensitive to some of the teratogenic and oxidative stress effects caused by exposure to these chemicals.

The objectives of these experiments were to determine if chronically contaminated killifish from these two populations were resistant to AHR-ligand induced toxicity (teratogenesis and ROS production), and to determine if PCB induction of ROS and deformities are mediated through AHR/CYP1A.

Materials and methods

Materials

7-Ethoxyresorufin and dihydroethidium (DHE) were purchased from Molecular Probes (Eugene, OR). PCB126 was purchased from AccuStandard (New Haven, CT), 3-methylcholanthrene, quercetin (QE), piperonyl butoxide (PBO), alpha naphthoflavone (ANF) and N-acetylcysteine (NAC) from Sigma (St. Louis, MO) and salt (Bio-crystals) for preparing 30ppt sea water was purchased from Marine Enterprises International (Baltimore, MD).

Killifish collection and maitenance

Adult killifish (*Fundulus heteroclitus*) were collected from reference sites: Kings Creek, (NC), Beaufort (NC), and West Island (RI) in June, August and October of 2003, respectively. Killifish from polluted sites: Newark Bay (NJ) and New Bedford Harbor (MA) were collected in April and August 2003, respectively. Animals were held under laboratory conditions for at least four months to depurate contaminant body burdens before initiating experiments. The half-life of PCBs in fish is approximately 4 months (Niimi, 1983). Killifish were maintained in 10 or 20-gallon recirculating tanks equipped with charcoal Whisper^{TM} filters, in 30 ppt artificial seawater at 24-25°C on a 16/8 L/D cycle. Fifty percent of the tank water was replaced every other week.

Killifish are batch spawners that release eggs on the new and full moons (Bradford and Taylor, 1987). Killifish embryos were collected during the weeks of new and full moon from during December 2003, and June and July 2004 and spawned in the laboratory as described previously (Nacci et al., 1998; Arzuaga et al., submitted). pawned eggs were collected and placed individually in 20 mL scintillation vials containing 3 ml of sea water (30ppt). On day two, eggs were examined microscopically for evidence of fertilization and assigned to treatment groups. Developmental stage was determined as described (Armstrong and Child, 1965a).

Exposure to test compounds

Killifish embryos were aqueously exposed to test compounds dissolved in acetone diluted in 30 ppt seawater. Treatments consisted of vehicle (0.001% acetone) or the AHR ligands, 3-methylcholanthrene, or PCB126, the AHR/CYP1A and P450 inhibitors alpha naphthoflavone (ANF) and piperonyl butoxide (PBO) alone, or in combination with PCB126. The AHR/CYP1A inhibitor quercetin (QE) and the antioxidant N-acetylcysteine (NAC) were dissolved directly in seawater. Chemical exposure began on day two post-fertilization (pf). Embryos were kept in 20 mL scintillation vials (one embryo per vial) containing 10 mL of exposure media and held at 25°C on a 12/12 L/D cycle until analysis.

In ovo superoxide detection assay (SoDA)

Dihydroethidium (DHE) is a fluorescent probe that has been previously used for measuring the ROS product, superoxide, in vitro and in cell culture (Andres et al., 2000; Schlezinger et al., 2000; Schlezinger et al., 1999; Vanden Hoek et al., 1997), and most recently in living fish embryos (Arzuaga et al., submitted). ROS production was measured on day five pf for 3-MC experiments and day seven pf for all other experiments. Before ROS measurement, the exposure media was replaced with 2 mL of fresh media containing DHE dissolved in seawater. DMSO (0.01%) was used as vehicle for DHE, and the DHE seawater solution was sonicated for 20 minutes prior to embryo incubation to dissolve the DHE entirely. Embryos were kept in the dark on a plate shaker (~70 rpm) for 1.5 hours, and the DHE solution was replaced with 10ml of clean artificial seawater. DHE staining of embryo tissues was visualized on a Nikon E800 fluorescence microscope (Melville, NY) equipped with a Nikon, 100W mercury lamp and digital camera (spot RT Diagnostic Instruments, Sterling Heights, MI). Images were captured using Spot RT software interfaced with Adobe Photoshop (San Jose, CA). Image exposure times were determined separately for each experiment, and were based on the amount of time needed to detect a signal in the vehicle controls. Exposure time in our experiments was 0.8 seconds for the ethidium signal (a measurement of superoxide) using a Texas Red filter (EX-560/55 EM-645/75 DM-595). Images were analyzed using Scion Image (Frederick, MD).

In ovo CYP1A activity

In ovo CYP1A activity (ethoxyresorufin-o-deethylase, or EROD) was conducted as described (Nacci et al., 1998). The exposure seawater contained 7-ethoxyresorufin (7-ER) dissolved in DMSO (0.001%), at a final concentration of 21 ug/L. On day 7 exposure media was replaced with clean seawater. *In ovo* EROD was measured at 9 dpf using a fluorescence microscope with Texas Red filters (as described above for SoDA assay). CYP1A induction was evaluated based on the accumulation of the metabolite resorufin (RR) in the animal's bladder and is presented as the intensity of the RR signal (Texas Red). The exposure times for the RR image in PCB exposure experiments was 700 ms when using West Island killifish as reference, and 70 ms when using Kings Creek or Beaufort killifish as reference.

Developmental deformities

Killifish embryos from Beaufort, Newark Bay, and New Bedford Harbor were treated with vehicle, 1.5 nM and 3.0 nM PCB126 on day 2 pf. Exposure medium was changed on day 7 pf and heart deformities were quantified on day 10 pf. Heart deformities were classified as described (Meyer and Di Giulio, 2002). Deformities were quantified using a scale of 0 to 2 where 0 corresponds to a normal heart, 1 corresponds to a mildly deformed heart where chambers are elongated, but blood flow is apparent and 2 corresponds to a severely deformed heart where the chambers are elongated and blood flow is not visible. Heart deformities were quantified using the following design. Scintillation vials housing individual embryos were labeled with the following information: 1) day of fertilization and 2) treatment. On day 10 pf, the information on killifish embryo vials was substituted with a randomly assigned number. After quantification, the numbers were matched with corresponding treatment groups for data analysis.

Statistical analysis

Statistical analyses were performed using SYSTAT version 10.0 and SigmaStat version 3.1 (Systat Software, Point Richmond CA). In ovo EROD and SoDA integrated density values were analyzed for normality and equal variances using Kormolov-Smirnov

test, and Bartletts test respectively. Since PCB and 3-MC treatments caused significant increases in sample variance in ovo EROD and SoDA, integrated density values were converted to their square root and further statistical analysis was performed on these values. A student t test was used for comparing control versus PCB induced ethidium (ROS production) signal in Beaufort, Newark Bay and New Bedford Harbor killifish embryos. Two way analysis of variance (ANOVA) followed by Fishers Least Significant Difference (LSD) test was used for comparisons between fish populations and PCB126, or 3-MC treatments. One way (ANOVA) followed by LSD test was used for analysis of in ovo EROD and SoDA integrated density values in experiments where killifish were exposed to PCB126 alone, or in combination with the AHR/CYP1A and P450 inhibitors ANF, QE , and PBO, or the antioxidant NAC. Data on PCB induced heart deformities were analyzed by Kruskal-Wallis non-parametric analysis of variance by ranks followed by Dunn's nonparametric test for multiple comparisons. Level of significance was 0.05.

Results

Polluted killifish embryos are resistant to PCB126 induction of in ovo EROD, ROS and deformities.

To determine if chronically contaminated killifish are resistant to PCB126 mediated induction of in ovo EROD, ROS production, and deformities, killifish embryos from the reference and contaminated sites were exposed to vehicle or PCB126 on day two post-fertilization (pf) and induction of CYP1A activity (in ovo EROD), ROS production and heart deformities were compared. In ovo EROD was assayed on day 9 pf, and ROS production on day seven pf and developmental deformities on day ten pf. Two-way ANOVA revealed significant treatment, population and population by treatment effects. Exposure to 0.3 nM PCB126 resulted in a 31.7 fold induction of in ovo EROD in reference site, West Island (WI), killifish embryos but failed to induce in ovo EROD in killifish embryos from the two contaminated populations: Newark Bay (NB) and New Bedford Harbor (NBH) (Figure 5-1a). These data corroborate previous findings demonstrating that NB and NBH killifish are resistant to PCB mediated induction of in ovo EROD during early development (Arzuaga et al., 2004; Elskus et al., 1999; Nacci et al., 1999).

While PCB126 induced ROS production in reference site, Beaufort (BC), killifish embryos; NB and NBH killifish were resistant to this effect (Figure 5-1b). This suggests that chronically contaminated killifish are resistant to co-planar PCB induction of ROS production.

To determine if chronically contaminated killifish embryos are resistant to PCB induced deformities, embryos from all 3 populations were exposed to vehicle or PCB126 (at 1.5 nM or 3.0 nM). PCB treatment significantly increased ($p < 0.05$) the frequency of heart deformities in reference site embryos, but not in NB and NBH killifish embryos (Table 5-1). As demonstrated in studies performed by other groups (Meyer and Di Giulio, 2002; Nacci et al., 1999), these findings suggests that chronically contaminated killifish have developed resistance to PCB induced developmental deformities.

To determine if the AHR pathway is functional in NB and NBH killifish embryos, a dose-response experiment using PCB126 was performed. Killifish embryos from NB and NBH did not respond to the PCB126 (0.3 nM) dose that caused maximum *in ovo* EROD induction in WI killifish embryos (Figure 5-2a). However, NB and NBH killifish embryos did respond to a 15 fold higher PCB126 dose (4.5nM), which was toxic to WI fish embryos (Figure 2b, and c). These results suggest that although NB and NBH killifish embryos have reduced sensitivity to PCB126, the AHR pathway is still functional.

Polluted killifish embryos are resistant to 3-MC induction ROS production.

To determine if chronically contaminated killifish are also resistant to PAH mediated induction of in ovo EROD and ROS production during early development, reference and contaminated site killifish embryos were exposed to vehicle or 33.3 nM 3-MC, a dose that causes maximum induction of in ovo EROD in reference site embryos (Nacci et al., 1998). Two-way ANOVA revealed a significant population, treatment and population by treatment effect. Treatment with 3-MC resulted in significant in ovo EROD induction in all three populations. However, in ovo EROD of 3-MC treated reference site (BT) killifish embryos was considerably higher than in NB and NBH embryos (Figure 5-3a), suggesting that reference site animals are more sensitive than contaminated site killifish embryos. In comparison to the response observed in the

PCB126 treated animals, these data suggest that 3-MC could be activating CYP1A induction through an alternative pathway.

Treatment with 3-MC resulted in induced liver ROS production in reference site animals ($p<0.05$), but contaminated site killifish were resistant to this effect (Figure 5-3b).

To further study the in ovo EROD response to PAHs in polluted site killifish embryos Newark Bay and Flax Pond (reference site) fish embryos were exposed to various concentrations of 3-MC (1.12, 11.2, and 112 nM). One-way ANOVA revealed that NB killifish embryos treated with 11.2 and 112 nM 3-MC had significantly higher in ovo EROD activity when compared with vehicle treated animals (Figure 5-4). In comparison with reference site animals, the magnitude of the in ovo EROD response in NB killifish embryos was lower than the one observed in reference site animals. In combination with the PCB126 dose response experiments (Figure 5-2) these results suggest that although polluted site killifish embryos have lower sensitivity to coplanar PCBs and PAHs, the AHR pathway continues to be functional in this developmental stage.

The role of resistance to AHR activation and CYP1A induction

As mentioned above, many of the toxicological effects caused by co-planar halogenated AHR ligands are dependent on activation of the AHR and induction of CYP1A1/2, among other genes. Based on this evidence we propose that by acquiring resistance to AHR activation, NB and NBH killifish are able to survive in polluted environments. To test this hypothesis, reference site animals were treated with PCB126 in combination with AHR/CYP1A, and P450 inhibitors.

Role of AHR pathway in PCB126 induction of developmental deformities.

To determine if AHR activation plays an important role in PCB mediated induction of heart deformities during early development; reference site, Beaufort (BT), killifish embryos were exposed to 1.5 nM PCB126 alone or in combination with 367 nM alphanaphthoflavone (ANF), an AHR/CYP1A inhibitor. This does of ANF has been previously shown to block PAH induction EROD in killifish embryos (Wassenberg and

Di Giulio, 2004). Treatment with ANF was able to block PCB126 induction of heart deformities ($p<0.05$) (Table 5-2). This suggests that resistance to PCB126 dependent AHR activation and CYP1A induction is protective against PCB126 induced developmental deformities.

Role of AHR pathway in PCB126 induction of in ovo EROD and ROS production.

To determine if PCB126 induction of in ovo EROD and ROS production are AHR/CYP1A mediated, reference site killifish embryos were treated with 0.3 nM PCB126 alone or in combination with the AHR/CYP1A inhibitory flavonoids ANF and quercetin (QE). Treatment with 367 nM ANF alone did not affect in ovo EROD (Figure 5-5a). Embryos treated with PCB126 in combination with ANF had significantly lower CYP1A activity than PCB treated animals ($p<0.05$) (Figure 5-5b), suggesting that ANF blocked PCB126 induction of CYP1A expression and activity. Treatment with ANF alone increased embryonic liver ROS production ($p<0.05$), and did not affect PCB126 induction of ROS production (Figure 5-6).

Treatment with QE (100 μM) was also able to significantly reduce PCB126 induction of in ovo EROD in reference site animals ($p<0.05$) (Figure 5-7a), but did not affect PCB induction of ROS production (Figure 5-7b).

Role of P450 activity in PCB126 induced ROS production.

To determine if P450 activity plays an important role in PCB126 induction of ROS production, reference site animals were treated with 0.3 nM PCB126 alone, or in combination with 1 μM piperonyl butoxide (PBO). PBO is a non-specific P450 inhibitor (Franklin, 1977) and this dose has been previously shown to block PAH mediated induction of in ovo EROD in killifish embryos (Wassenberg and Di Giulio, 2004). Treatment with PBO significantly reduced ($p<0.05$) PCB126 mediated induction of in ovo EROD (Figure 5-8a), suggesting that the chemical was able to block P450 activity. More importantly, PBO treatment significantly reduced ($p<0.05$) PCB126 mediated induction of ROS (Figure 5-8b). This suggests that altered P450 activity by PCB126 is a source of ROS production.

Antioxidants and PCB126 induction of ROS production.

As mentioned above, previous studies have shown that killifish from contaminated sites have increased antioxidant levels (Meyer et al., 2003). To determine if antioxidants can block PCB126 induced production of ROS, reference site, Kings Creek (KC) VA, killifish embryos were exposed to PCB126 (0.3 nM) alone or in combination with the antioxidant n-acetylcysteine (NAC) at 200 μM. In ovo EROD induction and ROS production were measured in separate experiments. To our surprise, treatment with NAC significantly blocked ($p<0.05$) PCB126 induction of in ovo EROD by 54% (Figure 5-9a).

Also, NAC treatment significantly increased ROS production when compared to controls ($p<0.05$) and ROS production was significantly higher in animals treated with NAC plus PCB126 than the ones treated with PCB126 alone ($P<0.05$) (Figure 5-9b).

Studies on cell culture models have shown that vitamin E can protect against PCB induced ROS production (Slim et al., 1999). To determine if vitamin E can also reduce PCB mediated induction of ROS production; reference site animals were exposed to PCB alone, or in combination with vitamin E. Although Vitamine E treatment resulted in a small reduction of PCB126 mediated ROS production (data not shown), it has also been shown that it can significantly reduce PCB induction of CYP1A activity (Wassenberg and Di Giulio, personal communication). It is possible that vitamin E is binding to the PCB126 molecule and preventing uptake by the embryo, or binding to the AHR.

Discussion

In these experiments we tried to determine if the resistance to AHR activation and CYP1A induction observed in polluted site killifish embryos is an important mechanism that allows them to survive in contaminated sites. Killifish embryos from the two polluted sites (Newark Bay, NJ and New Bedford Harbor) were resistant to PCB126 mediated induction of in ovo EROD, and heart deformities. These observations are in agreement with results obtained in previous studies (Arzuaga et al., 2004; Nacci et al., 1999). We also report that NB and NBH killifish embryos are resistant to PCB126 induction of ROS as measured by dihydroethidium (SoDA assay). Studies performed by others have shown that fish collected from contaminated sites have altered levels of

antioxidants and antioxidant enzymes (Ahmad et al., 2000; Meyer et al., 2003; Palace, 1998; Stephensen et al., 2000). It is possible that by having altered AHR function and increased levels of antioxidant, chronically contaminated killifish have gained resistance to the developmental effects of co-planar PCB exposure. To test this hypothesis, reference site animals were exposed to PCB126 in combination with AHR/CYP1A and P450 inhibitors, and antioxidants

Resistance to AHR activation and CYP1A induction protects killifish embryos against PCB126 induced teratogenesis. In this experiment we used ANF (an AHR and CYP1A inhibitor) to demonstrate that the toxic effects caused by PCBs are mediated through AHR activation and CYP1A induction. Treatment with ANF was able to block deformities caused by PCB126 exposure in reference site animals. These results are similar to those reported by other studies on the effects of TCDD exposure on zebrafish development (Dong et al., 2002), and suggest that AHR and CYP1A are associated with altered development caused by PCB exposure. However, studies on co-planar PAH induced deformities have generated conflicting results when compared to those obtained here. (Wassenberg and Di Giulio, 2004) ovserved that killfish embryos treated with a combination of PAH mixtures with ANF had significantly higher occurrence and more drastic deformities, when comparedn with animals tyreated with PCB mistures alone. As mentioned bellow, these results suggest that AHR activation and CYP1A induction may be beneficial after PAH expsure, but detrimental after exposure to chlorinated AHR ligands.

Activities of cytochrome P450s are a potential source of co-planar PCB induced ROS production (Bondy and Naderi, 1994; Kuthan and Ullrich, 1982). We used PBO to determine the role of total P450s in PCB126 induced ROS production. In reference site killifish PBO treatment decreased PCB126 mediated induction of in ovo EROD and ROS production. The AHR/CYP1A inhibitors ANF and QE were able to reduce PCB126 mediated induction of in ovo EROD, but did not affect PCB126 induction of ROS. These results suggest that instead of CYP1A, other AHR inducible P450s could be increasing ROS production in PCB126 treated killifish embryos. Hepatic cytochrome P450s can significantly contribute to formation of ROS (Bondy and Naderi, 1994), and AHR ligands can induce other P450s such as CYP1B1 (Spink et al., 2002; Wang et al., 2002) and

CYP2S1 (Rivera et al., 2002) in different vertebrate models. Future studies should be aimed at determining if other P450s can be induced by co-planar PCBs in killifish, if they can generate ROS, and if their expression is altered in chronically contaminated killifish.

Induction of ROS production by PCB126 is not associated with teratogenesis in reference site killifish. It was interesting to see that neither ANF nor QE were able to reduce PCB126 induction of in ovo EROD and heart deformities in reference site killifish, but did not affect PCB induction of ROS. These results are surprising because previous studies using cell culture models have found that ANF and QE decrease AHR ligand induction of oxidative stress (Fabiani et al., 1999; Park et al., 1996; Ramadass et al., 2003). These results suggest that ROS production is not associated with heart deformities in killifish embryos exposed to PCB126. However, PCB126 induced ROS production could be causing other harmful effects in developing killifish such as lipid peroxidation (Hilscherova et al., 2003), DNA damage (Cantrell et al., 1996; Hassoun and Stohs, 1996), and apoptosis (Dong et al., 2002; Toomey et al., 2001) or altered immune function (Duffy et al., 2003). Further studies are required to determine if exposure to co-planar PCBs can cause these effects in developing killifish embryos.

Paradoxically, treatment with ANF alone increased ROS production and failed to block PCB126 induced ROS production. It is possible that in our experimental model, ANF treatment activates alternative pathways that increase ROS production. For example, in addition to AHR and CYP1A inactivation, ANF can stimulate cytochrome P450 3A4 (CYP3A4) activity in vitro (Koley et al., 1997), which could also increase ROS production due to enzyme uncoupling. Also, ANF can induce nitric oxide (NO) production in rat endothelial cells and NO produced in the liver can react with superoxide to form the highly reactive peroxynitrite which causes oxidative stress (Cheng et al., 2003; Jaeschke et al., 2002). Another possibility is that interfering with AHR function could be harmful. It has been shown that the AHR is associated with stress signaling pathways (Matsumura, 2003) and complete elimination of the AHR has been shown to cause abnormal liver development in AHR knockout mice (Gonzalez and Fernandez-Salguero, 1998).

The antioxidant NAC was not protective against PCB126 induced ROS production in reference site killifish. Studies by other groups have shown that oxidative

stress and other toxic effects caused by PCBs can be reduced by co-treatment with antioxidants such as vitamin E, or NAC (Dong et al., 2002; Hilscherova et al., 2003; Slim et al., 1999). In this study, the effects caused by exposure to the antioxidant NAC were not expected. We observed that NAC treatment inhibited CYP1A induction by PCB126 and increased ROS production. Studies by other groups have shown that NAC treatment reduces toxicity of AHR ligands (Burdick et al., 2003; Cantrell et al., 1996; Dong et al., 2002), but contradicting results in experiments using antioxidants have also been documented by others (Santanam and Parthasarathy, 1995). It is possible that the NAC dose used in these studies were toxic, or that it increased ROS production by a mechanism that we are not aware of. To further study the role of increased antioxidant levels in resistance to AHR ligands, total oxygen scavenging capacity (TOSC) as well as the specific antioxidants that are potentially participating in resistance should be measured in NB and NBH killifish.

AHR independent signaling pathways could be controlling CYP1A induction in killifish. Contaminated site killifish embryos were resistant to PCB126 induction of in ovo EROD, but responsive to treatment with 3-MC. This was surprising because previous studies have shown that embryonic and adult killifish from NBH are resistant to PAH mediated induction of CYP1A catalytic activity (Nacci et al., 1999; Nacci et al., 2002). Our results are similar to those obtained from adult Atlantic tomcod (*Microgadus tomcod*) from the Hudson River (NY) which are resistant to CYP1A mRNA induction by halogenated AHR ligands (e.g. co-planar PCBs and TCDD) but respond to non-halogenated ligands such as benzo-a-pyrene (B[a]P) and beta-naphthoflavone (BNF) (Courtenay et al., 1999). Similar observations have also been made in studies using primary hepatocytes from NBH killifish (Bello et al., 2001), which revealed that resistance to halogenated AHR ligand induction of EROD was greater than the resistance to a non-halogenated ligand. One possibility is that 3-MC could be inducing CYP1A expression in NB and NBH killifish embryos through more than one signaling pathway (Brauze and Malejka-Giganti, 2000; Delescluse et al., 2000).

The CYP1A induction response to 3-MC observed in chronically contaminated killifish could be associated with adaptation to an environment containing complex mixtures of different kinds of AHR ligands. Recent in vivo studies have shown CYP1A1

activity is detrimental after exposure to halogenated AHR ligands (Uno et al., 2004b), but beneficial after exposure to non-halogenated AHR ligands (Uno et al., 2004a). This suggests that PAHs and PCBs could be causing toxic effects through different mechanisms associated the metabolic fate of these chemicals. Halogenated AHR ligands can increase ROS production through uncoupling of the P450 catalytic cycle (Schlezinger and Stegeman, 2001; Shertzer et al., 2004). These chemicals are resistant to metabolism and increase microsomal CYP1A expression and ROS production for extended periods of time (Buhler and Wang-Buhler, 1998; Celander and Forlin, 1995; Shertzer et al., 1998). On the other hand, PAHs induce CYP1A for shorter periods of time when compared to PCBs (Celander and Forlin, 1995). Although metabolic activation of PAHs can increase ROS production (Peters, 1996), excretion of PAHs is faster than PCBs. It has also been proposed that close coupling between phase I (CYP1A1) and phase II metabolism can increase excretion of PAHs (Nebert et al., 2004). GST is a phase II enzyme that can successfully conjugate toxic PAH metabolites (Sheweita, 2000; Sundberg et al., 2002) and expression of this enzyme is altered in fish collected from contaminated sites (Otto and Moon, 1996). Adult killifish from the contaminated Elizabeth River VA, and Newark Bay NJ, possess higher basal activity of glutathione-s-transferase (GST) than reference site animals (Armknecht et al., 1998). In NBH killifish, induction of GST by AHR ligands is greater than in reference site animals (Bello et al., 2001). It is possible that in chronically contaminated killifish, resistance to induction of CYP1A and other P450s by co-planar PCBs reduces PCB toxicity because there would be a smaller pool of uncoupled P450s generating ROS and causing oxidative stress. On the other hand, induction of CYP1A by PAHs, coupled to higher expression of GST could increase clearance of unstable Phase I metabolites that would otherwise cause damage.

In summary, the results presented in this study demonstrate that chronically contaminated killifish are resistant to PCB126 and 3-MC induction of ROS production, that PCB126 induction of developmental deformities and ROS production are mediated through the AHR pathway, and P450 activity, respectively, and that contaminated killifish embryos have different sensitivities to PAH and PCB induction of CYP1A expression. In combination with altered expression of phase II enzymes, these adaptations potentially increase survival of killifish inhabiting contaminated sites.

Figure Legends

Figures 5-1 (a and b). In ovo EROD and ROS production of killifish embryos treated with 0.3 nM PCB126.

a) In ovo EROD activity of killifish embryos from reference site: West Island (WI), and polluted sites: Newark Bay (NB) and New Bedford Harbor (NBH) treated with PCB126. Fish embryos were exposed to 0.3 nM PCB126 from day two to day seven post-fertilization (pf) and bladder resorufin fluorescence (in ovo EROD) measured on day nine pf. Digital images were taken under Texas red excitation/emission filters (EX-560/55 EM-645/75) and image exposure times was 700 milliseconds (ms). Bars represent mean ± standard error (SE) for n = 19 - 21 embryos per treatment group. * significant from respective control at $p < 0.05$.

b) Liver ROS production of killifish embryos from reference site: Beaufort (BT), and polluted sites: Newark Bay (NB) and New Bedford Harbor (NBH) treated with PCB126. Embryos were treated with vehicle or 0.3 nM PCB126 on day two pf and stained with 50 μM DHE on day seven pf. Digital images were taken under Texas red excitation/emission filters and the exposure time was 800 ms. Integrated density values were normalized to % of controls for each population. Bars represent mean % of control ± standard error (SE) for n = 16 - 20 embryos per treatment group. * significant from respective control at $p < 0.05$.

Figures 5-2 (a, b and c). In ovo EROD for WI, NB and NBH killifish embryos treated with PCB126 (0, 0.3, 1.5, and 4.5 nM). Fish embryos were exposed to PCB126 from day two to day seven post-fertilization (pf) and bladder resorufin fluorescence (in ovo EROD) measured on day nine pf. Digital images were taken under Texas red excitation/emission filters (EX-560/55 EM-645/75) and image exposure times was 700 milliseconds (ms). Bars represent mean ± standard error (SE). Different letters represent statistical difference from respective control at $p < 0.05$.

a) In ovo EROD activity of WI killifish embryos. N = 11 to 20 individuals per treatment group.

b) In ovo EROD activity of NBH killifish embryos. N = 19 to 20 individuals per treatment group.
c) In ovo EROD activity of NB killifish embryos. N = 17 to 21 individuals per treatment group.

Figures 5-3 (a and b). In ovo EROD and ROS production of killifish embryos treated with 33.5 nM 3-MC.

a) In ovo EROD activity of killifish embryos from reference site: Beaufort (BT), and polluted sites: Newark Bay (NB) and New Bedford Harbor (NBH) treated with 3-MC. Fish embryos were exposed to 33.5 nM 3-MC from day two to day seven pf and bladder resorufin fluorescence (in ovo EROD) measured on day nine pf. Digital images were taken under Texas red excitation/emission filters (EX-560/55 EM-645/75) and image exposure times was 70 milliseconds (ms). Bars represent mean ± standard error (SE) for n = 19 - 21 embryos per treatment group. * significant from respective control at $p < 0.05$.

b) Liver ROS production of killifish embryos from reference site: Beaufort (BT), and polluted sites: Newark Bay (NB) and New Bedford Harbor (NBH) treated with 3-MC. Embryos were treated with vehicle or 33.5 nM 3-MC on day two pf and stained with 50 μM DHE on day five pf. Digital images were taken under Texas red excitation/emission filters and the exposure time was 800 ms. Integrated density values were normalized to % of controls for each population. Bars represent mean % of control ± standard error (SE) for n = 15 - 17 embryos per treatment group. * significant from respective control at $p < 0.05$.

Figure 5-4. In ovo EROD for Flax Pond and Newark Bay (NB) killifish embryos treated with 3-MC (0, 1.12, 11.2, and 112 nM). Fish embryos were exposed to PCB126 from day two to day seven post-fertilization (pf) and bladder resorufin fluorescence (in ovo EROD) measured on day nine pf. Digital images were taken under Texas red excitation/emission filters (EX-560/55 EM-645/75) and image exposure times was 700 milliseconds (ms). Bars represent mean ± standard error (SE) for N = 10 to 23

individuals per treatment group. Different letters represent statistical difference from respective control at $p < 0.05$.

Figure 5-5 (a and b). In ovo EROD signal of reference site killifish embryos treated with vehicle, 367 nM ANF, 1.5 nM PCB126 and PCB126 plus ANF.

a) Reference site, West Island (WI), killifish embryos were exposed to vehicle, 0.3 nM PCB126 or 367 nM ANF from day two to day seven pf and bladder resorufin fluorescence measured on day 9 pf. Digital images were taken under Texas red excitation emission filters and the exposure time was 700 ms. Bars represent mean ± standard error (SE) for n = 13 - 14 embryos per treatment group. Different letters represent statistically significant differences at $p < 0.05$.

b) Reference site, Beaufort (BT), killifish embryos were exposed to vehicle, 0.3 nM PCB126 or 367 nM ANF plus 0.3 nM PCB126 from day two to day seven pf and bladder resorufin fluorescence measured on day 9 pf. Digital images were taken under Texas red excitation emission filters and the exposure time was 70 ms. Bars represent mean ± standard error (SE) for n = 17 - 24 embryos per treatment group. Different letters represent statistically significant differences at $p < 0.05$.

Figures 5-6. Liver ROS production in reference site, Beaufort (BT), killifish embryos treated with vehicle, 367 nM ANF, 0.3 nM PCB126 and PCB126 plus ANF. Embryos were treated with chemicals on day two pf and stained with 50 μM DHE on day seven pf. Digital images were taken under Texas red excitation/emission filters and the exposure time was 1.5 seconds. Integrated density values were normalized to % of controls for each population. Bars represent mean % of control ± standard error (SE) for n = 19 - 22 embryos per treatment group. * significant from respective control at $p < 0.05$.

Figure 5-7 (a and b). In ovo EROD and ROS production of reference site, West Island (WI) killifish embryos treated with vehicle, 100 μM quercetin (QE), 0.3 nM PCB126 and PCB126 plus QE.

a) In ovo EROD activity of killifish embryos. Fish embryos were exposed to chemicals from day two to day seven pf and bladder resorufin fluorescence (in ovo EROD) measured on day nine pf. Digital images were taken under Texas red excitation/emission

filters (EX-560/55 EM-645/75) and image exposure times was 700 milliseconds (ms). Bars represent mean ± standard error (SE) for n = 18 - 20 embryos per treatment group. Different letters represent statistically significant differences at $p < 0.05$.

b) Liver ROS production of killifish embryos. Embryos were exposed to chemicals on day two pf and stained with 50 μM DHE on day seven pf. Digital images were taken under Texas red excitation/emission filters and the exposure time was 800 ms. Integrated density values were normalized to % of controls for each population. Bars represent mean % of control ± standard error (SE) for n = 14 - 17 embryos per treatment group. Different letters represent statistically significant differences at $p < 0.05$.

Figure 5-8 (a and b). In ovo EROD and ROS production of reference site, Beaufort (BT) killifish embryos treated with vehicle, 1 μM piperonyl butoxide (PBO), 0.3 nM PCB126 and PCB126 plus PBO.

a) In ovo EROD activity of killifish embryos. Fish embryos were exposed to chemicals from day two to day seven pf and bladder resorufin fluorescence (in ovo EROD) measured on day nine pf. Digital images were taken under Texas red excitation/emission filters (EX-560/55 EM-645/75) and image exposure times was 70 milliseconds (ms). Bars represent mean ± standard error (SE) for n = 18 - 20 embryos per treatment group. Different letters represent statistically significant differences at $p < 0.05$.

b) Liver ROS production of killifish embryos. Embryos were exposed to chemicals on day two pf and stained with 50 μM DHE on day seven pf. Digital images were taken under Texas red excitation/emission filters and the exposure time was 800 ms. Integrated density values were normalized to % of controls for each population. Bars represent mean % of control ± standard error (SE) for n = 14 - 17 embryos per treatment group. Different letters represent statistically significant differences at $p < 0.05$.

Figure 5-9 (a and b). In ovo EROD and ROS production of reference site, Kings Creek (KC) killifish embryos treated with vehicle, 200 μM N-acetylcysteine (NAC), 0.3 nM PCB126 and PCB126 plus NAC.

a) In ovo EROD activity of killifish embryos. Fish embryos were exposed to chemicals from day two to day seven pf and bladder resorufin fluorescence (in ovo EROD)

measured on day nine pf. Digital images were taken under Texas red excitation/emission filters (EX-560/55 EM-645/75) and image exposure times was 70 milliseconds (ms). Bars represent mean ± standard error (SE) for n = 21 - 23 embryos per treatment group. Different letters represent statistically significant differences at $p < 0.05$.

b) Liver ROS production of killifish embryos. Embryos were exposed to chemicals on day two pf and stained with 50 μM DHE on day seven pf. Digital images were taken under Texas red excitation/emission filters and the exposure time was 800 ms. Integrated density values were normalized to % of controls for each population. Bars represent mean % of control ± standard error (SE) for n = 19 - 20 embryos per treatment group. Different letters represent statistically significant differences at $p < 0.05$.

Table 5-1: Heart deformities in BT, NB, and NBH killifish embryos exposed to 0, 1.5, and 3.0 nM PCB126.

Population	PCB dose (nM):	Normal:	Mild deformed:	Severely deformed:	N
BT	0	14	1	0	15
	1.5*	0	9	6	15
	3.0*	0	4	11	15
NB	0	13	1	0	14
	1.5	13	1	1	15
	3.0	12	3	0	15
NBH	0	10	3	0	13
	1.5	12	0	1	13
	3.0	8	1	3	13

Killifish embryos were exposed to vehicle, 1.5 and 3.0 nM PCB126 from day 2 to day 7 pf and heart deformities were quantified on day 10 pf. * significant treatment effects at $p<0.05$

Table 5-2: Heart deformities in BT killifish embryos exposed to 0, 1.5nM PCB126 and 1.5nM PCB126 plus 367nM ANF.

Population	Treatment:	Normal:	Mild deformed:	Severely deformed:	N
BT	Vehicle	12	1	0	13
	1.5 nM PCB126*	1	1	11	13
	367 nM ANF + PCB126	5	4	1	10

Killifish embryos were exposed to chemicals from day 2 to day 7 pf and heart deformities were quantified on day 10 pf. * significantly different from respective control group at $p<0.05$

Figure 5-1a. In ovo EROD of killifish embryos treated with 0.3 nM PCB126.

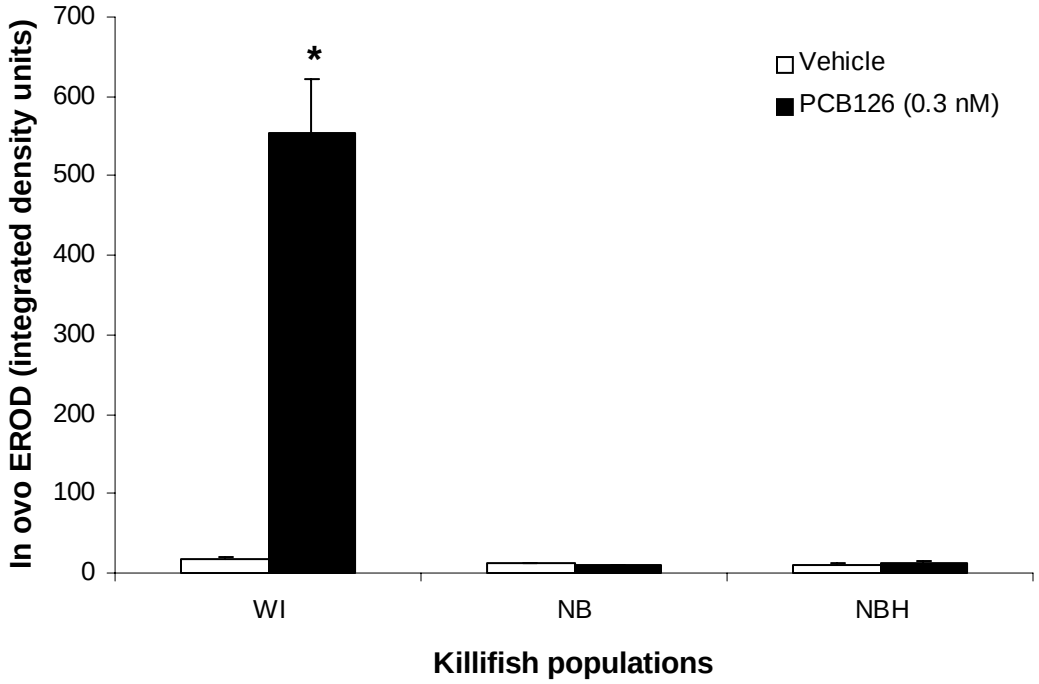


Figure 5-1b. ROS production of killifish embryos treated with 0.3 nM PCB126.

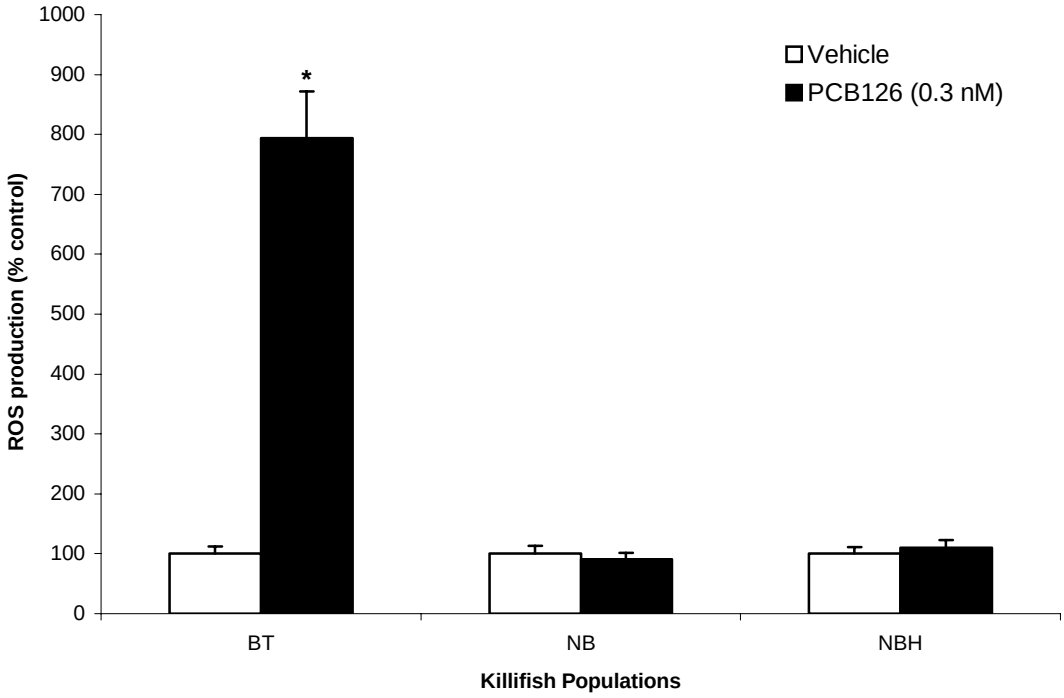


Figure 5-2a In ovo EROD for WI killifish embryos treated with PCB126

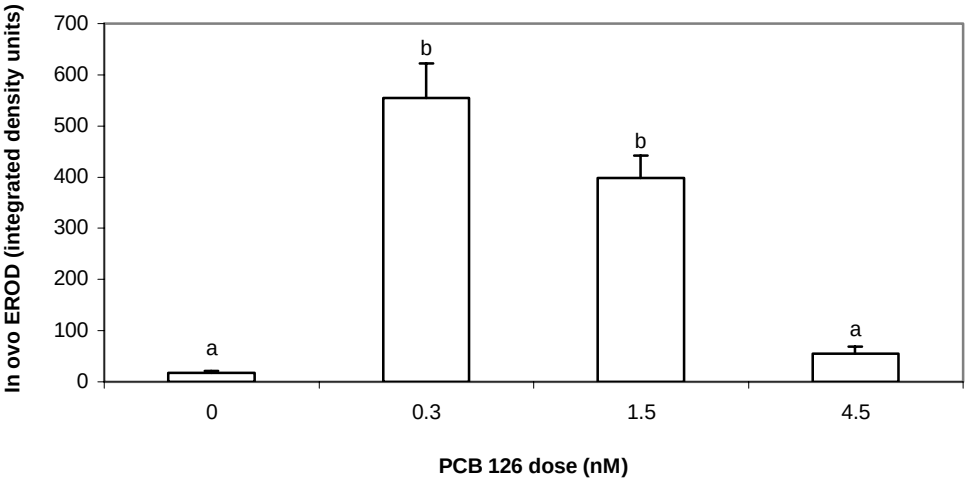


Figure 5-2b In ovo EROD for NBH killifish embryos treated with PCB126

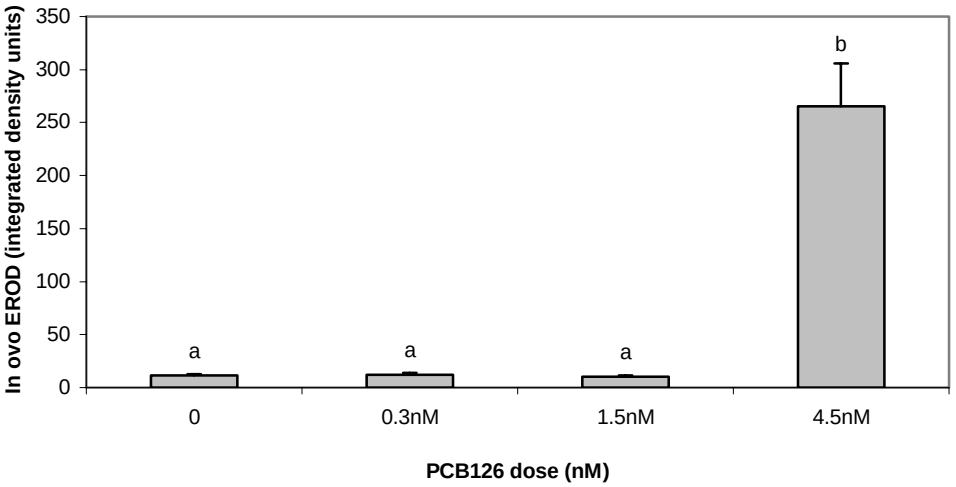


Figure 5-2c. In ovo EROD for NB killifish embryos treated with PCB126.

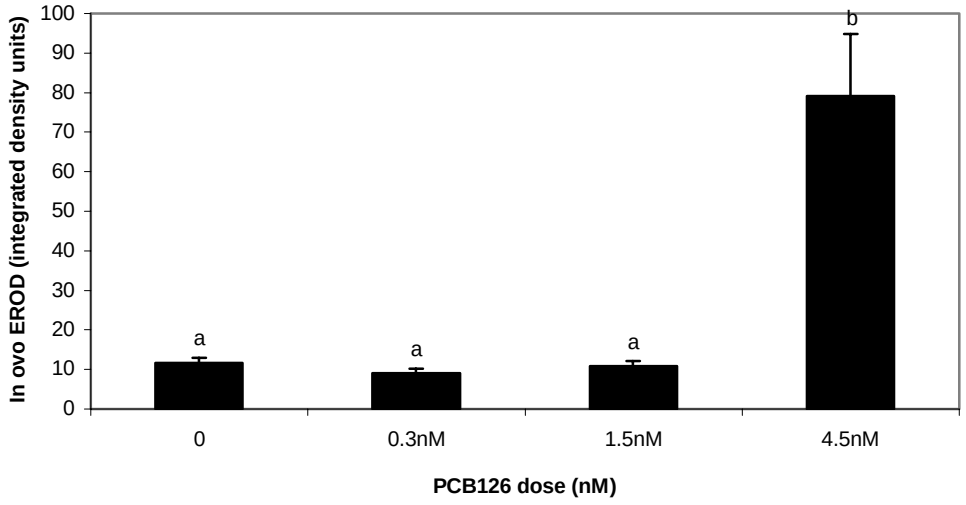


Figure 5-3a. In ovo EROD of killifish embryos treated with 33.5 nM 3-MC.

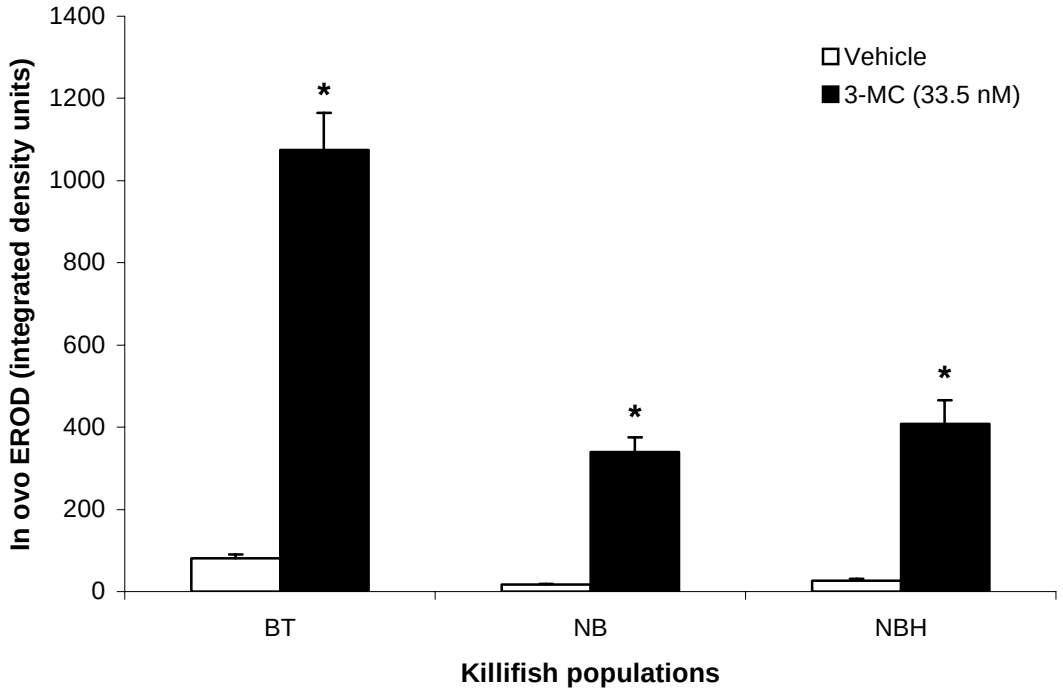


Figure 5-3b. ROS produciton of killifish embryos treated with 33.5 nM 3-MC.

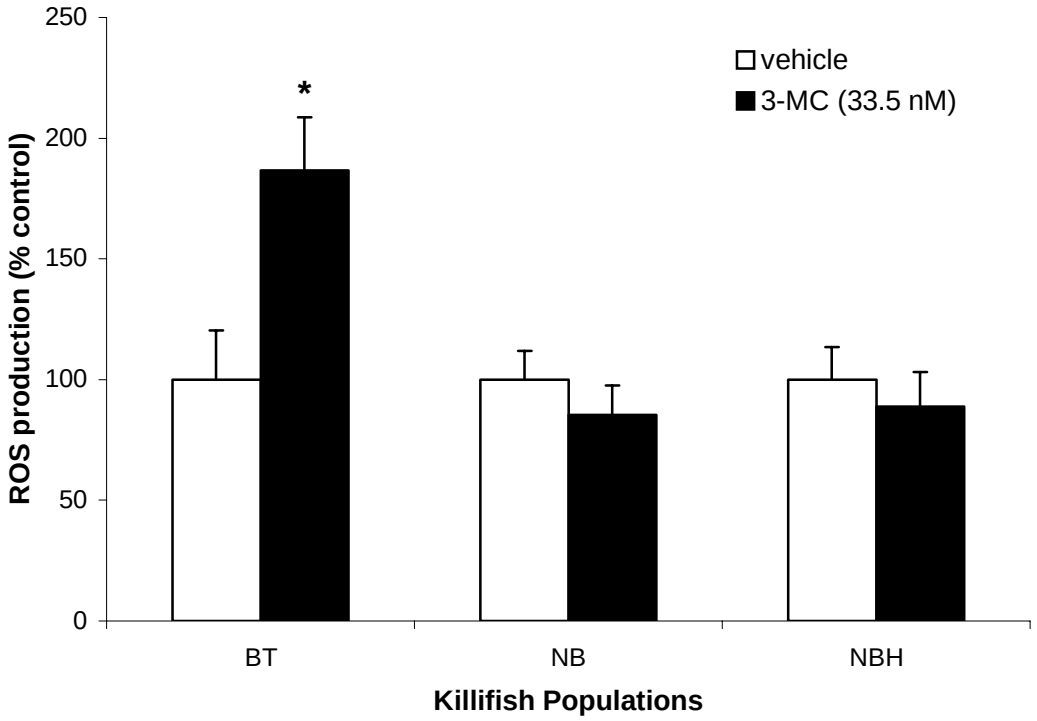


Figure 5-4. In ovo EROD for Flax Pond and Newark Bay killifish embryos treated with 3-MC (0, 1.12, 11.2, and 112 nM)

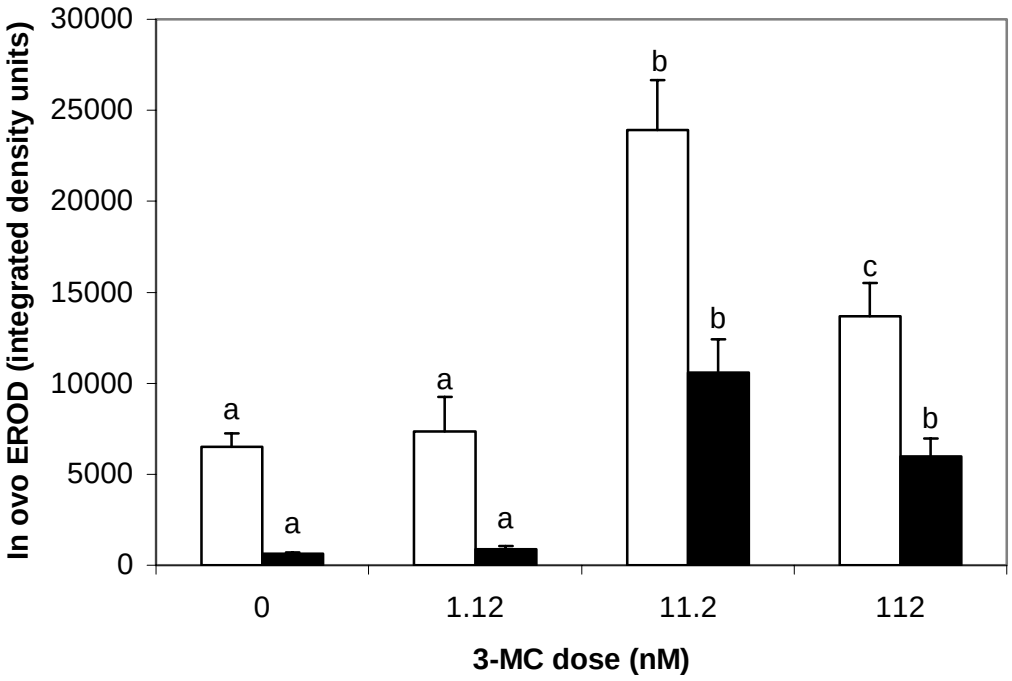


Figure 5-5a. In ovo EROD of killifish embryos treated with vehicle, 1.5 nM PCB126, or 367 nM ANF.

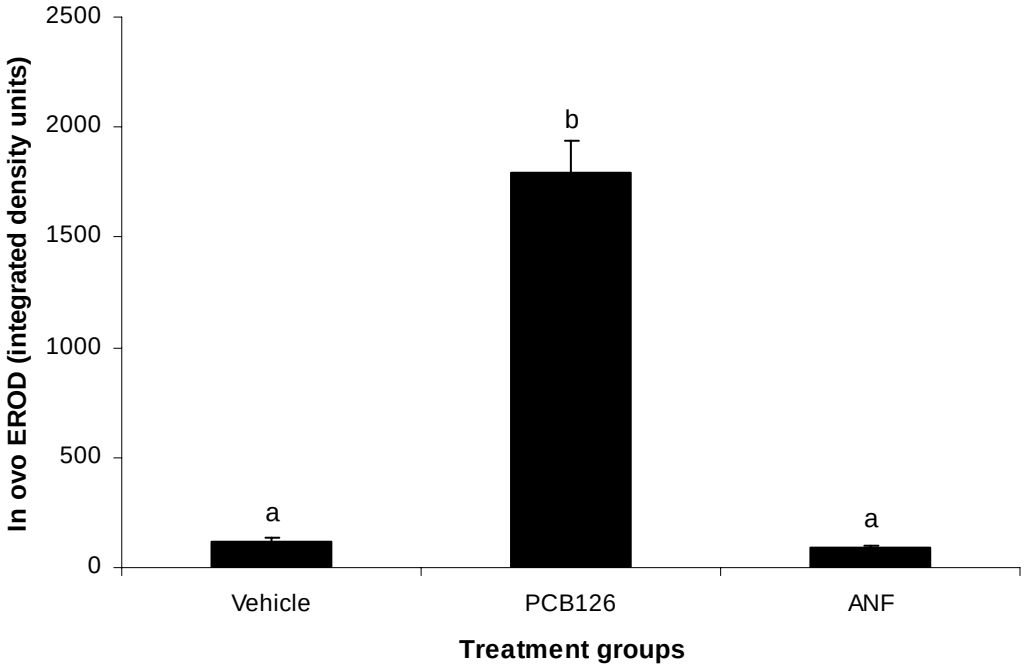


Figure 5-5b. In ovo EROD of killifish embryos treated with vehicle, 1.5 nM PCB126 and PCB126 plus ANF.

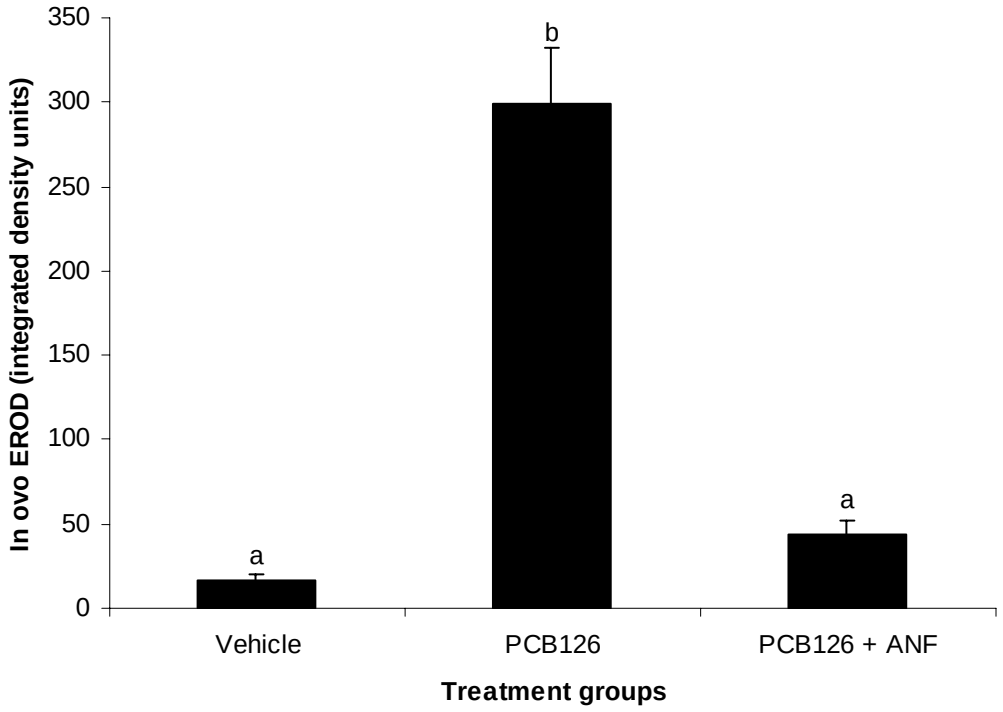


Figure 5-6. Liver ROS production in killifish embryos treated with vehicle, 367 nM ANF, 0.3 nM PCB126 and PCB126 plus ANF.

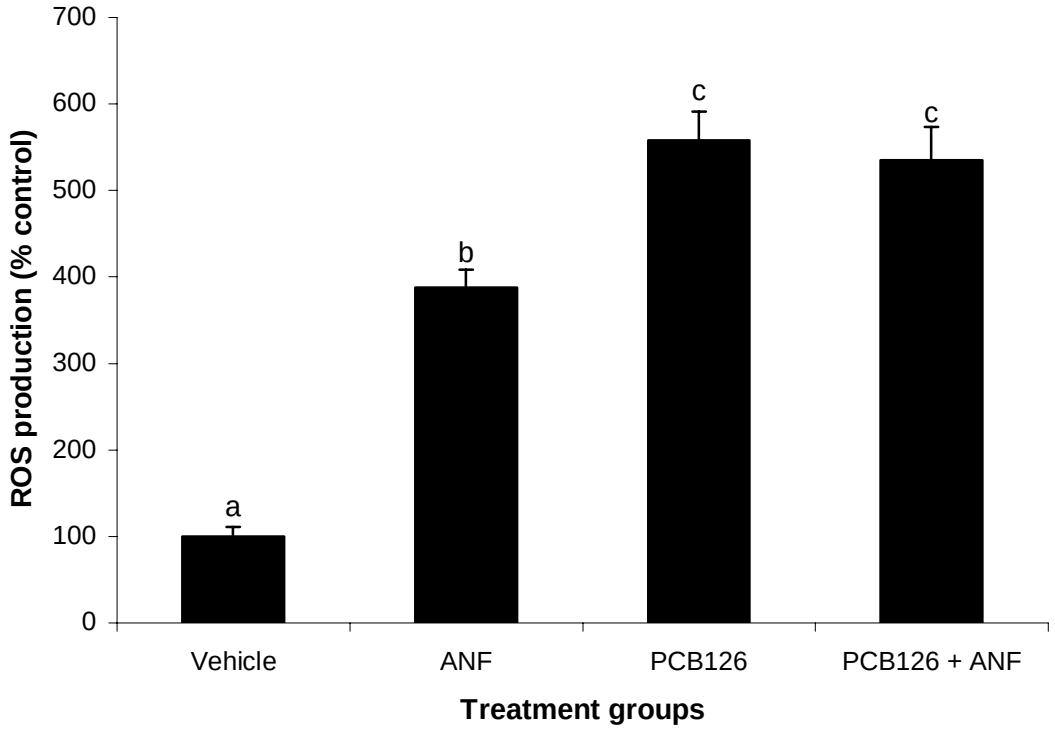


Figure 5-7a. In ovo EROD and of killifish embryos treated with vehicle, 100 μM quercetin (QE), 0.3 nM PCB126 and PCB126 plus QE.

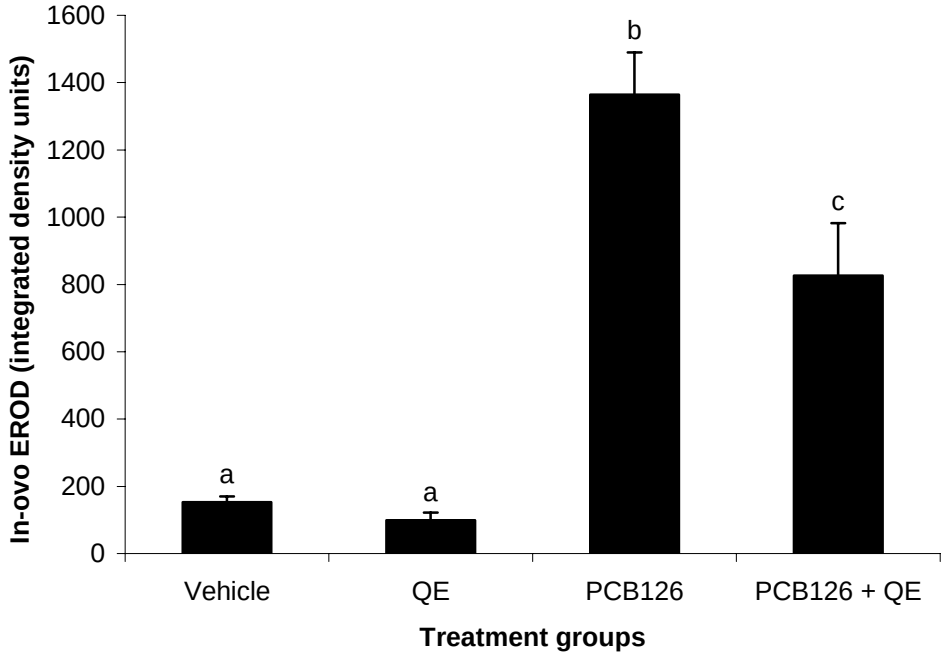


Figure 5-7b. ROS production of killifish embryos treated with vehicle, 100 μM quercetin (QE), 0.3 nM PCB126 and PCB126 plus QE.

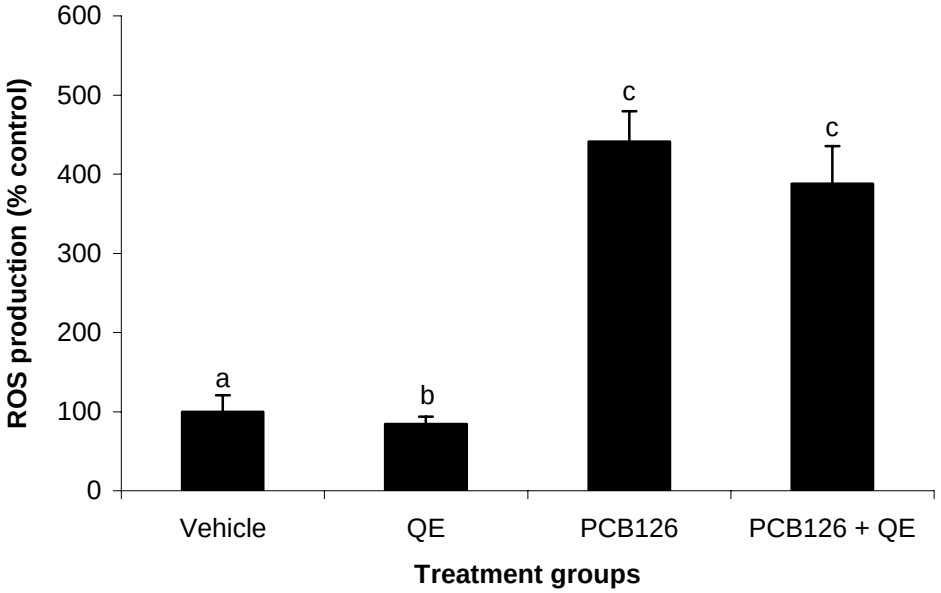


Figure 5-8a. In ovo EROD killifish embryos treated with vehicle, 1 μM piperonyl butoxide (PBO), 0.3 nM PCB126 and PCB126 plus PBO.

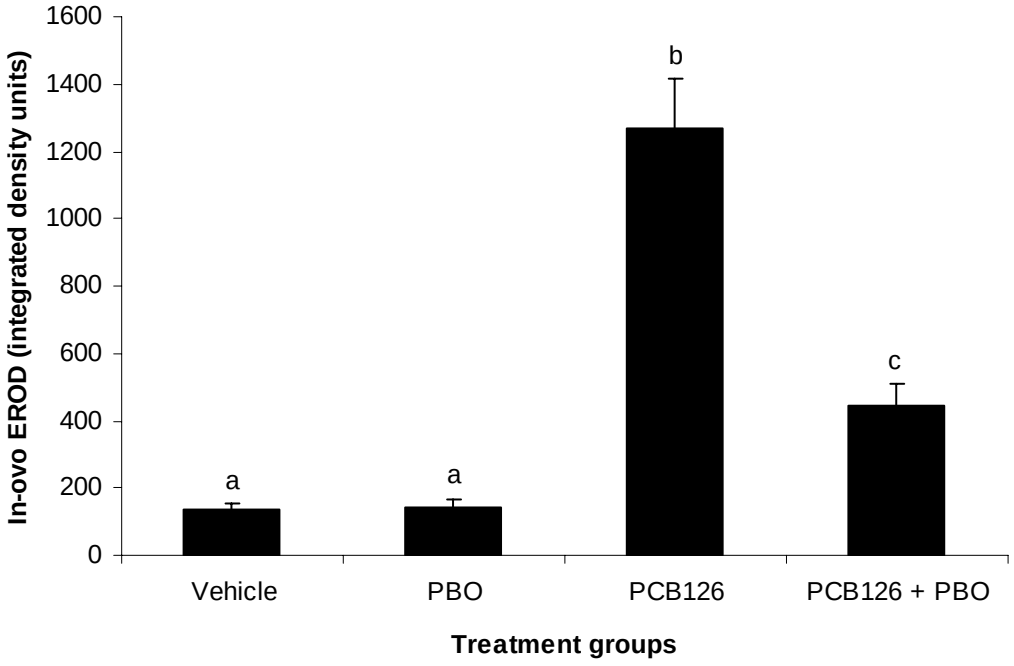


Figure 5-8b. ROS production of killifish embryos treated with vehicle, 1 μM piperonyl butoxide (PBO), 0.3 nM PCB126 and PCB126 plus PBO.

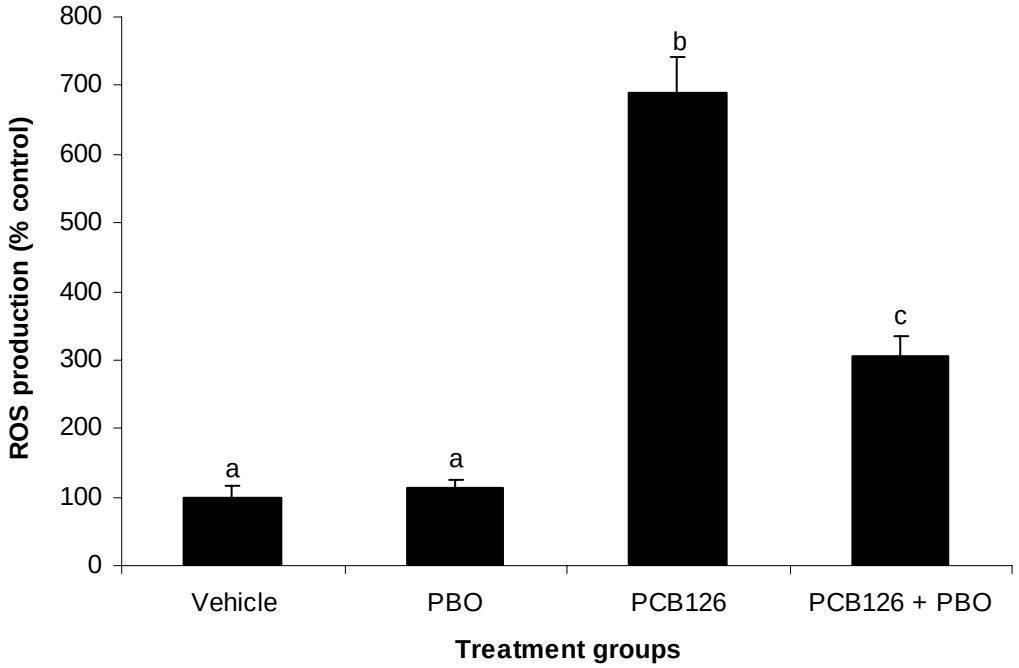


Figure 5-9a. In ovo EROD of killifish embryos treated with vehicle, 200 μM N-acetylcysteine (NAC), 0.3 nM PCB126 and PCB126 plus NAC.

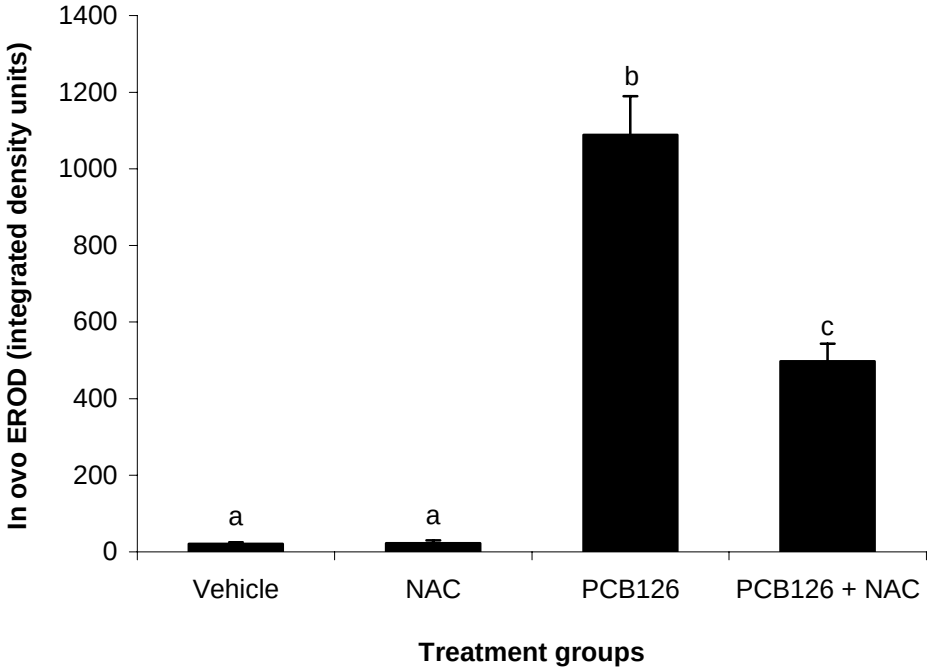
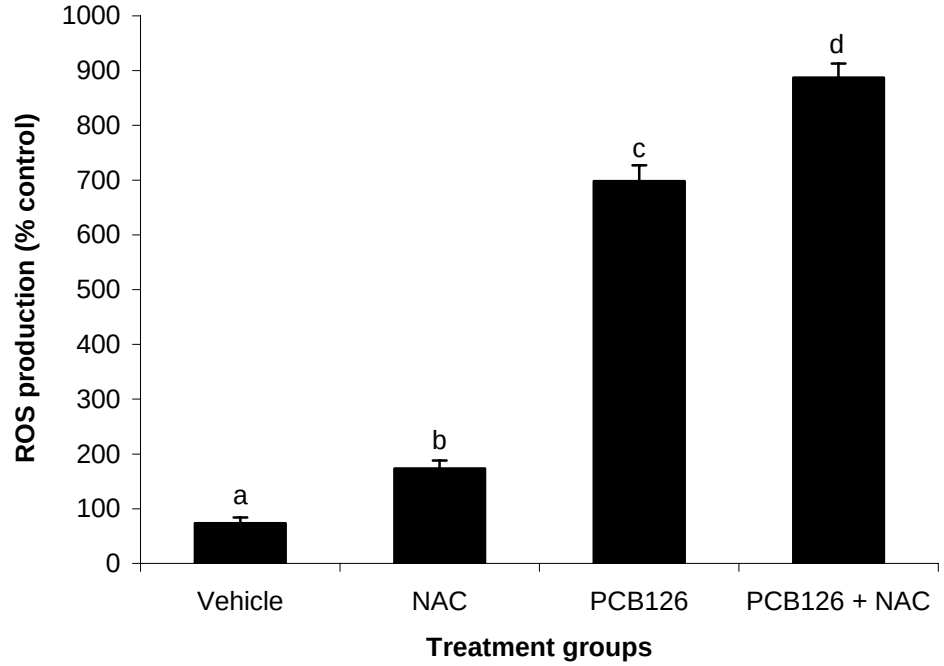


Figure 5-9b. ROS production killifish embryos treated with vehicle, 200 μM N-acetylcysteine (NAC), 0.3 nM PCB126 and PCB126 plus NAC.



Chapter Six

The DNA de-methylating agen 5-azacytidine does not restore CYP1A induction in PCB resistant Newark Bay killifish (*Fundulus heteroclitus*).

Abstract

Newark Bay (NB) killifish (*Fundulus heteroclitus*) have been chronically exposed to environmental contaminants that activate the aryl hydrocarbon receptor (AHR) -and are tolerant to toxic effects and CYP1A induction provoked by AHR ligands. Resistance to *CYP1A* induction could be due to an epigenetic mechanism such as DNA methylation. We measured in-ovo CYP1A catalytic activity (ethoxyresorufin-O-deethylase, EROD) in NB and reference site killifish embryos aqueously exposed to various concentrations of the DNA de-methylating agent 5-azacytidine, 5-AC (5, 50 and 500 μM) with or without 0.2μg/L of the CYP1A inducer 3,3',4,4',5-pentachlorobiphenyl (IUPAC PCB126). Neither PCB126 alone, nor PCB126 plus 5-AC induced EROD above control levels in vehicle treated Newark Bay fish. In reference site fish, the same PCB126 dose provoked a 7.4 fold EROD induction relative to controls. We conclude that Newark Bay killifish are resistant to CYP1A induction by co-planar PCBs during early embryological development and our data suggests that DNA methylation does not play a critical role in resistance to CYP1A induction in this model.

Introduction

Halogenated aromatic hydrocarbons (HAHs) such as dioxin and coplanar PCBs can cause a variety of toxic effects in the fish model *Fundulus heteroclitus.* These chemicals induce the monooxygenase cytochrome P450 1A (CYP1A) through the AHR pathway (Hahn, 1998a; Whitlock, 1999). Chronic exposure to these and other AHR ligands has resulted in resistance to CYP1A induction in a Newark Bay (NB) killifish (*Fundulus heteroclitus*) population (Elskus et al., 1999; Prince and Cooper, 1995a). Although the molecular mechanism that results in this phenotype is not currently known, epigenetic mechanisms such as DNA methylation and histone acetylation can alter induction of CYP1A induction by AHR ligands (Nakajima et al., 2003; Xu et al., 1997).

Cell culture studies have shown that DNA methylation of the CYP1A promoter can reduce its induction by AHR ligands. (Takahashi et al., 1998) demonstrated that in 3-methylcholanthrene (3-MC) resistant rabbit R9ab lung cells, the CpG site contained within the XRE sequence of the CYP1A promoter was highly methylated in both coding and non coding DNA strands. When these cells were treated with the DNA de-methylating agent 5-azacytidine (5-AC), CYP1A became inducible after treatment with 3-MC. A similar mechanism could also be taking place in the NB PCB-resistant killifish population.

Materials and methods

Exposure to test compounds and in ovo CYP1A activity

The same DNA de-methylating agent (5-AC) has been shown to reduce transgene methylation in zebrafish (*Danio rerio*) embryos (Collas, 1998). Based on the 5-AC doses used in that study, we treated Newark Bay killifish embryos with equal and higher concentrations of 5-AC with or without 3,3',4,4',5-pentachlorobiphenyl (PCB126), and measured CYP1A activity (EROD) prior to hatching.

To confirm that NB killifish embryos were resistant to CYP1A induction by AHR ligands, we exposed embryos from both NB and a reference site to PCB126 and measured CYP1A activity *in ovo* using established methods (Nacci et al., 1998). Briefly, NB (PCB-resistant) and Succotash Salt Marsh, SSM (reference site) killifish were individually exposed in 20 mL scintillation vials to 10 ml of seawater (30 ppt) containing the CYP1A substrate 7-ethoxyresorufin (21 μg/L) and either vehicle (acetone, 0.01% final concentration) or PCB126 (0.2μg/L) from day two to day seven post-fertilization at 25°C, 12/12 L/D. On day seven, the exposure water was changed to fresh seawater. This PCB126 dose has been previously shown to cause maximal induction of CYP1A activity in reference site animals (Nacci et al., 1999). CYP1A activity was measured on day ten post-fertilization. CYP1A induction is evaluated based on the accumulation of the metabolite resorufin (rr) in the animal's bladder relative to vehicle controls (Nacci et al., 1998).

Statistical analysis

Statistical analysis was performed using SYSTAT version 10 (Systat software Inc., Point Richmond CA). Two-way ANOVA followed by Fishers Least Significant Difference (LSD) was used to detect statistical differences between treatments and fish populations (Flax Pond and Newark Bay). One way (ANOVA) followed by LSD test was used for analysis of in ovo EROD integrated density values in experiments where Newark Bay killifish were exposed to PCB126 alone, or in combination with 5-AC. Level of significance was 0.05.

Results and Discussion

In reference site killifish treatment with PCB126 provoked a significant increase in CYP1A activity, but in NB fish treatment with PCB126 did not (Figure 1), demonstrating that the former are resistant to CYP1A induction by co-planar PCBs during embryological development.

To determine if DNA methylation is the molecular mechanism involved in suppressing CYP1A expression in resistant fish, NB killifish embryos were exposed to 5-AC (5, 50, and 500 μ(micro)M) with and without co-exposure to PCB126 (0.2 μg/L).

Treatment with PCB126 in combination with 5-AC did not relieve resistance to EROD induction by PCBs in NB killifish (Figure 2). This suggests that resistance to CYP1A induction may not be due to DNA methylation in this fish population. Yet future experiments should measure additional endpoints to assure that 5-AC reduces genomic DNA methylation in this fish model. After treating embryos with 5-AC, DNA methylation of the CYP1A gene promoter could be measured by bisulfate sequencing. Additional studies could also include the use of other agents that can alter the DNA methylation status in fish embryos. For example, sodium butyrate has also been shown to reduce transgene methylation in zebrafish embryos (Collas, 1998)

Figure legends:

Figure 6-1: CYP1A catalytic activity in Newark Bay and reference site (SSM) killifish embryos exposed to vehicle or PCB126. CYP1A induction is presented as the intensity of the resorufin (rr) signal emitted by the animal's bladder (excitation: 533-588mn; emission: 608-683nm), normalized to the signal emitted under ultraviolet (uv) light (excitation: 340-380mn; emission: 435-485nm). Photographic images were quantified using Scion Image software (http://www.scioncorp.com). Bars represent the mean and S.D. n = 20 individuals per treatment. * Significantly different from respective controls.

Figure 6-2: CYP1A catalytic activity in Newark Bay killifish embryos treated with PCB126 alone, or PCB126 plus 5-AC at different concentrations. Bars represent the mean ± S.D. n = 5 individuals per treatment group.

Figure 6-1. CYP1A catalytic activity in Newark Bay and reference site killifish embryos exposed to vehicle or PCB126.

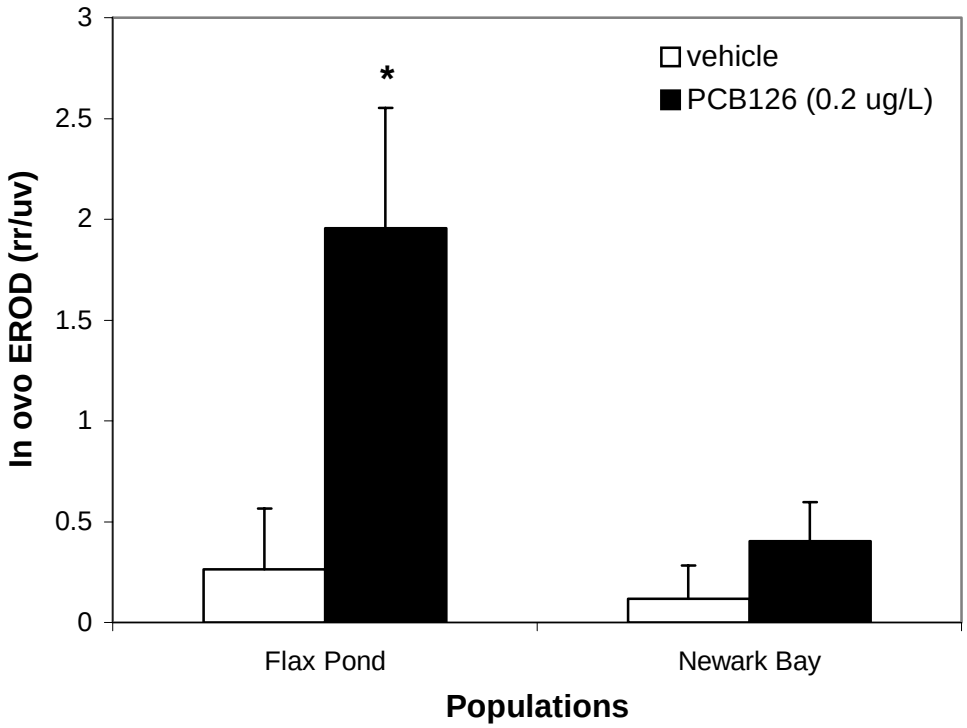
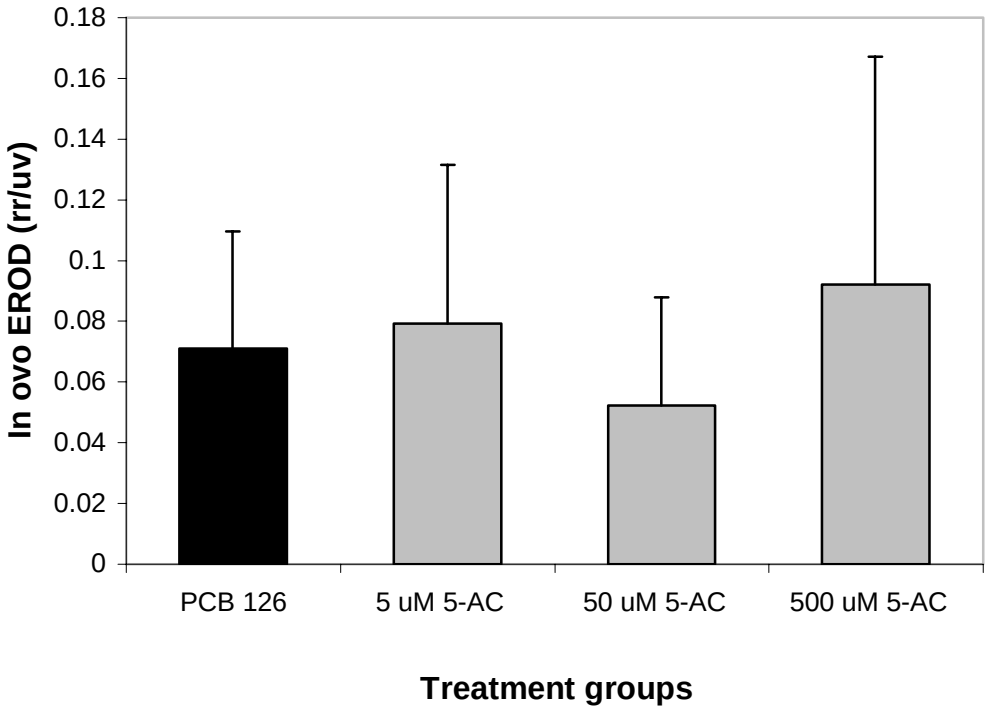


Figure 6-2. CYP1A catalytic activity in Newark Bay killifish embryos treated with PCB126 alone, or PCB126 plus 5-AC at different concentrations.



Chapter Seven

Cloning, sequence and methylation analysis of the CYP1A promoter region from polluted and reference site killifish (*Fundulus heteroclitus*).

Abstract

Xenobiotic-carcinogenic compounds such as coplanar polychlorinated biphenyls (PCBs) and polycyclic aromatic hydrocarbons (PAHs) induce xenobiotic metabolizing enzymes that form part of the aryl hydrocarbon receptor (AHR) gene battery. One of these enzymes is Cytochrome P450 1A (CYP1A). Various killifish (*Fundulus heteroclitus*) populations chronically exposed to these compounds have developed resistance to their toxic effects and induction of CYP1A. The molecular and biochemical mechanisms for resistance in killifish are not known yet. Cell culture studies have shown that methylation if the CYP1A promoter can block AHR dependent transcription. Variations in the sequence of regulatory regions and DNA methylation patterns of the *CYP1A* promoter could result in alteration of gene expression between resistant and responsive fish populations. Chronic exposure to these compounds may have resulted in survival of those animals that have a low activity promoter. Using a PCR approach we amplified a ~1.6 Kb portion of the *CYP1A* promoter of fish from contaminated (Newark Bay NJ, and New Bedford Harbor MA) and reference sites (Flax Pond NY, and Scorton Creek MA). Sequence analysis of the CYP1A promoter regions has revealed DNA binding motifs for the following transcription factors: AHR, AP-1, GRE, SP-1, and TATA box in all three populations. We also noted the presence of additional DNA fragments in the 5' region of the sequences we analyzed. The frequency of these fragments was studied using a PCR approach. Primers were designed for conserved regions of the promoter flanking the sites where these additional fragments are present. Depending on the presence or absence of these fragments, the size of the resulting PCR products were estimated to be 685, or 728 bp long. Our results suggest that the additional DNA fragments were more frequent in the reference site population when compared to contaminated site killifish. It is possible that the different distribution of these fragments may have been the result of selective pressure imposed by contaminant exposure. DNA methylation studies of the *CYP1A*

promoter region were carried out using the methylation sensitive restriction enzyme HpaII. There were no significant differences in the methylation status of reference and contaminated site killifish *CYP1A* promoters. Further experiments should focus on determining of the variable sequences in the upstream region of the CYP1A promoter are in fact functional.

Introduction

Coplanar plychlorinated biphenys (PCBs) and polycyclic aromatic hydrocarbons (PAHs) are commonly found in contaminated aquatic environments (O'Connor, 1988). These chemicals are toxic and exposure can cause teratogenesis, oxidative stress, altered expression of xenobiotic metabolizing enzymes, and cancer (Baumann, 1998; Faroon et al., 2001; Palace et al., 1996). Among vertebrates, various fish species have been shown to be vulnerable to these chemicals (Carlson et al., 2002; Dong et al., 2001; James et al., 2004; Toomey et al., 2001).

In vertebrates, most the toxic effects caused by PAHs and PCBs are mediated through the Aryl Hydrocarbon receptor (AHR). The AHR is a member of the basic helix-loop-helix/Per-ARNT-Sim (bHLH-PAS) family of proteins. In the absence of ligand the AHR remains in the cytosol in a stable complex with two heat chock proteins (HSP 90), X-associated protein (or Ara9) and the HSP90 co-chaperone p23 (Carlson and Perdew, 2002; Safe, 2001). Upon ligand binding, the AHR translocates to the nucleus where it forms a complex with its DNA binding partner, the AHR nuclear translocator (ARNT). The AHR-ARNT heterodimer recognizes specific DNA sequences known as the xenobiotic response elements (XREs), which are found in the enhancer region of target genes (Swanson, 2002; Whitlock, 1999). The AHR has been identified, cloned and sequenced, in various vertebrates including fish (Abnet et al., 1999; Hahn, 1998a; Hahn et al., 1994)

Killifish from various contaminated sites in the eastern coast of the United States have been shown to be resistant to the acute toxic effects caused by exposure to PAHs and PCBs (Weis, 2002). These sites are New Bedford Harbor (MA), which is heavily contaminated with PCBs; a portion of the Elizabeth River (VA) that is mostly contaminated with PAHs, and Newark Bay, where, among many kinds of contaminants, both PAHs PCBs and dioxins are present (O'Connor, 1988; Wenning, 1994). Killifish

from these sites display altered expression of phase I and phase II enzymes and are recalcitrant to CYP1A induction upon exposure to AHR ligands (Meyer et al., 2003; Nacci et al., 2002; Weis, 2002). Studies by different groups have demonstrated that this phenomenon is heritable in some populations, but not in others. Lab raised first generation and second-generation killifish from New Bedford Harbor and first generation killifish larvae from Newark Bay are resistant to CYP1A induction (Elskus et al., 1999; Nacci et al., 1999). This suggests that in these two populations resistance to CYP1A induction is heritable. But lab raised, second generation Elizabeth River killifish loose their resistance to CYP1A induction and their response is similar to reference (clean site) animals (Meyer et al., 2002). This suggests that resistance to CYP1A induction in killifish populations with different exposure scenarios arises through more than one mechanism. It is possible that inherited resistance to CYP1A induction in New Bedford Harbor and Newark Bay killifish is due to selection of a low activity CYP1A promoter.

In an effort to elucidate the mechanism for resistance to CYP1A induction in polluted site killifish, we cloned and analyzed the DNA sequence of the CYP1A promoter of reference (Flax Pond, NY and Scorton Creek, MA) and polluted site (Newark Bay, NY and New Bedford Harbor, MA) killifish. Our objectives were to determine if polluted site killifish have an altered CYP1A promoter in comparison to reference site animals. Our results suggest that there are sequence variations in the CYP1A promoter. Differential distribution of these variable DNA fragments in the CYP1A promoter of reference and polluted site killifish populations is also reported. More studies are required in order to determine the function of these sequences.

Materials and methods

Materials:

The DNA polymeases and restriction enzymes used in this study were: Vent DNA polymerase (New England Biolabs, Beverly MA), Pfu DNA polymerase (Stratagene, La Jolla CA), Amplitaq gold (Applied Biosystems, Foster City CA), and HpaII and MspI restriction enzymes from New England Biolabs (Beverly, MA). Pfu cloning kit was obtained from and Stratagene (La Jolla, CA.). Agarose gel DNA extraction, and plasmid

DNA midi prep kits were obtained from obtained from Quiagen (Valencia, CA). Primers used for DNA reactions were obtained from Sigma Genosys (Haverhill, United Kingdom). The PCR DNA ladder used for determination of PCR product size was the PCR DNA marker (Mid West Scientific, St Louis MO).

Killifish collection and maintenance

Killifish (*Fundulus heteroclitus*) were collected from four populations with different responses to CYP1A inducers. Resistant animals were obtained from Newark Bay (NJ), and New Bedford Harbor (MA), and responsive animals were obtained from Scorton Creek (MA) and Flax Pond (NY). Minnow traps were used to capture killifish. Fish were maintained in 10 or 20-gallon recirculating tanks equipped with charcoal Whisper^{TM} filters, in 30 ppt artificial seawater.

Amplification of the killifish CYP1A1 promoter:

DNA extraction: DNA was extracted from liver or gonad tissue using conventional Phenol:chloroform extraction. DNA purity and content was analyzed spectrophotometrically. For each DNA amplification reaction 100 ng of DNA was used.

PCR reactions:

Forward and reverse primers were designed from a previously cloned killifish *CYP1A* promoter (Powell et al., 2004).

Forward Primer 1: 5'-GGA CCA TCA ATT TCC ACA TGA AGT TTG-3'

Forward Primer 2: 5'-GTT ATA GCC ACA GCT CAG TCA TTT TCT CC-3'

Reverse Primer 1: 5'-AGC ACA CCG TCA CCA AGG CTA AAC C-3'

Reverse Primer 2: 5'-CAG ACA CCG AGA GTG CTC CAA TGA ATG G-3'

The expected size of the PCR product is 2.5Kb.

We used either Vent DNA Polymerase (New England Biolabs), or Pfu DNA Polymerase (Stratagene) for PCR reactions. Both enzymes have proofreading exonuclease activity. DNA polymerase and buffer solutions for PCB were used

according to the manufacturer instructions. Conditions for the PCR were: 95 °C (1 minute) 55 °C (1 minute) and 72 °C (2 minutes and 30 seconds) for a total of 32 cycles.

Cloning and sequencing: PCR products were separated on 1% agarose gels, and products purified using a QIAquick Gel Extraction following manufacturer instructions. Purified PCR products were cloned in a pPCR-Script™ Amp SK(+) Cloning Vector using a PCR Script Amp Cloning Kit and following manufacturer instructions. Transformed colonies containing inserts were grown on LB media containing 10mg/ml ampicillin (Amp). Plasmids were isolated using the Qiagen plasmid midi kit following instructions from manufacturer. Purified plasmids were sent to for sequencing at the University of Maine DNA Sequencing Facility.

Sequence analysis: Obtained sequences were analyzed using the GeneDoc (Nicholas, 1997) and Transcription Element Search System, or TESS (Petsko, 2002; Schug and Overton, 1997) software packages. Genedoc is a free software package that can be used for sequence visualization and analysis (download from: www.psc.edu/biomed/ genedoc/). Unfortunately, like most free things, it is not very powerful tool. For example: alignment of three or more sequences is difficult to perform. TESS is a web based search engine that identifies potential transcription factor binding sites in submitted DNA sequences using consensus strings found in the TRANSFAC, IMD, and CBIL-GibbsMat databases (found at: www.cbil.upenn.edu/tess/).

Determination of frequency of DNA sequence inserts in the killifish CYP1A promoter:

PCR reaction:

For amplification of the 5' end of the promoter sequence, the following set of primers were designed using Primer 3 (Rozen and Skaletsky, 2000):

Forward primer: 5'-CCA CAG TCC AGT CAT TTT CTC C-3'

Reverse primer: 5'-CAA GGG CAG ACG AGT TCA TT-3'

For these reactions we used the DNA polymerase Aplitaq-gold (Applied Biosystems) and provided buffer solutions according to the manufacturer's instructions. Conditions for PCR were: 95 °C (8 minutes), the 42 cycles of: 94 °C (30 seconds), 55 °C (30 sec.), 72 °C (60 sec.), followed by 72 °C (7 min) and 4 °C indefinitely. The products

were separated in 2% agarose gels, and band sizes were determined by using a DNA ladder (PCR DNA marker™, Mid West Scientific).

CYP 1A promoter methylation study:

Restriction enzyme digestions:

We used a protocol similar to one previously described (Hammons et al., 2001). There are two HpaII sites (5'-CCGG-3') flanking two of the XRE sites on the killifish *CYP1A* promoter (Figure 1). HpaII is a methylation sensitive enzyme, which means that it cannot digest its recognition site if it is methylated. MspI recognizes the same sites that HpaII cuts, but is methylation insensitive so it will be able to digest the site regardless of the methylation status. Here, MspI is used as a positive control to make sure that the restriction digestion takes place. Genomic DNA (500ng) from each extract was subjected to one of the following treatments for a period of sixteen hours: HpaII digestion, MspI digestion, and digestion buffer but no enzyme. Restriction digestion of genomic DNA was followed by PCR to amplify the region containing the restriction sites. We used 100 ng of DNA from each individual digestion for PCR using the following primers:

Forward Primer: 5'-AGT TTG GTG CGC TCA TTG TT-3'

Reverse Primer: 5'-GAC ATC AGG CAG ACG TTC AA-3'

Conditions for PCR were: 95 °C (8 minutes), the 42 cycles of: 94 °C (30 seconds), 55 °C (30 seconds), 72 °C (1 minute), followed by 72 °C (7 minute) and 4 °C indefinitely. The primers were designed using Primer 3 (Rozen and Skaletsky, 2000). The expected size of the PCR product is 403bp. If there is methylation, HpaII will not cut it's recognition site and a PCR product should be obtained, but if there is no methylation, HpaII cuts its recognition site and no PCR product is obtained because the DNA template is fragmented. Amplification of DNA digested with MspI should not generate a PCR product unless conditions are not optimal for this reaction to be completed. Amplification of DNA incubated with restriction buffer, but no enzyme is expected to generate a PCR product unless PCR conditions are not optimal. These last two digestions with MspI, or no enzyme are used as controls.

Statistical analysis:

Statistical analysis was performed using SYSTAT version 10 (Systat software Inc., Point Richmond CA). Chi-square statistic was used for analyzing the methylation status of Newark Bay and Flax pond killifish CYP1A promoters.

Results and discussion

Sequence analysis of the killifish CYP1A promoter

We were able to clone and sequence the CYP1A promoter region of two Newark Bay, two Flax Pond, one New Bedford Harbor and one Scorton Creek killifish. The killifish CYP1A promoter sequences were analyzed using TESS (Transcription Element Search Software). We also compared our sequences to another one that was previously reported (Powell et al., 2004). Analysis CYP1A promoter sequence revealed presence of three XREs, a TATA box and putative DNA motifs for glucocorticoid response element (GRE), SP1, and AP-1 (Figure 1 and Table 1). There were no differences in the number of XREs between the analyzed promoters.

When comparing CYP1A promoter sequences of fish from different populations, we noted variations consisting of short (16 to 35 bp) DNA fragments that were present in some promoters, but not in others. One of these segments contained a GRE site (Figure 1), which was present in one of the Newark Bay, the New Bedford Harbor, and Scorton Creek sequences, but absent in the Flax Pond and remaining Newark Bay promoters. It is possible that these fragments may contain DNA motifs that alter CYP1A induction, and exposure to AHR ligands has resulted in selection of lower activity CYP1A promoters in contaminated sites. Studies by other groups have shown that differential expression of the lactate dehydrogenase (Ldh-B) gene in killifish from northern and southern populations are due to altered promoter sequences and distribution of alleles of this gene. Their results demonstrate that Ldh-B promoters with higher transcriptional activity occur more frequently in northern populations (Schulte et al., 1997; Segal et al., 1996). To determine if there are differences in the distribution of the CYP1A promoter fragments between PCB resistant, and reference site animals, this region was analyzed using a PCR approach. Primers that amplify the variable region were designed, and depending on whether the fragments are present or not, the size of the PCR products would be 685, or

728 bp long (Figure 2). Sixteen killifish from Flax Pond and nineteen killifish from Newark Bay were checked for the presence of additional DNA segments. The frequency of the larger PCR products was higher in Flax Pond when compared to Newark Bay killifish. Out of the sixteen Flax Pond animals, four animals generated the larger (~728bp) PCR products. All of the Newark Bay killifish generated the smaller (~685bp) product (Table 2). The additional DNA fragments occured less frequently in the Newark Bay when compared to the Flax pond promoter region.

In order to determine if the additional DNA fragments in the killifish CYP1A promoter are functional, and if their distribution is significantly altered in contaminated sites, a more stringent approach is necessary. The different CYP1A promoters could be cloned in to vectors, upstream of a reporter gene, and transfected in to immortalized or primary cells. If the fragments are indeed functional, then induction of the reporter gene after exposure to AHR ligands should vary according to the presence or absence of these sequences. This approach would determine if the presence of these sequences alters CYP1A induction response to AHR ligands. Future studies should involve additional sites to rule out that CYP1A promoter differences are not geographical, but that they arose as a result of selective pressure imposed by AHR ligand exposure. Ideally, these experiments should involve sequencing these variable regions, instead of the PCR approach used here. This would determine if the additional DNA fragments sequences are conserved among the promoters containing them, and potentially discover additional regulatory motifs.

DNA methylation status of the killifish CYP1A promoter

Studies using cell culture models demonstrate that one potential mechanism for acquiring resistance to CYP1A induction by AHR ligands is through methylation of the CYP1A1 promoter region (Jana et al., 2000; Takahashi et al., 1998). To determine if a similar mechanism is taking place in Newark Bay killifish, hepatic DNA was extracted from liver tissue of Newark Bay and Flax Pond killifish and the methylation status of the CYP1A promoter was studied using the restriction enzymes HpaII and MspI. The killifish CYP1A promoter has two HpaII/MspI restriction sites. As mentioned above HpaII is methylation sensitive and is used to detect methylation status, and MspI is

methylation insensitive and is used as a control. This analysis was originally performed on eleven animals from Flax Pond and eleven animals from Newark Bay. MspI was able to fully digest DNA from four Flax pond animals, and seven Newark Bay killifish. PCR products were obtained from the remaining samples digested with MspI, suggesting that the conditions of the restriction digestions were not optimal in these cases. Only data from the full MspI digestions is presented on Table 3 and Figure 3. One out of four Flax Pond killifish had methylated HpaII sites in the CYP1A promoter and two out of six Newark Bay killifish had methylated HpaII sites in the CYP1A promoter. Additional experiments are required to determine the optimal conditions for the DNA restriction digestions in Newark Bay and Flax Pond killifish. An alternative to the use of methylation sensitive restriction enzymes is to perform bisulfate sequencing. This method is more powerful would yield more information on the methylation status of the killifish CYP1A promoter since it analyzes complete sequence of regions containing XREs. It is also a more expensive technique.

Overall these results suggest that there are there are no differences in the number of XREs in the CYP1A promoters of reference and polluted site killifish and that variations in the 5' end of the CYP1A promoter were more frequent in the reference site animals. It is possible that these additional sequence fragments have an effect on CYP1A induction and their altered distribution in reference and contaminated site killifish is a result of selective pressure imposed by contaminant exposure.

Figure Legends

Figure 7-1: Alignment of killifish CYP1A promoter sequences from contaminated (Newark Bay NJ) and reference (Flax Pond NY) site individuals. Motifs for XREs TATA box, GRE, Sp1 and AP-1 labeled bellow the consensus sequence. The transcriptional start site is labeled bellow as Exxon 1. Approximately 1.6 Kb of the killifish CYP1A promoter is presented.

Figure 7-2: Additional DNA fragments in the killifish CYP1A promoter results in 728, or 685bp PCR products. PCR products were separated in 2% agarose gels and stained with ethidium bromide. Agarose gel contents are DNA molecular weight markers (lanes 1 and 7), PCR products of individual Flax Pond killifish CYP1A promoter (lanes 2, 3, 4, , 10, 11, and 12), and PCR products of individual Newark Bay killifish CYP1A promoters (lanes 6, 8, and 9). The size of the molecular weight markers (bp) is presented on the left side of the gel.

Figure 7-3: Ethidium bromide-stained agarose gels of PCR products derived from HpaII and MspI restriction digestions of hepatic DNA. Gel a) representative Flax Pond sample and b) representative Newark Bay sample. Hepatic DNA was isolated and subjected to DNA restriction digestion with HpaII, MspI or no enzyme. Restriction digestion was followed by PCR for amplification of the CYP1A promoter area containing the methylation sensitive sites. On gels a and b lanes correspond to PCR DNA ladder (lane 1), HpaII digestion (lane 2) MspI digestion (lane 3) and DNA incubation with no restriction enzymes (lane 4). The size of the molecular weight markers (bp) is presented on the left side of the gel.

Table 7-1: DNA motifs identified on the killifish CYP1A promoter using TESS (Petsko, 2002).

Motif	Sequence	Reference
XRE	5'-CACGCNA-3'	(Powell et al., 2004; Saatcioglu et al., 1990)
SP1	5'-CCCCTCCC-3'	(Lee et al., 1994; Ohtaka-Maruyama et al., 1998)
SP1	5'-GGGCGG-3'	(Segal et al., 1999)
AP-1	5'-TGATGTCA-3'	(Cockerill et al., 1995)
GRE	5'-TGTACA-3'	

DNA motifs recognized by transcription factors are presented along with references found in databases used by TESS.

Table 7-2: Additional DNA fragments in Flax Pond and Newark Bay killifish CYP1A promoters is reflected in variable size PCR products.

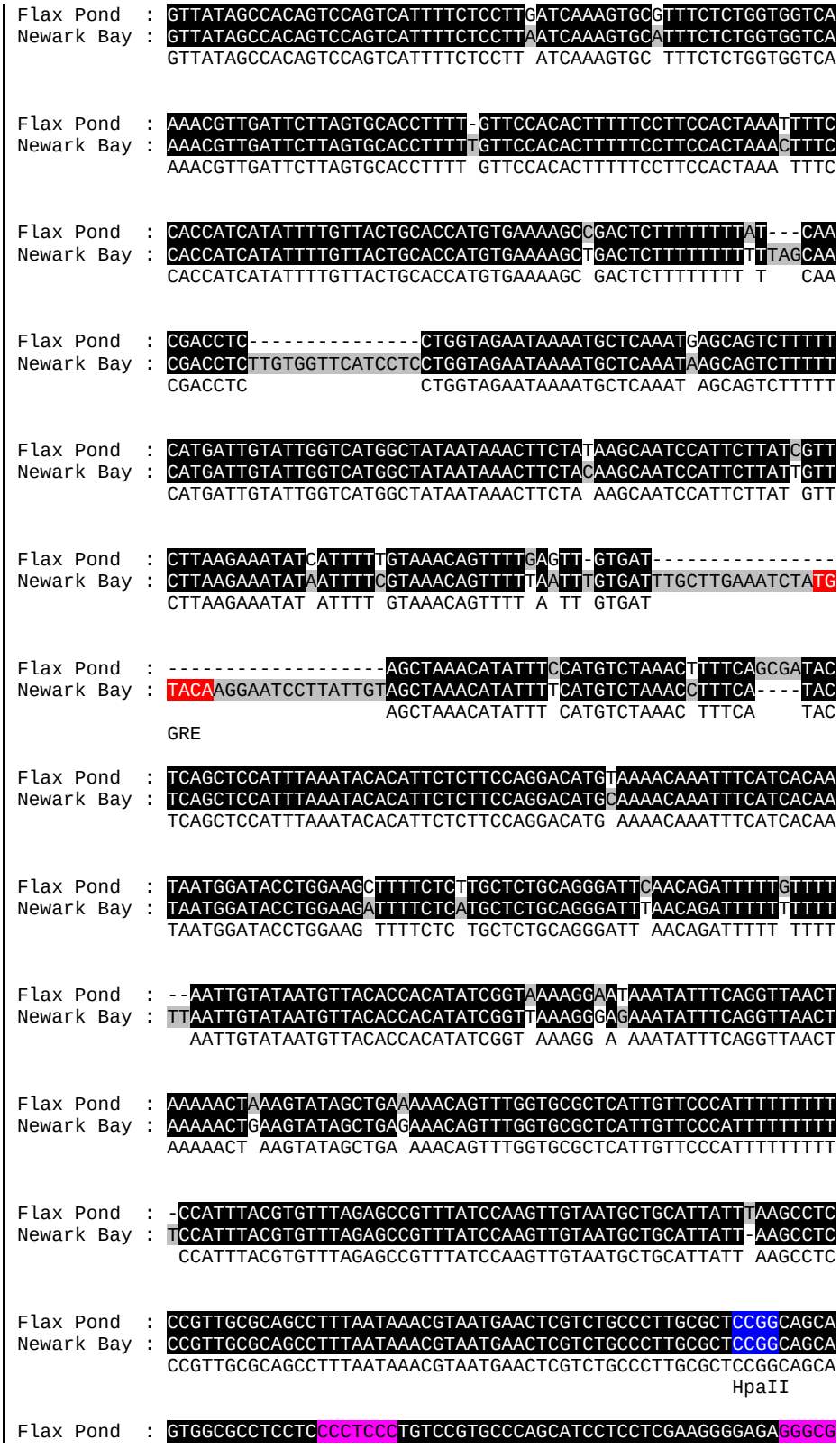
	728pb PCR product	685pb PCR product	Total
Flax Pond NY	4	12	16
Newark Bay NJ	0	19	19

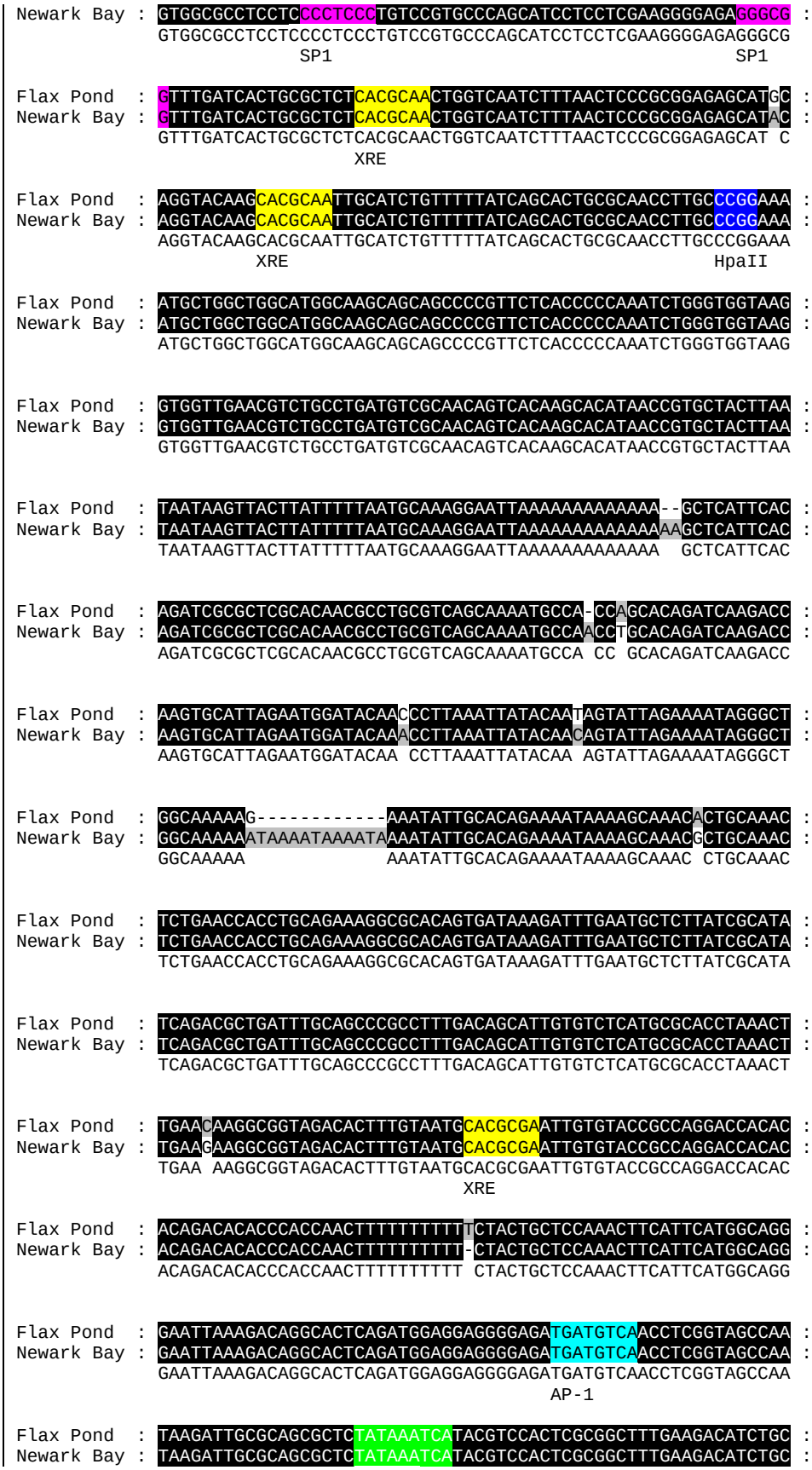
Table 7-3: DNA methylation status of the Flax Pond and Newark Bay killifish promoters

	Methylated	Non-methylated	Total
Flax Pond NY	1	4	5
Newark Bay NJ	2	6	8

There were no significant differences in the DNA methylation status between Flax Pond Newark Bay killifish CYP1A promoters. χ^2_c=0.043, P>0.05

Figure 7-1. Alignment of killifish CYP1A promoter sequences from contaminated (Newark Bay NJ) and reference site fish.




Newark Bay : GTGGCGCCTCCTCCCCTCCCTGTCCGTGCCCAGCATCCTCCTCGAAGGGGAGAGGGCG :
GTGGCGCCTCCTCCCCTCCCTGTCCGTGCCCAGCATCCTCCTCGAAGGGGAGAGGGCG
SP1
SP1
Flax Pond : GTTTGATCACTGCGCTCTCACGCAACTGGTCAATCTTTAACTCCCGCGGAGAGCATGC :
Newark Bay : GTTTGATCACTGCGCTCTCACGCAACTGGTCAATCTTTAACTCCCGCGGAGAGCATAC :
GTTTGATCACTGCGCTCTCACGCAACTGGTCAATCTTTAACTCCCGCGGAGAGCAT C
XRE
Flax Pond : AGGTACAAGCACGCAATTGCATCTGTTTTTATCAGCACTGCGCAACCTTGCCCGGAAA :
Newark Bay : AGGTACAAGCACGCAATTGCATCTGTTTTTATCAGCACTGCGCAACCTTGCCCGGAAA :
AGGTACAAGCACGCAATTGCATCTGTTTTTATCAGCACTGCGCAACCTTGCCCGGAAA
XRE
HpaII
Flax Pond : ATGCTGGCTGGCATGGCAAGCAGCAGCCCCGTTCTCACCCCCAAATCTGGGTGGTAAG :
Newark Bay : ATGCTGGCTGGCATGGCAAGCAGCAGCCCCGTTCTCACCCCCAAATCTGGGTGGTAAG :
ATGCTGGCTGGCATGGCAAGCAGCAGCCCCGTTCTCACCCCCAAATCTGGGTGGTAAG
Flax Pond : GTGGTTGAACGTCTGCCTGATGTCGCAACAGTCACAAGCACATAACCGTGCTACTTAA :
Newark Bay : GTGGTTGAACGTCTGCCTGATGTCGCAACAGTCACAAGCACATAACCGTGCTACTTAA :
GTGGTTGAACGTCTGCCTGATGTCGCAACAGTCACAAGCACATAACCGTGCTACTTAA
Flax Pond : TAATAAGTTACTTATTTTTAATGCAAAGGAATTAAAAAAAAAAAAAA--GCTCATTCAC :
Newark Bay : TAATAAGTTACTTATTTTTAATGCAAAGGAATTAAAAAAAAAAAAAAAGCTCATTCAC :
TAATAAGTTACTTATTTTTAATGCAAAGGAATTAAAAAAAAAAAAA GCTCATTCAC
Flax Pond : AGATCGCGCTCGCACAACGCCTGCGTCAGCAAAATGCCA-CCAGCACAGATCAAGACC :
Newark Bay : AGATCGCGCTCGCACAACGCCTGCGTCAGCAAAATGCCAACCTGCACAGATCAAGACC :
AGATCGCGCTCGCACAACGCCTGCGTCAGCAAAATGCCA CC GCACAGATCAAGACC
Flax Pond : AAGTGCATTAGAATGGATACAACCTTAAATTATACAATAGTATTAGAAAATAGGGCT :
Newark Bay : AAGTGCATTAGAATGGATACAAACCTTAAATTATACAACAGTATTAGAAAATAGGGCT :
AAGTGCATTAGAATGGATACAA CCTTAAATTATACAA AGTATTAGAAAATAGGGCT
Flax Pond : GGCAAAAAG------------AAATATTGCACAGAAAATAAAAGCAAACACTGCAAAC :
Newark Bay : GGCAAAAAATAAAATAAAATAAAATATTGCACAGAAAATAAAAGCAAACGCTGCAAAC :
GGCAAAAA AAATATTGCACAGAAAATAAAAGCAAAC CTGCAAAC
Flax Pond : TCTGAACCACCTGCAGAAAGGCGCACAGTGATAAAGATTTGAATGCTCTTATCGCATA :
Newark Bay : TCTGAACCACCTGCAGAAAGGCGCACAGTGATAAAGATTTGAATGCTCTTATCGCATA :
TCTGAACCACCTGCAGAAAGGCGCACAGTGATAAAGATTTGAATGCTCTTATCGCATA
Flax Pond : TCAGACGCTGATTTGCAGCCCGCCTTTGACAGCATTGTGTCTCATGCGCACCTAAACT :
Newark Bay : TCAGACGCTGATTTGCAGCCCGCCTTTGACAGCATTGTGTCTCATGCGCACCTAAACT :
TCAGACGCTGATTTGCAGCCCGCCTTTGACAGCATTGTGTCTCATGCGCACCTAAACT
Flax Pond : TGAACAAGGCGGTAGACACTTTGTAATGCACGCGAATTGTGTACCGCCAGGACCACAC :
Newark Bay : TGAAGAAGGCGGTAGACACTTTGTAATGCACGCGAATTGTGTACCGCCAGGACCACAC :
TGAA AAGGCGGTAGACACTTTGTAATGCACGCGAATTGTGTACCGCCAGGACCACAC
XRE
Flax Pond : ACAGACACACCCACCAACTTTTTTTTTTTCTACTGCTCCAAACTTCATTCATGGCAGG :
Newark Bay : ACAGACACACCCACCAACTTTTTTTTT-CTACTGCTCCAAACTTCATTCATGGCAGG :
ACAGACACACCCACCAACTTTTTTTTTT CTACTGCTCCAAACTTCATTCATGGCAGG
Flax Pond : GAATTAAAGACAGGCACTCAGATGGAGGAGGGGAGATGATGTCAACCTCGGTAGCCAA :
Newark Bay : GAATTAAAGACAGGCACTCAGATGGAGGAGGGGAGATGATGTCAACCTCGGTAGCCAA :
GAATTAAAGACAGGCACTCAGATGGAGGAGGGGAGATGATGTCAACCTCGGTAGCCAA
AP-1
Flax Pond : TAAGATTGCGCAGCGCTCTATAAATCATACGTCCACTCGCGGCTTTGAAGACATCTGC :
Newark Bay : TAAGATTGCGCAGCGCTCTATAAATCATACGTCCACTCGCGGCTTTGAAGACATCTGC :

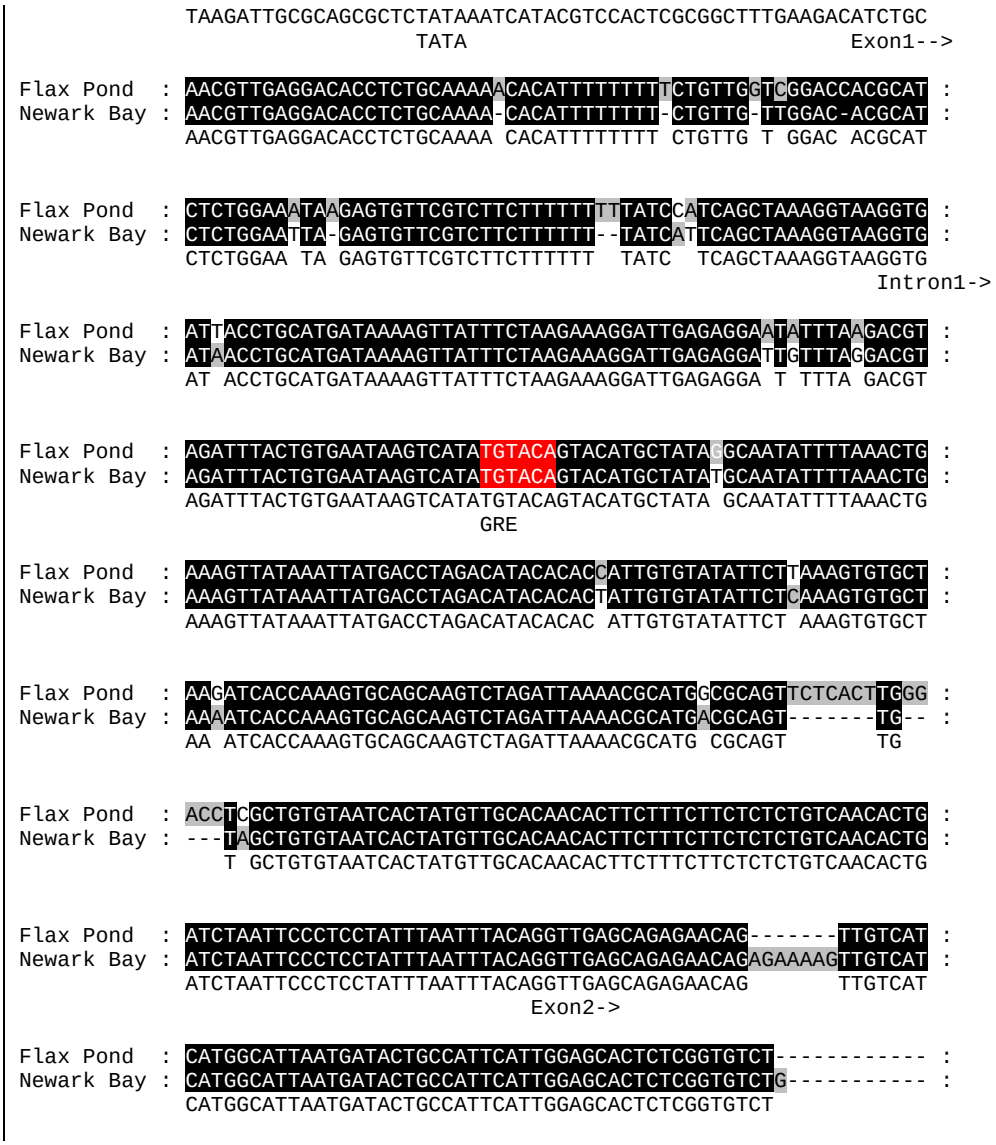

TAAGATTGCGCAGCGCTCTATAAATCATACGTCCACTCGCGGCTTTGAAGACATCTGC
TATA
Exon1-->
Flax Pond : AACGTTGAGGACACCTCTGCAAAAACACATTTTTTTTTCTGTTGGTCGGACCACGCAT :
Newark Bay : AACGTTGAGGACACCTCTGCAAAA-CACATTTTTTTT-CTGTTG-TTGGAC-ACGCAT :
AACGTTGAGGACACCTCTGCAAAA CACATTTTTTTT CTGTTG T GGAC ACGCAT
Flax Pond : CTCTGGAAATAAGAGTGTTCGTCTTCTTTTTTTTTATCCATCAGCTAAAGGTAAGGTG :
Newark Bay : CTCTGGAATTA-GAGTGTTCGTCTTCTTTTTT--TATCATTCAGCTAAAGGTAAGGTG :
CTCTGGAA TA GAGTGTTCGTCTTCTTTTTT TATC TCAGCTAAAGGTAAGGTG
Intron1->
Flax Pond : ATTACCTGCATGATAAAAGTTATTTCTAAGAAAGGATTGAGAGGAATATTTAAGACGT :
Newark Bay : ATAACCTGCATGATAAAAGTTATTTCTAAGAAAGGATTGAGAGGATTGTTTAGGACGT :
AT ACCTGCATGATAAAAGTTATTTCTAAGAAAGGATTGAGAGGA T TTTA GACGT
Flax Pond : AGATTTACTGTGAATAAGTCATATGTACAGTACATGCTATAGGCAATATTTTAAACTG :
Newark Bay : AGATTTACTGTGAATAAGTCATATGTACAGTACATGCTATATGCAATATTTTAAACTG :
AGATTTACTGTGAATAAGTCATATGTACAGTACATGCTATA GCAATATTTTAAACTG
GRE
Flax Pond : AAAGTTATAAATTATGACCTAGACATACACACCATTGTGTATATTCTTAAAGTGTGCT :
Newark Bay : AAAGTTATAAATTATGACCTAGACATACACACTATTGTGTATATTCTCAAAGTGTGCT :
AAAGTTATAAATTATGACCTAGACATACACAC ATTGTGTATATTCT AAAGTGTGCT
Flax Pond : AAGATCACCAAAGTGCAGCAAGTCTAGATTAAAACGCATGGCGCAGTTCTCACTTGGG :
Newark Bay : AAAATCACCAAAGTGCAGCAAGTCTAGATTAAAACGCATGACGCAGT-------TG-- :
AA ATCACCAAAGTGCAGCAAGTCTAGATTAAAACGCATG CGCAGT TG
Flax Pond : ACCTCGCTGTGTAATCACTATGTTGCACAACACTTCTTTCTTCTCTCTGTCAACACTG :
Newark Bay : ---TAGCTGTGTAATCACTATGTTGCACAACACTTCTTTCTTCTCTCTGTCAACACTG :
T GCTGTGTAATCACTATGTTGCACAACACTTCTTTCTTCTCTCTGTCAACACTG
Flax Pond : ATCTAATTCCCTCCTATTTAATTTACAGGTTGAGCAGAGAACAG-------TTGTCAT :
Newark Bay : ATCTAATTCCCTCCTATTTAATTTACAGGTTGAGCAGAGAACAGAGAAAAGTTGTCAT :
ATCTAATTCCCTCCTATTTAATTTACAGGTTGAGCAGAGAACAG TTGTCAT
Exon2->
Flax Pond : CATGGCATTAATGATACTGCCATTCATTGGAGCACTCTCGGTGTCT---------- :
Newark Bay : CATGGCATTAATGATACTGCCATTCATTGGAGCACTCTCGGTGTCTG---------- :
CATGGCATTAATGATACTGCCATTCATTGGAGCACTCTCGGTGTCT

Figure 7-2. Additional DNA fragments in the killifish CYP1A promoter results in 728, or 685bp PCR products.

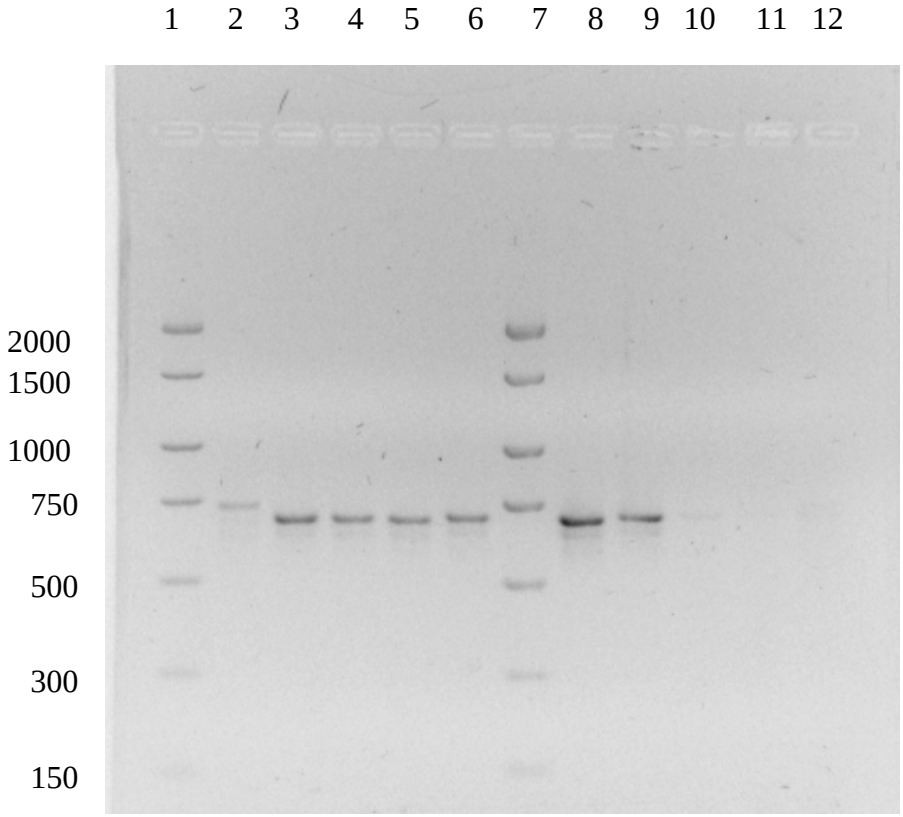
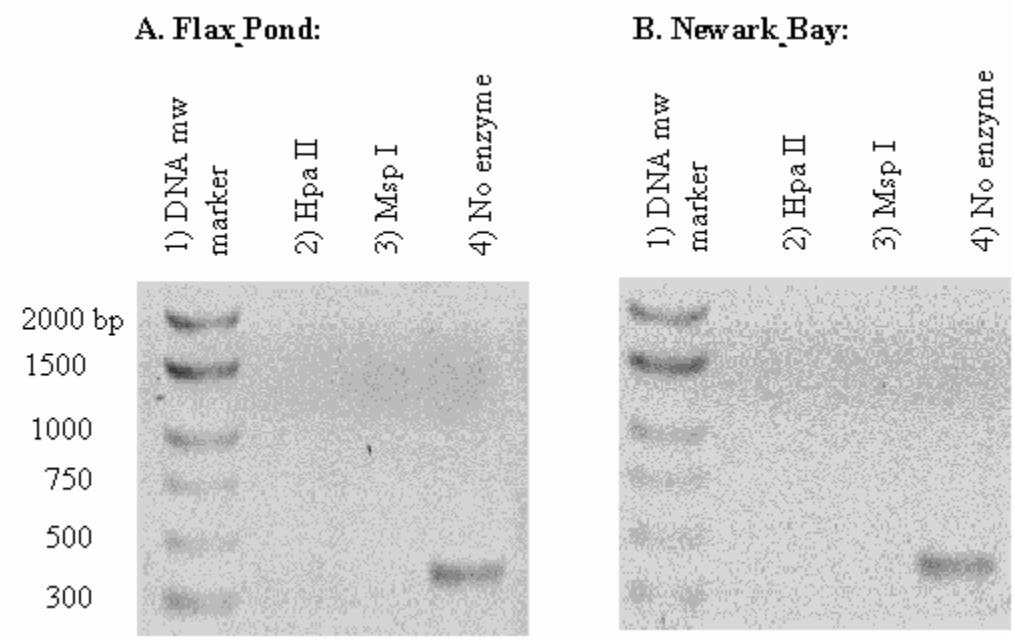


Figure 7-3. Ethidium bromide-stained agarose gels of PCR products derived from HpaII and MspI restriction digestions of hepatic DNA.



Chapter Eight

Summary and future directions

The results obtained from the experiments described in this thesis add further knowledge about the potential molecular and biochemical mechanisms involved in chemical resistance. Chronically contaminated killifish populations were shown to be resistant to AHR ligand induction of CYP1A and CYP3A expression, ROS production and developmental deformities (Chapters 2, 3, and 5). Other important findings were that polluted site killifish had higher activity of the phase II xenobiotic metabolizing enzyme GST and lower hepatic levels of functional AHR, when compared to reference (clean site) fish (Chapter 2).

It is possible that the changes in expression and induction of these enzymes could be essential for the survival of killifish populations in contaminated sites. Normally, exposure to halogenated aromatic hydrocarbons causes a variety of toxic effects, activation of the aryl hydrocarbon receptor and induction of responsive genes, including CYP1A. Studies by other groups suggest that CYP1A activity is associated with ROS production and metabolism of parent xenobiotics in to unstable intermediates (Dalton et al., 2002). As demonstrated in the killifish embryo model (Chapters 4 and 5), AHR activation and increased expression and activity of CYP1A is an important mechanism mediating the toxic effects caused by co-planar AHR ligands. The data presented in this thesis suggests that reduced sensitivity to activation of the AHR/CYP1A response is protective against the toxic effects caused by co-planar PCBs and PAHs and could increase killifish survival in polluted environments.

The implications of these studies, and possible future directions are presented below.

Resistance to activation of the AHR pathway and induction of responsive genes.

The resistance to CYP1A induction observed in contaminated site killifish could be the result of one or more different molecular mechanisms. Contaminated site killifish had reduced sensitivity to CYP1A induction by AHR ligands, but the embryo experiments described on Chapter 5 demonstrate that the AHR pathway is functional in

these organisms. Three different molecular mechanisms for resistance to CYP1A induction were considered in this thesis. They were: alterations in the DNA sequence of the CYP1A promoter region, altered methylation status of the CYP1A promoter and AHR expression measured by photoaffinity labeling. Differences in the CYP1A promoter region were observed between reference and polluted site killifish, but to determine if these alterations can truly alter promoter activity, functional studies are required (Chapter 7). The studies presented on Chapters 6 and 7 suggest that DNA methylation is not involved in resistance to CYP1A induction. CYP1A plays an important role in the toxic effects caused by AHR ligands, but since AHR plays a central role in co-planar PCB and PAH toxicity, it is possible that the molecular mechanism for resistance is at the level of the AHR signaling pathway. Lower AHR expression or activity possibly contributes to resistance in Newark Bay killifish (Chapter 2), but further studies should be conducted to determine if this adaptation is conserved in Newark Bay killifish. AHR expression should be studied in first generation (F1) and second generation (F2) Newark Bay killifish. Previous studies by (Elskus et al., 1999) as well as the results presented on Chapters 5 and 6 suggest that resistance to CYP1A induction in Newark Bay killifish is heritable. Since there are no available AHR antibodies for killifish, AHR expression could be measured at the level of mRNA (Karchner et al., 1999; Powell et al., 2000) or by photoaffinity labeling (Chapter 2).

Co-planar PCB and PAH resistant killifish could also have functional alterations the AHR, or transcription factors that participate in the AHR signaling pathway. An encouraging finding is that the frequency of killifish AHR1 alleles differs between reference site and New Bedford Harbor fish (Hahn et al., 2004). Although the different killifish AHR1 alleles have equal ability to bind to TCDD and the mammalian xenobiotic response element (Karchner et al., 1999), it is possible that differences in the association of AHR1 alleles with cytosolic proteins contribute to resistance to PCBs.

Unfortunately studying the two killifish AHRs has been a difficult task because of various technical issues. One is reason is that the killifish AHRs are very liable to degradation in tissue preparations. Also, attempts to produce an AHR antibody have been unsuccessful. These problems need to be solved in order to study AHR function in polluted site and reference site killifish. Once antibodies are available, co-

immunoprecipitation experiments could be performed to identify other transcription factors that interact with the killifish AHR. Another approach would be to use genomics to study the expression of transcription factors and signaling proteins that are involved in AHR signaling, such as nuclear factor 1 (Morel et al., 1999), protein kinase C, AP1, and other coactivators (Carlson and Perdew, 2002). These hypothesis generating experiments would first require for these regulatory factors to be isolated (amplified and cloned cDNA) and sequenced, followed by analysis of their expression in reference and polluted site killifish.

Phase II xenobiotic metabolizing enzymes.

In addition to resistance to CYP1A induction adult killifish from Newark Bay had higher basal activity of glutathione-s-transferase (GST) (Chapter 2). Similar observations have been made for other fish population that inhabit environments contaminated with AHR ligands (Armknecht et al., 1998; Otto and Moon, 1996). In vitro and cell culture studies have shown that GSTs can successfully conjugate unstable xenobiotic metabolites and protect against prooxidant chemicals (Sundberg et al., 2002; Zimniak et al., 1997). It is possible that, in combination with reduced sensitivity to CYP1A induction, having elevated GST expression and activity protects Newark Bay killifish against the toxic effects caused by AHR ligands.

There are few studies on the expression and function of GSTs in killifish. Future studies should be aimed at determining the specific GSTs whose expression is altered in polluted site killifish, their function and potential involvement in resistance to AHR ligands. The next steps could involve the cloning and sequencing of cDNAs coding for the GSTs whose expression is altered in Newark Bay killifish, and studying their expression by Northern Blots, or rt-PCR. Once the identity of these GST is known, their function can be studied using an immortalized cell culture or transgenic fish embryo model. Cells or fish embryos could be transfected with vectors containing actively transcribed cDNAs coding for the up-regulated Newark Bay killifish GSTs and toxicological endpoints monitored in the presence of co-planar PCBs, and/or PAHs.

These studies would help determine the specific role of GST activities in killifish survival under constant exposure to contamination. If GSTs are shown to have an

important role in reducing AHR ligand toxicity in killifish, these results would increase our understanig the mechanisms involved in chemical resistance. This would demonstrate that Newark Bay killifish are able to survive in polluted environments through alterations of multiple molecular and biochemical pathways.

Resistance to PCB induced oxidative stress.

Polluted site killifish were resistant to PCB126 and 3-MC induced ROS production. As mentioned above reduced P450 activity could be a possible mechanism that grants resistance to these effects (Chapter 5). However, it is possible that additional antioxidant mechanisms are also participating in resistance to ROS induction by AHR ligands and other xenobiotics present in contaminated sites. Various studies using different fish models suggest that chronic exposure to pollutants results in altered levels of antioxidants and antioxidant related enzymes. Some of the adaptations observed in fish collected from contaminated sites include increased hepatic levels of the antioxidant alpha-tocopherol (vitamin E) and expression in the antioxidant enzymes glutathione peroxidase, catalase, superoxide dismutase, and glutathione reductase (Otto and Moon, 1996; Palace, 1998; Stephensen et al., 2000). Recent studies have shown that killifish from PAH polluted areas in the Elizabeth River VA have higher levels of non-enzymatic antioxidants and increased expression of antioxidant enzymes in comparison to clean site animals (Meyer et al., 2003).

It is not yet known if changes in antioxidant related pathways are present in the Newark Bay and New Bedford Harbor killifish. Future experiments should include determination of both enzymatic and non-enzymatic antioxidants in Newark Bay and New Bedford Harbor killifish. These experiments could include the cloning and sequencing of cDNAs coding for enzymes such as superoxide dismutase, glutathione reductase, and glutathione peroxidase, followed by determination of RNA expression by northern blots or rt-PCR. As in the GST experiments described above, the function of potentially induced or down-regulated antioxidant enzymes could be further studied using a cell culture, or transgenic fish embryo models. Altered expression of antioxidant related enzymes, up-regulation of GST expression and activity, and resistance to

AHR/CYP1A activation, could increase tolerance to PCB and PAH induced toxicity in Newark Bay and New Bedford Harbor killifish.

The results presented on Chapter 5 suggest that activation of the AHR pathway by PCB 126 is associated with the induction of developmental deformities. This is supported by studies using zebrafish morpholino oligonucleotides, where the AHR pathway and not CYP1A mediated the teratogenic effects caused by exposure to dioxins (Carney et al., 2004). These zebrafish experiments did not focus on the cellular and tissue effects caused by dioxin exposure, such as ROS production. As mentioned earlier, CYP1A and other P450s are involved in the metabolism and excretion of xenobiotics, but can also cause damage by converting xenobiotics in to unstable intermediates or generate ROS. The results presented in Chapter 5 suggest that inhibition P450 activity is protective against PCB induction of ROS. Future studies should be aimed at determining if AHR ligands can induce other P450s, in addition to CYP1A, and their involvement in activation of xenobiotics and ROS production. Two P450s that deserve more detailed attention are CYP3A and CYP1B1. As shown in Chapter 3 CYP3A is inducible in reference site animals, but not in Newark Bay killifish. Like CYP1A, CYP1B1 also forms part of the AHR gene battery and its activity is also associated the metabolic activation of xenobiotics (Nebert et al., 2004). The expression of these two P450s after AHR ligand exposure can be studied in more detail by measuring enzyme mRNA expression and activities in reference and polluted site killifish.

From the embryo experiments using AHR/CYP1A inhibitors (Chapter 5), we concluded that there was no correlation between AHR induced deformities and ROS production. These results were surprising since previous studies using different model organisms suggest that AHR activation is associated with ROS production and developmental deformities (Hilscherova et al., 2003; Jin et al., 2001). Also, studies using killifish embryos have demonstrated that ROS producing agents can affect development (Meyer et al., 2003). It is possible that in our model, PCB induced ROS production is not associated with teratogenesis in killifish embryos, but could cause other harmful effects such as DNA damage (Park et al., 1996; Telli-Karakoc et al., 2002) or altered immune function (Duffy et al., 2003). Additional studies should be conducted to corroborate the results presented on chapter five and determine the physiological effects of PCB induced

ROS production. Morpholino oligonucleotides technology could be used on killifish embryos to study the roles of AHR and CYP1A. The advantage of using morpholinos is that they are more specific than chemical AHR/CYP1A inhibitors, and permit researchers to study the molecular components of co-planar PCB activated pathways in more detail. Additional toxicological endpoints, such as oxidative DNA damage, and altered immune function can be added to these experiments. Such studies would help understand the physiological effects and mechanisms involved in co-planar PCB induced oxidative stress.

Possible costs and outcomes of xenobiotic resistance.

It has been previously proposed that the adaptations observed in chronically contaminated animal populations might come at a cost (Weis, 2002). For example: killifish from the contaminated Elizabeth River VA, are more sensitive to low oxygen conditions than reference site animals (Wirgin and Waldman, 2004), and killifish eggs collected from the contaminated Piles Creek NJ, have lower fertilization success in comparison to reference site fish (Weis, 2002). We have observed that the Newark Bay killifish used in these studies were often infected with parasites, suggesting that they have a compromised immune system. This is possibly the result of constant exposure to PAHs and PCBs that can alter immune function (Carlson et al., 2002; Duffy et al., 2003). The molecular and biochemical differences between reference and polluted site killifish suggest that PCBs and other contaminants have acted as a selective pressure for low AHR/CYP1A responders. Constant exposure to pollution could have altered the overall genetic composition of exposed populations. The loss of genetic variability in these populations could make them more vulnerable to other kinds of stress encountered in unaltered environments. Future experiments could focus on determining if polluted and reference site killifish are equally vulnerable to "natural" forms of stress, such as changes in water salinity and dissolved oxygen. In the long term, a gradual decrease in the pollution levels of these contaminated sites in combination with exchange of genetic material from neighboring populations could slowly restore the genetic variability lost due to contaminant exposure.

Another aspect that should be considered is the possible role of resistant killifish in the transportation of xenobiotics. Although these animals are resistant to the immediately toxic effects of pollution, they can bio-accumulate large amounts of pollutants (Monosson et al., 2003). As mentioned earlier (Chapter 1), killifish are intermediates in the trophic structure of their community and other organisms that consume killifish could migrate to other a reas and introduce contaminants in to new environments. Such events could also have important implications to human health since killifish, are consumed by commercially relevant fish.

In conclusion, a description of some of the possible mechanisms that allow chronically polluted killifish to survive under constant exposure to environmental xenobiotics is presented in this thesis. Studies on polluted killifish can further our understanding of the molecular, biochemical and organismal pathways associated with the long-term toxic effects caused by xenobiotic AHR ligands and how the introduction of human made pollutants can alter aquatic environments.

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