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CHARACTERIZATION OF POLLUTANT RESPONSE IN TELEOSTS WITH VARYING DEGREES OF POLLUTANT SENSITIVITY

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ABSTRACT OF DISSERTATION

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ABSTRACT OF DISSERTATION

A dissertation submitted in partial fulfillment of the
requirements for the degree of Doctor of Philosophy in the
College of Arts and Sciences
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By
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Lexington, Kentucky

Director: Dr. Adria A. Elskus, Professor of Biology and Toxicology

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CHARACTERIZATION OF POLLUTANT RESPONSE IN TELEOSTS WITH VARYING DEGREES OF POLLUTANT SENSITIVITY

Cytochrome P4501A (CYP1A), a xenobiotic metabolizing enzyme found in all vertebrates, is highly induced following exposure to a number of organic contaminants. Several populations of teleosts residing in highly contaminated areas have been found to exhibit resistance to the toxic effects of contaminants, a condition characterized by reduced expression of CYP1A.

Within this work I demonstrated that expression of CYP1A mRNA, protein, and activity in caged rainbow trout (*Oncorhynchus mykiss*) was an effective biomarker of polychlorinated biphenyl (PCB) contamination. Furthermore, through the use of both laboratory and field studies, I demonstrated that several species inhabiting a PCB contaminated site exhibited either acquired (*Ameiurus natalis*) or natural (*Lepomis cyanellus*) resistance to the CYP1A inducing effects of PCBs. Further studies characterized the response of several other *Lepomis* species to CYP1A inducing compounds, demonstrating that the natural resistance of *L. cynaellus* is a characteristic shared by at least two other members of the genus. *Lepomis* species were relatively insensitive to CYP1A induction following PCB exposure yet exhibited highly induced CYP1A levels following exposure to another CYP1A inducer, the model polyaromatic hydrocarbon benzo[a]pyrene (BaP), suggesting a number of species within the genus *Lepomis* may display natural resistance to certain classes of CYP1A inducing compounds.

Additional studies using responsive and resistant populations of killifish were used to examine the consequences of resistance on fish physiology. Thyroid hormones, known to be altered by PCBs in mammals, were variable but did not differ significantly between responsive and resistant fish following PCB exposure. Treatment with PCBs suppressed production of the egg yolk precursor protein vitellogenin in primary hepatocytes of responsive fish. Studies examining the developmental impacts of toxicant exposure demonstrated altered aspects of development in PCB responsive but not resistant *Fundulus heteroclitus* embryos exposed to polybrominated diphenyl ethers (PBDEs), compounds structurally related to PCBs. PBDE exposure in juvenile *Ictalurus punctatus* failed to induce CYP1A or uridine diphosphate glucuronyltransferase (UDPGT) activity indicating PBDEs do not impact these commonly measured toxicological endpoints. The findings of this work describe novel pollutant responses in a number of species with varying degrees of pollutant sensitivity and contribute to the understanding of toxicant induced alterations in teleost physiology.

Key words: CYP1A, Resistance, Biomarker, PCB, Contaminant

Benjamin Frederick Brammell

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1.1 Introduction

The last century has seen an unprecedented proliferation in the number of chemical compounds manufactured for a number of industrial, agricultural, and biomedical applications. While these compounds provide benefits they also pose risks, to both humans and to wildlife, as a result of unintended biological effects. Although some of these effects are dramatic, such as the reproductive failure in birds resulting from DDT exposure [1], others are more subtle and in some cases their effects have only been recently recognized. Endocrine disruption, the ability of chemicals to mimic hormones in the body disrupting normal hormone function, is one of these recently recognized and less overt modes of chemical toxicity. Another relatively recently recognized effect of pollution is the artificial selection of resistant organisms, resulting in populations that are resistant to the pollutant but may have reduced genetic variability and/or other detracting characteristics. An additional problem related to the proliferation of chemicals released into the environment is that the biological effects of a number of compounds remain largely unknown. The studies presented in this dissertation provide novel information contributing to a greater understanding of the effects of both well characterized and emerging environmental pollutants.

1.2 Biotransformation

All organisms are constantly exposed to a number of compounds present in the environment through the diet, skin, and inhalation. Many of these compounds are absorbed through the skin, lungs, or gastrointestinal tract because of their lipophilicity, a physical property that prevents their rapid elimination from the body as a result of their tendency to move into lipid matrices [2]. The elimination of these xenobiotics from the body depends on a process known as biotransformation, the result of which is the transformation of a xenobiotic from a lipophilic to a hydrophilic state. Biotransformation typically results in more polar, less biologically active metabolites that are more readily excreted from the body [3].

The reactions catalyzed by biotransformation enzymes are generally divided into two groups, referred to as phase I and phase II enzymes. Phase I biotransformation enzymes generally introduce a small, water soluble functional group into a xenobiotic resulting in a small increase in hydrophilicity [2]. Phase I reactions include hydrolysis, reduction, and oxidation [2].

Phase II reactions generally insert a larger, hydrophilic group onto a xenobiotic, typically reacting with functional groups that are either already present on the xenobiotic or were introduced during the phase I reaction [3]. Phase II reactions include glucoronidaton, sulfation, acetylation, methylation, and conjugation with glutathione and amino acids [3]. Xenobiotics typically have greatly increased hydrophylicity following phase II reactions enabling the excretion of the compound from the body [2].

P450 Enzymes

The cytochrome P450 enzymes are a superfamily of phase I biotransformation enzymes found in both prokaryotic and eukaryotic organisms including vertebrate and invertebrate animals, plants, yeast, fungi, and bacteria [4]. P450 enzymes play a role in the biotransformation of a number of both endogenous and exogenous compounds [4]. In addition a number of essential physiological processes such as the synthesis and metabolism of steroids, fatty acids, and prostaglandins are performed by various P450 enzymes [5]. While the highest concentration of these enzymes are typically found in the liver endoplasmic reticulum they are present at some concentration in virtually all tissues of most vertebrates [2]. P450 enzymes also play key roles in determining the intensity and action of drugs and in the detoxification of xenobiotics [2].

P450 enzymes typically work by inserting an oxygen atom onto the structure of substrates, leaving the product relatively hydrophilic and generally, although not always, less toxic [6]. All P450 enzymes are heme containing proteins [6], the iron atoms of which are typically in the ferric (Fe3+) state [2]. When reduced to the ferrous (Fe2+) state by the addition of a single electron from the coenzyme, cytochrome P450 reductase, the iron has the capacity to bind oxygen [2]. Following binding of first the substrate and then an oxygen atom, the Fe2+O2 complex is converted to a Fe^{2+} OOH by the addition of another electron and a hydrogen atom by either cytochrome P450 reductase or cytochrome b5 [2, 7]. Addition of one more proton cleaves the Fe^{2+} OOH complex producing water and a $(FeO)^{3+}$ compound that transfers the O atom to the substrate which is then released, returning cytochrome P450 to its original state [2].
The somewhat more hydrophilic substrate may then undergo phase II reactions which lead to dramatically more polar conjugates which are excreted from the body [4]. Alternatively, some carcinogens and other chemicals are converted by P450 enzymes to more toxic products (bioactivation) [8].

Approximately 500 P450 isoforms in 74 gene families are currently recognized [9] although some predictions indicate at least 100 will eventually be recognized in each mammalian species and perhaps as many as 1000 in all phyla [10]. As previously discussed, P450 enzymes perform a number of tasks related to both endogenous and exogenous substrates. Certain isoforms, such the enzyme designated cytochrome P450 1A, are involved primarily in the biotransformation of xenobiotic compounds.

Cytochrome P450 1A

Cytochrome P450 1A (CYP1A) is a P450 isozyme involved in the detoxification of a number of organic xenobiotic compounds, including many environmental pollutants [4]. CYP1A utilizes a number of environmental pollutants as metabolic substrates including polyaromatic hydrocarbons (PAHs) and polychlorinated biphenyls (PCBs), leading to both excretion and bioactivation [11].

CYP1A is regulated as part of the aryl hydrocarbon gene battery [12], a group of hundreds of genes activated in response to binding of the aryl hydrocarbon receptor (AHR) [13]. The AHR is a cytosolic receptor bound to two heat shock proteins until activation [14]. Upon activation the AHR dissociates from the heat shock proteins and moves to the nucleus where it forms a complex with the aryl hydrocarbon nuclear transloactor [15]. The bound complex then acts as a transcription factor, binding to regions of DNA known as xenobiotic response elements found in the promotor regions of responsive genes [15]. Activation of the AHR leads to the production of a host of both phase I and II enzymes, including CYP1A.

CYP1A is a widely used biomarker of exposure to planar aromatic hydrocarbon compounds, including environmental contaminants such as PCBs [16-18]. While endogenous levels of the enzyme are relatively low, strong and rapid induction of CYP1A mRNA and catalytically active protein occurs in response to inducing compounds [19], making CYP1A an ideal biomarker of exposure to such pollutants. Quantification of CYP1A mRNA, protein, and activity (measured as ethoxyresorufin-O-deethylase, EROD) levels are often used as biomonitoring tools [20, 21].

Glutathione S-transferase

The glutathione *S*-transferases (GSTs) are a family of enzymes involved in the conjugation and excretion of a number of both endogenous and exogenous ligands [22]. GSTs are phase II biotransformation enzymes that transfer the tripeptide glutathione to electrophilic centers of a wide variety of xenobiotic compounds providing protection against oxidative damage [23]. GSTs are divided into four classes, all of which are located primarily in the cytosolic fraction of hepatocytes [24]. GSTs are involved in the removal of a number of xenobiotics from the body and the relative levels of various GST isoforms are believed to greatly influence the sensitivity of organisms to environmental chemical toxicities [25]. GSTs are inducible following substrate exposure although the elevation is generally very slight relative to CYP1A elevation [26]. GST enzymes are present in teleosts and a number of studies demonstrate elevated GST activity following exposure to environmental contaminants [26-28]. However, this response appears to be highly species specific and the majority (67%) of fish studies to date do not demonstrate a significant increase in GST exposure following pollutant exposure [29].

Uridine diphosphate glucuronyltransferase

The uridine diphosphate glucuronyltransferases (UDPGTs) are another group of phase II enzymes that comprise a major pathway for the inactivation and excretion of a number of both endogenous and exogenous organic compounds [24, 30]. Uridine 5′- diphosphoglucuronic acid is formed and attached to a wide variety of substrates by UDPGT, increasing their hydrophilicity thereby enabling excretion [30]. Glucoronidation of endogenous compounds in teleosts is important in functions such as regulating hormone action via excretion (thyroid and steroid hormones)[31] and in the elimination of bilirubin [32]. Glucuronide conjugates of a number of structurally diverse xenobiotic compounds have been detected in the bile, urine, and tissues of teleosts indicating the importance of UDPGT mediated biotransformation and elimination in these species [33]. Unlike GST, UDPGT activity is reported to increase following pollutant exposure in the majority of fish studies (52%) and is considered the phase II enzyme of teleosts most responsive to pollutant exposure [29].

1.3 The Endocrine System

The endocrine system consists of a number of ductless glandular tissues that secrete specific chemical messengers or hormones into the blood. Hormones circulate through body fluids and elicit responses in target cells that possess specific receptors. Most organisms have a variety of endocrine glands that control a wide array of body functions including cellular metabolism, growth, osmoregulation, reproduction, cardiovascular function, digestion, and even coloration [34]. Endocrine regulation is typically relatively slow and sustained and facilitated by low concentrations of hormones [34]. The endocrine system is regulated, in many cases, by negative feedback loops and therefore represents a system in a delicate balance, one that is easily disturbed by foreign compounds.

Thyroid Hormones

Thyroid hormones are released by the thyroid gland and circulate through the body and may control more physiological processes than any other hormone. Thyroid hormone function can be divided into two broad categories: controlling development, especially of the central nervous system, and maintaining metabolic homeostasis by affecting the function of virtually all organ systems [35]. Thyroid hormones act on the liver, kidney, heart, nervous system, and skeletal muscle, sensitizing these organs to epinephrine and stimulating respiration, oxygen consumption, and metabolic rate. Thyroid regulation is responsible for metamorphosis, growth rate, limb regeneration, nervous system function, as well as metabolism in nearly all vertebrates [34].

The thyroid gland in vertebrates is under control of the hypothalamic/pituitary axis [36]. Iodine is actively accumulated from the blood by the thyroid gland and incorporated into iodinated precursors in the follicles of the gland. Stimulation of thyroid follicles by TSH released by the anterior pituitary stimulates the production and release of two major thyroid hormones: 3,5,3'-triiodothyronine (T_3) and thyroxine (T_4). The production of thyroid stimulating hormone is in turn regulated by release of TSH-releasing hormone (TRH) produced and released by the hypothalamus [36]. Production of thyroid hormone is ultimately regulated via negative feedback exherted by T3 and T4 on both the hypothalamic neurons that secrete TRH and the TSH-secreting cells of the anterior pituitary [36].

Although T3 is the most active form of thyroid hormone, binding to the thyroid hormone receptor with the greatest affinity, relatively little T3 is released from the thyroid tissue of most vertebrates including teleosts [37]. The major hormone secreted by the thyroid gland is T4 which is transported through the blood primarily bound to a transport protein such as transthyretin, the major TH transport protein in non-mammalian vertebrates [38], or thyroid binding globulin, the major TH hormone transport protein in humans [39]. The thyroid gland also secretes small amounts of T3 along with T4 which also travels through the blood bound to thyroid hormone transport proteins [37, 39]. The affinity of such proteins for T3 is typically less than that observed for T4, resulting in higher unbound plasma concentrations of T3 relative to T4 [39]. Although some T3 is released by the thyroid gland the primary source of T3 is the enzymatic conversion of T4 to T3 by removal of iodide from the outer ring of T4 by 5'monodeiodination in extrathyroidal tissues [37]. The advantage to such regulation lies in partitioning the regulation of plasma levels of the prohormone and tissue levels of the active form of the hormone, plasma T4 levels may fluctuate without affecting tissue levels of T3 [37].

Estrogen

Sex steroid hormones are produced by nearly all vertebrates and have widespread metabolic effects on the growth and differentiation of tissues, particularly the reproductive organs [40]. Androgens are predominant in males while female sex steroids include both estrogens and progesterone. While both estrogens and androgens are trophic hormones and have anabolic effects, estrogens primarily have anabolic effects on mammary glands and reproductive organs while androgens have anabolic effects on skeletal muscle [34, 40]. Both hormones are hydrophobic and as such are often transported through the blood via plasma binding proteins [34]. Humans and some other primates have a sex hormone-binding globulin that binds both estrogens and androgens [40]. A similar protein is found in teleost fish except that it binds corticosteroids along with estrogens and androgens [40].

The production of sex steroids is under the control of the hypothalamic/pituitary axis and involves both negative and positive feedback. The release of sex steroid hormones in mammals is stimulated by release of follicle stimulating hormone (FSH) and lutenizing hormone (LH) from the anterior pituitary [36]. Release of FSH and LH is in turn stimulated by release of gonadotropin-releasing hormone (GnRH) from the hypothalamus [36]. The release of GnRH is

regulated by negative feedback exherted by the sex steroid hormones on the hypothalamic neurosecretory cells which simulataneously exhert negative feedback on the anterior pituitary endocrine cells that produce FSH and LH [36]. In primates, prior to ovulation increasing secretion of estradiol by the ovaries stimulates production of increasing amounts of LH and FSH from the anterior pituitary [34]. This example of positive feedback culminates in a sudden surge in LH and FSH production closely following a peak in estradiol production and leading to rupture of the follicle and release of the ovum [36]. In teleost fish the system is similar although gonadotropin I (GTH I) and gonadotropin II (GTH II) are the equivalents of mammalian FSH and LH [40]. Gonadotropin release from the pituitary in teleosts is stimulated by release of hypothalamic GnRH, a process affected by a wide array of factors, dependent on species, including photoperiod, temperature, availability of food, proximity of a mate, and, in some cases, lunar cycles [41]. The release of estrogen has both physiological and behavioral effects that prepare organisms for reproduction.

In oviparous species, including teleosts, an important role of estrogen is to promote the formation of a particular lipoprotein in the liver (vitellogenin) which is incorporated into the yolk of the developing eggs [34, 40]. In mature females vitellogenin production is under the control of estradiol, increasing levels of estradiol in the blood which is transported through the bloodstream to the ovaries [42]. Vitellogenin enters the ovary via receptor mediated endocytosis [43] and is then cleaved into the smaller yolk proteins, phosvitin, lipovitellin, and beta-component, which accumulate in the yolk of the developing oocytes [44]. Although plasma vitellogenin levels are normally non-existent to low in males and immature females [45], production of vitellogenin in these groups occurs upon exposure to estrogen or an estrogen mimic [45].

Endocrine Disruption

Many environmental contaminants are known to interfere with regulation of the endocrine system thereby disrupting normal development and reproduction. A striking example is the reproductive dysfunction in populations of American alligators in Lake Apopka, Florida [46]. These alligators displayed low clutch viability, decreased juvenile population densities, and adult mortality following a pesticide spill in the Lake [47]. Additional studies discovered alligators

exhibited abnormal circulating hormone levels as well as gonadal abnormalities, symptoms consistent with endocrine disruption [46, 48].

Fish are also known to be sensitive to endocrine disrupting compounds in the environment. Numerous studies in Europe have reported induction of vitellogenin, the egg yolk precursor protein, in male fish and the development of intersex gonads in fish exposed to synthetic or natural estrogens released into waterways via municipal sewage [49-51]. Other studies have reported symptoms of endocrine disruption in fish including depressed levels of sex hormones [52], lower gonadosomatic index, altered thyroid hormone levels, and increased plasma testosterone concentrations in males [53]. Several recent studies have correlated reproductive dysfunction in wild fish with halogenated aromatic hydrocarbons found in the environment, possibly the result of endocrine disruption [54, 55]. These data indicate that fish are sensitive to endocrine disruption, the consequences of which are not known.

1.4 Contaminants

Anthropogenic impacts on the environment take on a number of forms. One of the most disruptive is the release of manufactured chemicals, either intentionally or unintentionally, into the environment. Often the impact of manufactured chemicals on both wildlife and humans are difficult to ascertain and frequently are only fully known following years of production and use. Synergistic and antagonistic interactions among chemicals further complicate the situation making understanding of the ecotoxicological implications difficult. The contaminants examined in this dissertation are briefly reviewed below.

PCBs

Polychlorinated biphenyls (PCBs) are members of a class of halogenated aromatic compounds produced by the chlorination of biphenyl. The physiochemical properties of these compounds, such as their low flammability and high electric conductivity, make them desirable for a number of commercial uses. Commercial production of PCBs began in the U.S. in 1929 when they were used in transformers and capacitors, pesticide extenders, adhesives, dedusting agents, cutting oils, flame retardants, heat transfer fluids, hydraulic lubricants, sealants, paints, and in carbonless copy paper [56]. PCBs were also widely produced in other countries including France, Germany, Italy, and Japan [57]. Widespread environmental PCB contamination was first

recognized in the mid-1960s [58]. Further investigation revealed the presence of PCBs in nearly every aspect of the global ecosystem [57]. When the harmful effects of PCBs were realized, production in the U.S. slowed and was eventually banned by the EPA in 1979 [56].

The total amount of PCBs produced worldwide has been estimated at 1.5 million tons [59]. While some uses of PCBs resulted in their direct introduction into the environment; a large portion of the PCBs in the environment are present as a result of careless disposal practices, accidents, and leakage from disposal sites [57]. In 1987 alone it was estimated that about 400,000 tons of PCBs were released into the environment [56].

Toxicity of PCBs is structure dependent. Coplanar PCB congeners, congeners lacking chlorines or possessing only one chlorine at the ortho position, are potent agonists of the AHR and are often referred to as the "toxic" PCB congeners [60]. These congeners are structurally homologous to 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) [61] and mimic the highly toxic effects of this compound [60]. The three most toxic PCB congeners are those lacking chlorine atoms at the ortho position, congeners 77, 126, and 169 [60]. Mono-ortho congeners, congeners possessing one chlorine atom at the ortho position, are also AHR agonists and many exhibit toxic effects [60].

A standardized system has been developed to rank the relative toxicity of various PCB congeners. The Toxic Equivalency Factor (TEF) system is now used by most regulatory agencies in risk assessment [57]. Individual PCB congeners are assigned a value that is the fractional toxicity of the congener in relation to TCDD [60]. These values are obtained from in vivo and in vitro studies [56]. TEFs can be used to calculate the toxic potential of a mixture of congeners, the Toxic Equivalent (TEQ) [60].

PCBs have achieved a ubiquitous distribution due, in part, to the gaseous transport of these contaminants [62]. Primarily lower chlorinated PCB congeners can volatilize and are subject to atmospheric transport [56], resulting in the ubiquitous distribution of PCBs [62]. A general trend in which compounds are evaporated in latitudes with warmer temperatures and then condense and fall out closer to the poles results in the accumulation of PCBs and other pollutants in these areas [63]. The accumulation of even low levels of atmospherically delivered PCBs in the food chain, referred to as biomagnification, can result in PCB levels in biota that exceed guidelines for human consumption [64].

PCBs have very high octanol-water (K_{ow}) values indicating they preferentially accumulate in lipid matrices in the environment and organic carbon in soil [57]. As a result PCBs, like a number of other lipophilic environmental pollutants, tend to move into lipid rich biological tissues and remain there, a process known as bioaccumulation. The PCBs with the highest rates of bioaccumulation are the moderately chlorinated congeners containing five to seven chlorine atoms per molecule [65]. More highly chlorinated congeners are usually less available to organisms because they are tightly bound with soils and sediments, found in lower concentrations in the environment, and less able to cross the plasma ammbrane and enter the cell [65] while congeners with less chlorination are more readily metabolized and eliminated [65].

Aquatic organisms can acquire pollutants through two principal routes, through the uptake of pollutants directly from the water, referred to as bioconcentration, or through biomagnification, the accumulation of pollutants through contaminated food [66]. The sum of these processes is referred to as bioaccumulation. Pollutants are also lost through two mechanisms in aquatic organisms; either through excretion or through diffusion across the gills [57]. The degree to which an organism bioaccumulates a compound depends on the balance between uptake and elimination. PCBs are highly hydrophobic, typically resulting in dissolved water concentrations on the order of pg/L to ng/L [67], and as a result dietary accumulation of PCBs frequently is the dominant route of PCB exposure in aquatic organisms [68]. However, several studies have demonstrated that aqueous exposure to PCBs can also serve as a significant route of PCB exposure in aquatic organisms [67, 69].

The toxic effects of PCBs in a number of vertebrates including teleosts are well documented. Effects observed in various teleosts species following PCB exposure include increased incidence of hepatic tumors [70], alteration of thyroid hormone levels [71, 72], alterations in sex steroid levels [73, 74], and adversely impacted reproduction [75-78]. CYP1A plays a central role in the toxicity of PCBs inducing toxicity via uncoupling of the enzyme-substrate complex during the catalytic cycle resulting in release of reactive oxygen species from the enzyme's active site [79, 80]. The ubiquitous distribution of PCBs coupled with their potent toxic effects make them an environmental contaminant of great concern.

PBDEs

PBDEs are manufactured for use as flame-retardants and have become widespread environmental contaminants with relatively high concentrations found in biological samples from around the globe. PBDEs are included as additive flame retardants in products such as plastics, electrical components, building materials, and synthetic textiles [81]. Global production of PBDEs was 40,000 tons per year in 1990 and has remained relatively constant since [81]. PBDEs enter the environment through a variety of routes including disposal of PBDE containing products, leachate from landfills, emissions from production plants, and volatilization [81, 82]. Due to their lipophillic nature, PBDEs bioaccumulate in tissue and relatively high levels of PBDEs have been detected in biota [81], including fish [83], fish eating birds [84], marine mammals [85], and humans [86]. The distribution of PBDEs in the environment coupled with their tendency to accumulate in animals has led to widespread PBDE exposure.

Studies examining temporal trends of organohalogenated contaminants reveal that while most other organohalogen contaminant levels are decreasing, PBDE levels are either remaining constant or increasing. The total concentrations of several PBDE congeners in guillemot eggs from the Baltic Sea increased from 1970 to 1989 [84]. Data from a Swedish River (River Viskan) indicates the concentrations of PBDEs in pike muscle increased from 1974 to 1991 [87]. PBDE concentrations in eel from the River Roer increased from 1983 to 1993 [88]. Perhaps of most concern are data from Sweden that indicate an exponential increase in PBDE levels in human breast milk between 1972 and 1996 [89]. Clearly, PBDEs are a growing threat to wildlife and humans. Studies detailing the effects of PBDE exposure are greatly needed.

Although much remains unknown regarding the biological effects of PBDEs the most commonly observed endpoints of PBDE toxicity examined to this point are thyroid effects in rats and mice, primarily the alteration of thyroid hormone levels. Fowles et al. [90] reported that mice orally dosed with the commercial PBDE mixture DE-71 displayed decreased plasma T_4 levels in a dose dependent manner. Similarly Hallgren and Darnerud [91] reported rats administered PBDEs through gastric intubation exhibited a significant reduction in T_4 levels. Weanling rats exposed to the commercial PBDE mixtures DE-71 and DE-79 demonstrated dose dependent reduction of T_4 levels and three to four fold induction of UDGPT [92]. Although the effects of PBDEs on aquatic organisms remains largely unexplored, effects similar to those

observed in mammalian systems might be anticipated based on physiological similarity of the affected systems.

PAHs

Polyaromatic hydrocarbons (PAHs) are mutibenzene ring compounds that are widespread environmental pollutants originating from both natural and anthropogenic sources [93]. PAH emission sources include incomplete fossil fuel combustion, wood burning, and gaseous waste from both industrial and private combustion [94]. Oil spills as well as natural oil seeps are also a common source of PAHs [95]. PAHs compose 20% of the hydrocarbons in crude oil and are the most bioactive of all petroleum compounds [95]. The formation and release of PAHs from both anthropogenic and natural sources results in widespread exposure of aquatic organisms to these contaminants.

Although PAHs are lipophilic and tend to move into lipid matrices in organisms, elimination of these compounds is very efficient and generally no bioaccumulation is observed in fish [29]. PAHs are easily transformed by phase I enzymes such as CYP1A to more hydrophillic products such as phenols, quinines, and epoxides [96]. The reported half-lifes of a number of PAH compounds in rainbow trout ranged from one to nine days [97]. Aside from biotransformation and elimination, PAHs may also undergo bioactivation, a process in which the parent product is transformed into a more toxic metabolite. A number of PAHs may be transformed into carcinogenic metabolites by the action of phase I enzymes [2]. PAHs are also known to exert a number of other toxic effects on fish including altered levels of sex steroid hormones [98], reduced larval viability [99], and alterations in egg size and number [54].

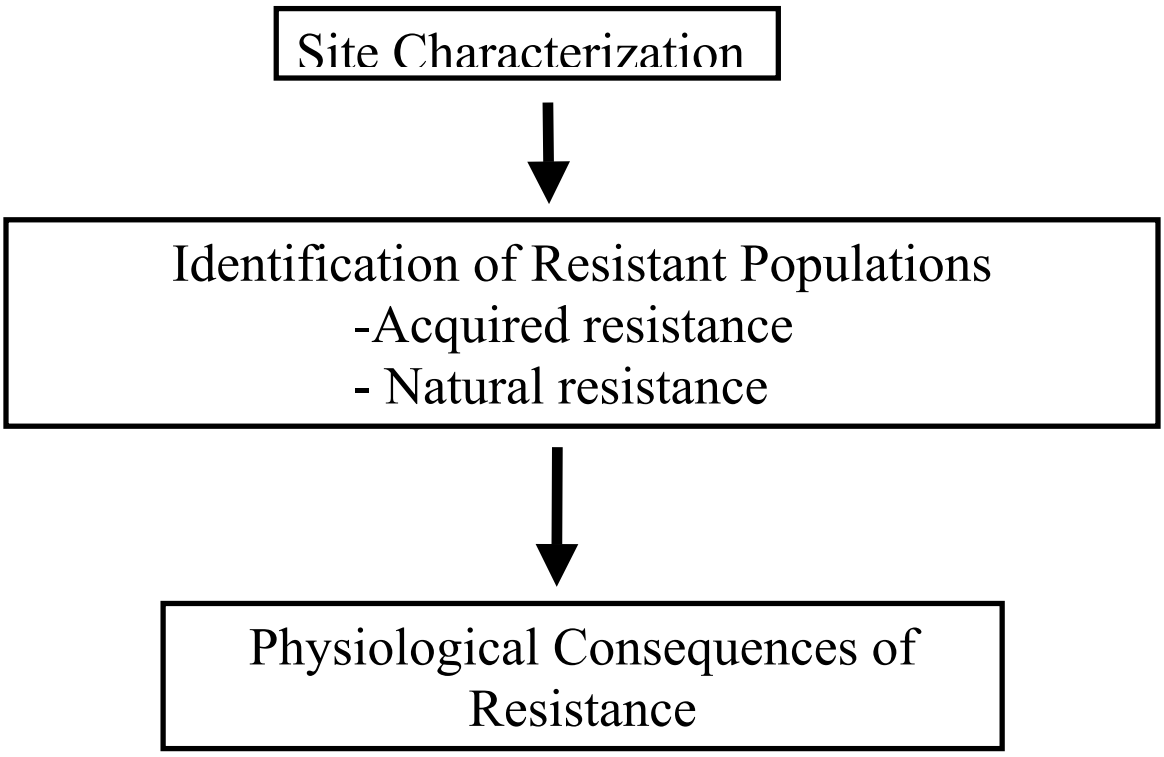
1.5 Resistance

Numerous studies have demonstrated that fish populations living in chronically contaminated habitats can develop resistance to pollutants [100-103], a condition typically associated with reduced response of CYP1A [103]. Fish from populations resistant to organic contaminants fail to express elevated levels of CYP1A following contaminant exposure and experience lower rates of mortality and developmental deformities relative to reference fish when exposed to inducing compounds [104], seemingly demonstrating enhanced fitness in contaminated habitats. Acquired resistance has been reported in a number of fish species that

reside in chronically contaminated habitats. Marsh minnows (*Fundulus heteroclitus*) from areas heavily contaminated with organic pollutants are reported to express lower levels of CYP1A activity in response to PCB exposure relative to reference populations [105, 106]. Acquired resistance has also been reported in Atlantic tomcod (*Microgadus tomcod*) [107] and largemouth bass (*Micropterus salmoides*) [108, 109] inhabiting the PCB contaminated Hudson River. Yellow perch (*Perca flavescens*) inhabiting a lake chronically contaminated by PCBs have also been reported to display acquired resistance [110]. Chemical resistance appears to be a population level response to pollutant exposure enabling the persistence of populations in highly contaminated areas [111].

While the mechanism underlying chemical resistance remains unclear, it may be at least partially explained by a shift in metabolic pathways [103]. *F. heteroclitus* resistant to PAHs demonstrate constant levels of CYP1A following pollutant exposure but elevated levels of constitutive GST [112, 113], a phase II enzyme involved in the conjugation and elimination of xenobiotics. Other studies have demonstrated the role of GST in tumor resistance in fish [114], suggesting a protective role of GST in the chemical resistant fish. An increase in metabolic pathways leading to detoxification as opposed to bioactiviation may provide at least a partial mechanistic explanation for the observation of chemical resistance.

1.6 Specific Aims



While many of the effects of chemical resistance are well documented, others, such as endocrine effects in resistant organisms exposed to pollution, are completely unexplored. The central focus of this dissertation was to examine instances of chemical resistance and the consequences of this resistance. The studies included in chapter two established the presence of bioavailable contaminants at the study site. Studies outlined in chapters three and four idenitifed populations of fish demonstrating both natural and acquired resistance to organinc contmainants. Research reported in chapters five through seven examines the physiological consequences of both natural and acquired resistance. The following questions are addressed:

1. Are PCBs present and biologically available in a remediated system?
2. Are resident species in this system resistant to chemical contaminants?
3. Are certain species naturally chemically resistant?
4. Are chemically resistant organisms immune to endocrine disrupting effects of pollution?
5. Do PBDEs exhert toxicity in a manner similar to PBDEs?

Chapter 2: CYP1A expression in caged rainbow trout discriminates among sites with varying degrees of PCB contamination

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2.1 Abstract

It has become increasingly apparent that resident fish can develop resistance to chemicals in their environment, compromising their usefulness as sentinels of site-specific pollution. To detect site differences in bioavailable polychlorinated biphenyls (PCBs) in a contaminated stream system (whose resident fish appear to have acquired tolerance to PCBs [115]), we evaluated the ability of the PCB-inducible biomarker, CYP1A, to detect site differences using field caged juvenile rainbow trout (*Oncorhynchus mykiss*). CYP1A assays are much less expensive than PCB chemical analyses and, unlike PCB body burdens, provide a measure of biological response to contaminant exposure. Trout were caged in the Town Branch/Mud River system (Logan County, KY), a site currently undergoing remediation for PCBs, in remediated (Town Branch), unremeditated (Mud River), and reference sites for two weeks during the spring of 2002. To evaluate the relative PCB exposure of caged trout, and provide a reference point against which to calibrate CYP1A response, PCB levels were measured in sediments and in non-migratory resident fish from each site. CYP1A expression in caged trout clearly detected the presence of PCBs in the Town Branch/Mud River stream system. Sediment PCB levels, resident fish PCB body burdens, and CYP1A expression in caged trout all produced identical pollution rankings for the study sites. All measures of hepatic CYP1A (mRNA, enzyme activity, and protein concentration) easily detected significant differences between fish caged at the most polluted site and the reference sites. However, hepatic CYP1A activity (measured as ethoxyresorufin o-deethylatse, EROD), was the only CYP1A endpoint to significantly discriminate among sites with the lowest PCB contamination levels. Unlike resident fish, which fail to show site differences in CYP1A expression in this waterway [115], caged fish proved to be a robust and sensitive discriminator of relative PCB contamination in this system. In sum, we determined that, unlike resident species, CYP1A expression in caged fish consistently reflects

relative in situ pollutant levels, two weeks appears to be an optimal caging period for CYP1A response, tissue-specific CYP1A expression can provide insights into route of exposure, and CYP1A in caged trout can detect aqueous exposure to even highly hydrophobic contaminants. We conclude that CYP1A expression in caged trout is a reliable, robust and inexpensive alternative to chemical analysis that can be used for first pass determination of relative environmental pollution and pollutant bioavailability in aqueous systems.

2.2 Introduction

Biomarkers are an effective monitoring tool in toxicology, allowing researchers to assess the biological response to pollutants in both aquatic and terrestrial organisms [16]. Unlike measurements of contaminants in tissues or sediments which provide no information on biological effect, alterations in biomarkers indicate that chemicals present in the system are biologically active. The xenobiotic metabolizing enzyme, cytochrome P-4501A (CYP1A), is a widely used biomarker of exposure to planar aromatic hydrocarbon compounds, including environmental contaminants such as PCBs [16-18]. CYP1A is the major pollutant inducible cytochrome P-450 isoform in fish [116]. While endogenous levels of the enzyme are relatively low, strong and rapid (within hours) induction of CYP1A mRNA and catalytically active protein occurs in response to inducing compounds [19], making CYP1A an ideal biomarker of exposure to such pollutants. Following their uptake across the cell membrane, inducing compounds, such as planar PCBs, bind to the cytosolic aryl-hydrocarbon receptor, initiating transcription of a number of genes, including CYP1A [20]. Quantification of CYP1A mRNA, protein, and activity (measured as ethoxyresorufin-O-deethylase, EROD) levels are often used as biomonitoring tools and are evaluated here as measures of exposure to organic contaminants [20, 21].

PCBs are well-characterized inducers of fish CYP1A in both laboratory and field settings. Significant elevation of CYP1A has been observed in laboratory studies following exposure to industrial PCBs [117]. Numerous field studies report induced CYP1A in feral fish collected from PCB contaminated sites [118, 119] and several studies have detected elevated CYP1A levels in fish caged in areas contaminated heavily, although not necessarily exclusively, with PCBs [21, 120].

While PCB exposure most often leads to elevated levels of CYP1A, inhibition of CYP1A protein expression and activity has been reported following exposure to high concentrations of PCBs [121] or following long term PCB exposure [122]. Chronic exposure to PCBs can lead to genetically heritable resistance to CYP1A induction by these chemicals [104], which compromises the usefulness of resident species as sentinels of pollutant effects [103, 106]. Thus, while elevated CYP1A expression is generally a reliable bioindicator of short-term PCB exposure, certain exposure situations can inhibit expression of this biomarker.

In addition to the possibly compromised nature of resident fish as pollutant indicators, in situ caging offers several advantages as a biomonitoring tool over the collection of feral animals. As noted by Nyholm [123], caging allows both the exposure time and exposure location to be explicitly defined, whereas analysis of feral organisms does not. Caging also allows complete knowledge of an organism's contamination history and provides the option to use individuals of particular sizes, gender, or age, and to choose species best suited to study goals. Caging studies with a number of different fish species have been used to assess environmental pollution with varying levels of sensitivity and accuracy [21, 120].

Rainbow trout are excellent candidates for biomonitoring studies as they are both amenable to caging [21] and sensitive to CYP1A inducers [4]. Exposure to industrial mixtures of PCBs induces CYP1A expression in rainbow trout [117] and rainbow trout CYP1A protein concentration and activity levels in liver, gill and other tissues have been validated as biomonitoring tools [124-126]. Thermal restrictions prevent rainbow trout survival in most southeastern U.S. streams during the warmest summer months, therefore eliminating the possibility of inadvertently establishing rainbow trout as a non-native species if caged animals were to escape. Rainbow trout are also widely cultured for recreation and consumption and are therefore both readily available and acclimatized to confinement in relatively small areas.

The Town Branch/Mud River system in Southwestern Kentucky has been contaminated since the 1960s with PCBs from a local manufacturing plant [127], including Aroclor 1260, a PCB mixture that induces CYP1A in fish [128]. Only certain sections of this waterway have been remediated, providing an ideal system for investigating the ability of CYP1A expression in caged fish to detect and discriminate among sites with varying levels of PCB contamination.

The objective of this study was to assess the presence of biologically available contaminant concentrations in the Town Branch/Mud River system using CYP1A expression in caged trout as a biomonitoring tool. We hypothesized that relative expression levels of CYP1A in caged trout would reflect relative levels of bioavailable PCBs among the study sites. CYP1A expression in gill and liver was measured to compare the relative sensitivity of these tissues, to evaluate the possibility of aqueous routes of exposure to CYP1A inducers, and to assess the relative sensitivity of CYP1A to the highly hydrophobic inducers present in this system, PCBs. In addition, we compared CYP1A protein, catalytic activity, and mRNA expression for their relative sensitivity as biomonitoring metrics for the in situ rainbow trout model. PCB analysis of

sediment and resident fish was conducted to evaluate PCB residues in this system, their bioavailability to fish, and to provide a chemical measure of relative PCB exposure to the caged fish. There are few studies measuring both in situ PCB concentrations and the biochemical response of fish caged in PCB-contaminated freshwater sites [21, 119]. This study makes a substantial contribution to the biomonitoring field by establishing a robust model for pollution monitoring that is supported by chemical data.

2.3 Methods

Materials

7-Ethoxyresorufin and resorufin were obtained from Molecular Probes (Eugene, OR, USA). The monoclonal antibody made against scup CYP1A protein, MAb 1-12-3, was a generous gift of Dr. John Stegeman (Woods Hole Oceanographic Institution). Cy^{TM}5-conjugated affinipure goat anti-mouse IgG was obtained from Jackson Immunoresearch Laboratories Inc. (West Grove, PA, USA) and precast polyacrylamide gradient gels were from Invitrogen (Carlsbad, CA, USA). Nitrocellulose membrane (0.45 μm) was obtained from Schleicher and Schull (Keene, NH, USA). The Bio-Dot SF Microfiltration Apparatus was obtained from Bio Rad (Hercules, CA, USA). Tri-Reagent was from Sigma (St. Louis, MO, USA), DNAse I and QuantumRNA^{tm} Classic internal standard were from Ambion (Austin, TX, USA), and cDNA synthesis kit was from Amersham Biosciences (Uppsala, Sweden). All other reagents were obtained from Sigma, Fisher Scientific (Fair Lawn, NJ), or Invitrogen.

Animals

Juvenile (12-13 month old) rainbow trout (*Oncorhynchus mykiss)* (weight range 20-100 g) were obtained from Wolf Creek National Fish Hatchery in Jamestown, KY and held in the laboratory for seven weeks prior to caging. Fish were held in 948 l (250-gallon) flow-through tanks at 9^0C, 14/10 light/dark, and fed Purina Trout Chow ad libitum every other day.

Study Site: Town Branch/Mud River

The Town Branch/Mud River system (Figure 2.1) has been contaminated since the 1960s with PCBs from a local manufacturing plant. For over 20 years PCBs were released into a lagoon behind the plant that leaked waste containing high concentrations of PCBs (as high as

332,500 ppm) into Town Branch approximately 8 km (5 miles) upstream of its confluence with the Mud River [127]. Sediment PCB concentrations of 280 ppm (dry sediment, clay-silt fraction) (Table 1) [129, 130] were documented in Town Branch in 1986, initiating remediation efforts on Town Branch that began in 1997. Removal of contaminated sediments from both the streambed and floodplain of Town Branch was completed in July 2001 (Michael Mills, KY Division of Water, personal comm.). No remediation has been conducted in the Mud River section downstream of the Town Branch confluence, and relatively high levels of PCBs continue to be found in sediments in this area (5.9 ppm dry sediment, clay-silt fraction) [130]. We chose caging sites in the remediated section of Town Branch, the unremediated Mud River, and two reference sites located upstream of these areas.

Cage Design

Cages were octagonal, 61 cm in diameter (24 inches), 31.5 cm (12 inches) in height and constructed of 1/2 inch PVC and heavy-duty garden mesh sewn together with galvanized wire and plastic locking ties. Cages were completely submerged and tethered via nylon rope to an iron fence post driven into the streambed.

Caging Experiment

Juvenile rainbow trout were caged at different locations spread over four sites (10-12 fish per cage, 5 cages per site) in the Town Branch/Mud River system (Figure 1) during April and May, 2002, a time when water temperatures and water levels are optimal for cage deployment of this species. Cages were positioned in four sites: the remediated section of Town Branch (TB remediated), in a clean section of Town Branch upstream from the original source of PCB contamination (TB reference), in the Mud River just below the confluence with the contaminated section of Town Branch (MR unremediated), and in a clean section of the Mud River well above the confluence with Town Branch (MR reference). Fish were held in cages for 14 days in the Mud River and for 15 days in the Town Branch sites. Trout held in the laboratory and sacrificed on day 16 served as uncaged, laboratory held controls. Temperature measurements were recorded at each site on days 0, 14, 15 and pH measurements recorded on day 14-15 following cage deployment (Table 2.1).

On day 14-15, trout were removed from cages and immediately sacrificed. Fish were weighed, and liver and gill tissue removed and flash frozen in liquid nitrogen (-192°C). Carcasses were kept on wet ice until returned to the laboratory (within 36 hours) and stored at -80 ^{0}C for later analysis of gut contents.

Liver Microsomal Protein Isolation

Livers were removed from liquid nitrogen, weighed, and sub-sectioned. Aliquots for RNA analysis (0.07-0.2 g) were refrozen in liquid nitrogen. Aliquots for microsome preparation were immediately homogenized in 10 volumes (weight:volume) of ice-cold 50 mM Tris buffer (pH 7.4). Microsomal fractions were obtained by differential centrifugation as previously described [131]. The final 100,000 x g microsomal pellets were resuspended in 50 mM Tris buffer (pH 7.4) containing 1mM EDTA, 1 mM DTT and 20% glycerol at a 1:1 ratio (liver weight: resuspension buffer volume). Microsomal samples were stored in liquid nitrogen until analyzed for catalytic activity and CYP1A protein content (within three weeks).

RNA Isolation and Analysis

Total RNA was isolated from a 150 mg medial section of gill or liver tissue, respectively, using Tri-Reagent™ [132]. Total RNA was dissolved in molecular grade water and quantified by spectroscopy at 260 nm. A 5 μg aliquot of total RNA was treated with 1 unit of DNAse I for 10 min. at 37 ^{o}C to remove genomic DNA. DNAse was rendered inactive prior to cDNA synthesis with 1 μl of a 20mM solution of ethylenediaminetetraacetic acid. The same 5 μg aliquot of total RNA was then reverse transcribed with Moloney Murine Leukemia Virus reverse transcriptase and random hexamer primers as part of the 1st strand cDNA synthesis kit. A 2 μL aliquot of a 15 μL cDNA synthesis reaction was PCR amplified for 26 cycles using the protocol and primers for the target gene, CYP1A, in rainbow trout as described in McClain et al. 2003 [126]. An 18S universal ribosomal RNA primer (QuantumRNA™ Classic 18S internal standard) was used to co-amplify (in a separate reaction) a reference transcript for all samples. The 18S PCR product (amplicon) expression value was used to normalize all CYP1A raw values by calculating CYP1A expression as a ratio of (measured CYP1A amplicon) ÷ (measured 18S amplicon) for each sample. This ratio of CYP1A/18S was determined for each sample and is referred to as a normalized expression (NE) value. All precautions for verifying template

integrity and quality control of the mRNA quantification were the same as described in McClain et al 2003 [126].

Catalytic and Protein Assays

Ethoxyresorufin -O-deethylase (EROD) activity, a reaction catalyzed exclusively by CYP1A [4], was measured fluorometrically in 48 well CoStar™ plates using the method of Hahn et al. [133]. The reaction mixture contained 50 mM Tris, pH 7.4, 0.1 M NaCl, 2μM 7-ethoxyresorufin, and 100 to 300 ug of microsomal protein in a final volume of 200 μl. The reaction was initiated by the addition of 1.34 mM NADPH, run at room temperature (28-29^{0}C), and production of the fluorescent product, resorufin, measured for 13 minutes. The linear portion of the curve was used to evaluate the rate of the reaction. All EROD assays were run in triplicate on a PerSeptive Biosystems Cytofluor Series 4000™ multi-well plate fluorometer.

Microsomal protein was measured fluorometrically using the method described by Lorenzen and Kennedy [134] with bovine serum albumin as the standard. All protein assays were run in triplicate.

Immunoblotting Procedures

CYP1A protein was quantified by immuno-blotting using a Bio-Dot SF™ microfiltration slot-blot apparatus. Twenty micrograms of microsomal protein diluted in 200 μL buffer (20 mM Tris, 0.5 M) was loaded into each well and vacuum transferred onto a nitrocellulose membrane (0.45 μM). The membrane was incubated in TBS-5% milk at 4^{o}C overnight to block non-specific binding, followed by incubation with MAb 1-12-3, a monoclonal antibody which recognizes CYP1A in multiple vertebrate species [19], essentially as described [106]. CYP1A signal was detected using Cy^{TM}5-conjugated affinipure goat anti-mouse IgG as the secondary antibody and blots were scanned at 633 nm excitation/670 NM emission using a Typhoon 8600 scanner (Molecular Dynamics) and quantified using Image Quant (Molecular Dynamics). Liver microsomes from trout treated with the CYP1A model inducer, ß-naphthoflavone, were loaded in seven concentrations ranging from 0.1 to 7 μg of protein diluted in 200 μL buffer to evaluate linearity of the CYP1A signal on each blot. All samples were run at least in triplicate.

Stomach Content Survey

Fish carcasses were removed from the -80·C freezer in the laboratory and allowed to thaw. At least 5 caged fish from each site were dissected and contents of the stomach and digestive tract examined to determine if caged fish were eating.

Resident Fish Collection and PCB Analysis

Resident fish were collected from caging sites approximately five months after completion of the caging study. Fish were collected by use of backpack shocker (Smith-Root, Inc., Model 12-B) or by a large portable floating electroshocking unit described by Price et al. 2003 [135] and were either sacrificed in the field or returned to the lab and sacrificed within 24 hours. Livers were flash frozen in liquid nitrogen. Carcasses were wrapped in aluminum foil, tagged, and stored at -20 ^{o}C until PCB extraction.

Fish were measured for length and whole body weight before fillets were taken with solvent-cleaned surgical instruments. Yellow bullhead fillets were analyzed after removal of skin while all other fish were scaled and fillets analyzed with skin intact. PCBs in fish fillets were extracted and analyzed using standard U.S. EPA methods [136]. The fillet samples were weighed, then ground with 20g anhydrous sodium sulfate and extracted with petroleum ether in a Soxhlet apparatus for 5-h. The extracts were concentrated to near dryness in a Roto-evaporator (Buchi Model RE121). Lipid and pesticides were removed from the reconstituted samples (5.0 mL in iso-octane) as described [136] and then analyzed by gas chromatography. A 1.0 mL sub-sample was taken for lipid determinations prior to clean-up. Elemental sulfur was removed by shaking the extract with 2-propanol (2 mL) and tetrabutylammonium sulfite (2 mL), adding ultra-pure water (8 mL) and reshaking. The organic phase was removed and mixed with 2.0-mL concentrated sulfuric acid [136], SW-846 Method 3660B, sulfur cleanup]. A 4µL aliquot was then analyzed by gas chromatography.

Samples were analyzed for Aroclors 1248, 1254, and 1260 according to SW-846 Method 8082 (polychlorinated biphenyls by gas chromatography; [136]). Analysis was performed using a Hewlett-Packard (HP) Model 5890A gas chromatograph equipped with an electron capture detector and an HP Model 7673A Automatic Sampler. Samples were analyzed using a 60m X 0.53mm ID SPB-5 (0.5µm film) fused silica megabore column (Supelco, Inc.) with ultra-high purity helium and nitrogen as carrier and makeup gases, respectively. The temperature program

was 160 °C (6 min); 10 °C/min-235 °C (0 min); 0.9 °C/min-260 °C (10 min); injector temperature, 280 °C; detector temperature, 300 °C. PCB peak heights were quantified using an HP Model 3396A integrator and multiple-peak linear regression analysis was performed with Lotus-123® software. Five external standards for each Aroclor were used for calibration curves and for every tenth sample, either a solvent blank or a standard was analyzed. Detection limits for tissue samples ranged from 12 to 160 µg PCB/kg tissue. Reported values are corrected for recovery using an internal recovery standard.

Sediment Collection and Extraction for PCB Analysis

Sediment samples were collected from caging sites in Fall 2002, approximately five months after completion of the caging study. Samples were restricted to the upper 5-10 cm of stream sediment. All sediment samples were collected in acetone-rinsed 0.47 L glass jars with Teflon-lined lids with stainless steel spoons and scoops. Collected sediments were placed on ice for transport to the laboratory where they were refrigerated.

Wet sediment extractions of PCBs were performed following U.S EPA SW-846 Method 3540C [136]. Weighed sub-samples (61.5 ± 12.9 g wet wt.; 42.7 ± 8.9 g dry wt.) were extracted with 300 mL of acetone/methylene chloride (1:1 v:v) in a 500-mL Soxhlet extractor for 15 h. The extract was concentrated, cleaned, and analyzed as previously described [136]. Detection limits for sediment samples ranged from 8 to 17 µg PCB/kg sediment. Reported values are corrected for recovery using an internal recovery standard.

Statistical Treatment of Data

Statistical analyses were performed using SYSTAT Version 10. For the EROD and protein data, a mean was taken of all fish analyzed within a cage and that mean used as one replicate. Only one replicate was available for laboratory held fish and they were therefore excluded from the analysis. mRNA analysis was conducted on a total of five fish per site, including laboratory held fish. The fish were held in cages one and two in the TB remediated site, cages one through five in the TB reference site, and cage one in both the Mud River contaminated and reference sites. All data were transformed (log 10) before analysis. CYP1A data were analyzed using one-way analysis of variance (ANOVA). Differences in means were tested using the Bonferroni test. All differences were considered significant at $p <$ or $= 0.05$.

2.4 Results

Fish Survival

Seventeen of the twenty cages deployed in the experiment were recovered, with cage loss likely due to two high flow events that occurred during the two-week caging period. Some mortality of caged fish did occur. Overall survival of caged fish at sites ranged from approximately 42% in the Mud River unremediated section to approximately 73% in the Town Branch remediated section. Fish survival appeared to relate directly to the flow regime at individual cages, with fish in cages exposed directly to swift current during high flow events experiencing higher mortality than fish caged in slower flow areas. Water quality parameters were similar among sites (Table 2.1), and well within the range of tolerance of rainbow trout [137, 138] making it unlikely that pH or temperature confounded the results of this experiment.

PCB Analysis

PCB concentrations in Mud River reference sediment were below detection. Somewhat unexpectedly, PCB levels in the Mud River unremediated sediment were fairly low. In stark contrast to our expectations, PCB sediment concentrations in the Town Branch remediated section were quite high (Table 2.2).

PCB body burdens in fish mirrored those in sediment (Table 2.3). PCB concentrations in both sediment and green sunfish (*Lepomis cyanellus*) and creek chubs (*Semotilus atromaculatus*) from the Town Branch reference site were significantly lower than PCB body burdens in fish collected in the Town Branch remediated site.

Liver CYP1A expression

All three measures of hepatic CYP1A1 expression reflected the relative degree of PCB exposure among the sites. Liver CYP1A mRNA levels in fish caged in the remediated portion of Town Branch were significantly elevated (8-fold) over levels in caged Town Branch reference fish (Figure 2.2A). In contrast, no differences were observed in hepatic CYP1A mRNA expression between fish caged at the Mud River unremediated and reference sites (Figure 2.2A).

Levels of hepatic CYP1A catalytic activity, measured as EROD, were significantly higher in trout caged in the remediated sections of Town Branch than EROD levels in fish caged in the upstream Town Branch reference site and both Mud River sites (Figure

2.2B). EROD activity in trout caged in the unremediated section of the Mud River was significantly higher than EROD activity in trout caged in the Mud River reference section (Figure 2.2B), but did not differ significantly from EROD levels in Town Branch reference or laboratory held fish. Fish caged at the Mud River reference site had lower EROD activity levels than fish caged at any other site.

Hepatic CYP1A protein levels in fish caged at the remediated section of Town Branch were significantly elevated (5-fold) over CYP1A levels in fish caged in Town Branch reference sites (Figure 2.2C). Fish caged in the unremediated section of the Mud River had CYP1A protein levels statistically indistinguishable from those in fish caged at the reference sites (Figure 2.2C).

Gill CYP1A expression

Unlike liver CYP1A, gill CYP1A levels did not differ significantly among sites, however, the general trend was similar to the hepatic response. Gill CYP1A mRNA levels in fish from the Town Branch remediated section tended to be higher relative to gill CYP1A mRNA levels in reference site fish (Figure 2.3). A power analysis [139]of these data indicate a sample size of n=19 would be needed to detect a 50% change in gill CYP1A mRNA levels among fish from these sites.

Condition Factor

The Fulton-type condition factor $[W(g)/L(cm)^3]x100,000$ [140] of trout caged in all sites was similar and ranged from 0.79 (± 0.07) to 0.86 (± 0.21) (mean(±S.D)). The condition factor of laboratory held control fish (1.04 (± 0.04)) was significantly higher than that of caged fish.

Stomach Content Examination

Analysis of the stomach contents of caged rainbow trout in this study revealed the presence of a limited number of invertebrates. Nearly all fish examined had some prey items in their stomach with no significant differences observed between sites. However, the stomach content of nearly all fish was sparse, consisting of only a few, very small (5-10mm) invertebrates, an indicator of limited feeding during the exposure period.

2.5 Discussion

This study is the first to measure CYP1A expression in caged fish at the level of mRNA, protein, and activity in a site highly contaminated by PCBs. CYP1A expression in caged rainbow trout produced pollution rankings among study sites with varying degrees of PCB contamination that were identical to rankings produced by sediment PCB concentrations and resident fish PCB body burdens. Liver CYP1A proved to be more sensitive than gill CYP1A in detecting site contamination, suggesting that liver may be the better indicator for highly hydrophobic compounds such as PCBs. Among the various assays used to measure CYP1A expression, only CYP1A activity (EROD) was able to discriminate among the less contaminated sites; CYP1A mRNA and CYP1A protein detected contamination only at the most highly polluted site (although with a higher 'n' value, hepatic CYP1A mRNA may have also detected the less contaminated sites). Although this study was not designed to distinguish among routes of exposure, evidence of limited feeding, together with CYP1A expression in fish gill, suggests pollutants are present in the aqueous phase. The results of this study illustrate the utility of caging studies and demonstrate that bioavailable PCBs are present in the Town Branch/Mud River system. Importantly, this study establishes that CYP1A expression in caged trout consistently reflects PCB concentrations in contaminated waterways, can help to determine routes of exposure, and is a robust and inexpensive alternative to chemical analysis for first pass determination of relative environmental pollution in aqueous systems.

The results of this study demonstrate the successful utilization of a caging study to detect biochemical response to PCBs in a site where the use of resident fish failed to indicate the presence of contamination. Previous work in our laboratory found no difference in hepatic CYP1A activity in resident fish species collected from the contaminated and the reference sites in the Town Branch/Mud River system, despite the presence of high PCB levels in fish from the impacted sites [115]. It is likely that the Town Branch/Mud River resident fish have become resistant to PCB induction of CYP1A. Laboratory challenge experiments with resident fish (Chapter 3) and previous studies by others [104, 106], demonstrate the development of resistance in fish residing in sites chronically impacted with these and other CYP1A inducing chemicals. The present study highlights the advantages of using caged fish over the collection of resident fish in biomarker studies.

The relative levels of CYP1A induction observed in caged rainbow trout were comparable to those reported in other studies of fish caged in polluted areas. For hepatic EROD, our observation of a 2.5-fold increase in rainbow trout caged at a PCB-remediated site relative to caged controls agreed well with the 2-fold induction of EROD activity reported in juvenile rainbow trout caged for two weeks (12-15^{0}C) in a site contaminated by industrial and municipal effluents [125]. Our results were also similar to the 8-fold EROD induction observed in juvenile rainbow trout caged for three weeks (4.7-10.5^{0}C) in a harbor contaminated by poorly treated industrial and municipal sewage as well as oil from commercial fishing vessels [124]. For hepatic CYP1A protein, the 5.5-fold induction of CYP1A protein in fish caged at the remediated site was somewhat higher than the 2.4-fold and 2.1-fold increase in hepatic CYP1A protein in largemouth bass and channel catfish, following 7 and 14 day caging experiments [120]. Sediment PCB levels in that river system (5-20 ppm dry weight [120]) were somewhat lower than those in the Town Branch remediated site (46 ppm dry weight). The differences in relative CYP1A protein induction among these studies may be due to species and/or exposure differences. For CYP1A mRNA, McClain et al. [126] reported 4- and 11-fold induction of hepatic CYP1A mRNA in juvenile rainbow trout caged for 8 days (15-20^{0}C) in a site contaminated by creosote as well as a mixture of other industrial and agricultural contaminants relative to fish caged at a control site. We found an 8.2-fold increase in hepatic CYP1A mRNA in trout caged in the Town Branch remediated section relative to levels in caged reference fish. While direct comparisons of CYP1A expression among different field studies are difficult to make since exposure conditions are necessarily diverse, relative comparisons among studies are useful for indicating the range of CYP1A responses likely to be observed in the field. Together these studies demonstrate the utility and sensitivity of CYP1A expression, measured either as mRNA, protein, or catalytic activity, as an indicator of pollutant response.

It is important to note that the ability to acquire resistance to CYP1A inducers is not limited to chronically exposed resident fish, but may develop in caged fish as well, depending on exposure time and type of inducer present. Rainbow trout caged for three weeks in a PCB contaminated river exhibited little to no CYP1A induction relative to caged controls [141], suggesting caged fish may have developed tolerance to these inducers. In the laboratory, rainbow trout injected with PCBs were less responsive to CYP1A induction by a second PCB dose administered 5 to 20 weeks later, demonstrating decreased sensitivity to inducers may occur

within weeks. We found significant induction of CYP1A in caged rainbow trout after two weeks of exposure in a PCB contaminated system, suggesting the optimal caging time may be one to two weeks.

In addition to duration of exposure, the type of inducer polluting the habitat may influence the development of resistance. Although fish can develop resistance to readily metabolized non-halogenated hydrocarbon pollutants, such as polynuclear aromatic hydrocarbons, this resistance appears to be transient [142], while prolonged exposure to halogenated pollutants, such as PCBs, may lead to genetically heritable resistance in some species [104]. Thus exposure time and type of chemical contaminants present in the system should be considered when optimizing caging study experimental design for biomonitoring purposes.

The PCB levels in the Town Branch remediated section are at the upper end of those measured in fish from sites considered highly contaminated with these chemicals. Median total PCB concentrations in fish from New Bedford Harbor (NBH), Massachusetts, considered one of the most highly contaminated PCB Superfund sites in the U.S., ranged from 5.5 ppm wet weight, edible flesh in flounder up to 24 ppm in American eel [143]. Total mean PCB levels in edible flesh of resident fish caught in the Town Branch remediated site ranged from 16.7 ppm wet weight in longear sunfish to 75.2 ppm in creek chubs. Although the PCB concentrations in caged fish were not determined, sediment PCB levels exceeded those measured in previous studies in which induced CYP1A was detected in caged fish [21, 120]. These high levels of PCBs in both sediment and biota confirm the presence of contaminant levels capable of inducing the observed biomarker response.

Although gill CYP1A mRNA can be elevated in response to contaminant exposure through either aqueous or dietary routes, aqueous exposure is known to result in greater gill CYP1A expression relative to dietary exposure [144]. Gill CYP1A expression, in conjunction with evidence that caged fish consumed little food, suggests that caged fish were exposed to contaminants in part, if not mainly, via an aqueous route. Although statistical significance was not observed, the near 4-fold elevation of CYP1A mRNA levels in gills of fish caged in the Town Branch remediated site relative to those caged at the Town Branch reference site suggests biologically effective pollutant exposure was present at the remediated Town Branch site. Although PCBs are strongly hydrophobic, with dissolved water concentrations on the order of

pg/L to ng/L, aqueous exposure to PCBs can nevertheless be a significant route of PCB exposure to aquatic organisms [69, 145]. The high ventilation rate of fish makes them particularly efficient at extracting hydrophobic organic pollutants from the water column [146]. The decline in condition factor and the limited stomach contents of caged fish during the experiment relative to laboratory controls further indicates that dietary exposure was not a major route of exposure. Exposure via aqueous routes, possibly coupled with exposure to suspended sediment during two high water events occurring during the experiment, seems the most likely route of contaminant exposure to the caged fish.

The prolonged elevation of CYP1A mRNA observed in the present study indicates that caged fish were likely responding to the presence of chlorinated inducers at the study sites. In scup (*Stenotomus chrysops*) exposed to 2,3,7,8 – tetrachlorodibenzofuran, CYP1A mRNA, EROD, and CYP1A protein concentrations all remained elevated and highly correlated during a two-week period following a single intraperitoneal injection [147], likely reflecting the resistance of halogenated inducers to metabolism and excretion [121]. This contrasts with the shorter duration of CYP1A mRNA induction following exposure to rapidly metabolized, non-halogenated inducers, such as polynuclear aromatic hydrocarbons (PAHs). Kloepper-Sams and Stegeman [148] reported that CYP1A mRNA concentrations in killifish returned to control levels five days following a single intraperitoneal injection of the PAH, ß-naphthoflavone while CYP1A protein and EROD activity levels remained elevated for at least 13 days. Similarly, Levine and Oris [149] reported that in trout aqueously exposed to the PAH, benzo[150]pyrene, hepatic CYP1A mRNA concentrations returned to control levels within 72 to 120 hours while EROD activity remained elevated through 120 hours. The temporal pattern of CYP1A mRNA induction by chlorinated compounds described above, together with the prolonged elevation of CYP1A mRNA in the caged trout, and our analyses demonstrating that PCBs are present and bioavailable in this system, supports our conclusion that CYP1A levels in caged trout likely reflect their exposure to PCBs.

Confinement coupled with relatively high water levels likely induced stress in caged fish during the course of this study. Stress hormones including cortisol are known to modulate CYP1A enzyme activity in both mammals [151] and cultured fish hepatocytes [152, 153]. In a study examining the effects of caging stress on EROD, tilapia exposed to 3,4,5,3',4' – pentachlorobiphenyl (PCB 126) had significantly higher hepatic and head kidney EROD activity

following a 2-hour confinement relative to unconfined, PCB treated fish [154]. Jorgensen et al. [155] demonstrated that chronically high cortisol levels resulted in a downregulation of induced CYP1A protein levels in arctic charr (*Salvelinus alpinus*), although both protein and EROD levels were still induced significantly over untreated controls. While stress hormones do modulate CYP1A, this modulation does not appear to either mask exposure effects or to threaten CYP1A's usefulness as a biomarker.

The condition factor of caged fish in this study approximated the condition factor observed in healthy wild salmonids, including rainbow trout [156]. This indicates that the condition of caged fish was not out of the normal range observed in salmonids. Moreover, complete lack of feeding (starvation) in rainbow trout for periods up to three weeks has been reported to enhance CYP1A response to inducers [157]. Based on these studies, it is highly unlikely that inadequate feeding compromised the results of the present experiment. .

In summary, we found CYP1A expression in caged trout to consistently reflect PCB concentrations in contaminated waterways, that two weeks may be an optimal exposure period (longer may lead to resistance), and that CYP1A can help determine route of exposure. Moreover, we found the use of caged fish to be preferable to resident fish for biomarker-based pollution evaluations. CYP1A expression in caged rainbow trout successfully discriminated among varying levels of contamination (this study) in sites where CYP1A expression in resident fish was homogenous [115]. We conclude that CYP1A expression in caged trout is a reliable, robust and inexpensive alternative to chemical analysis that can be used for first pass determination of relative environmental pollution and pollutant bioavailability in aqueous systems.

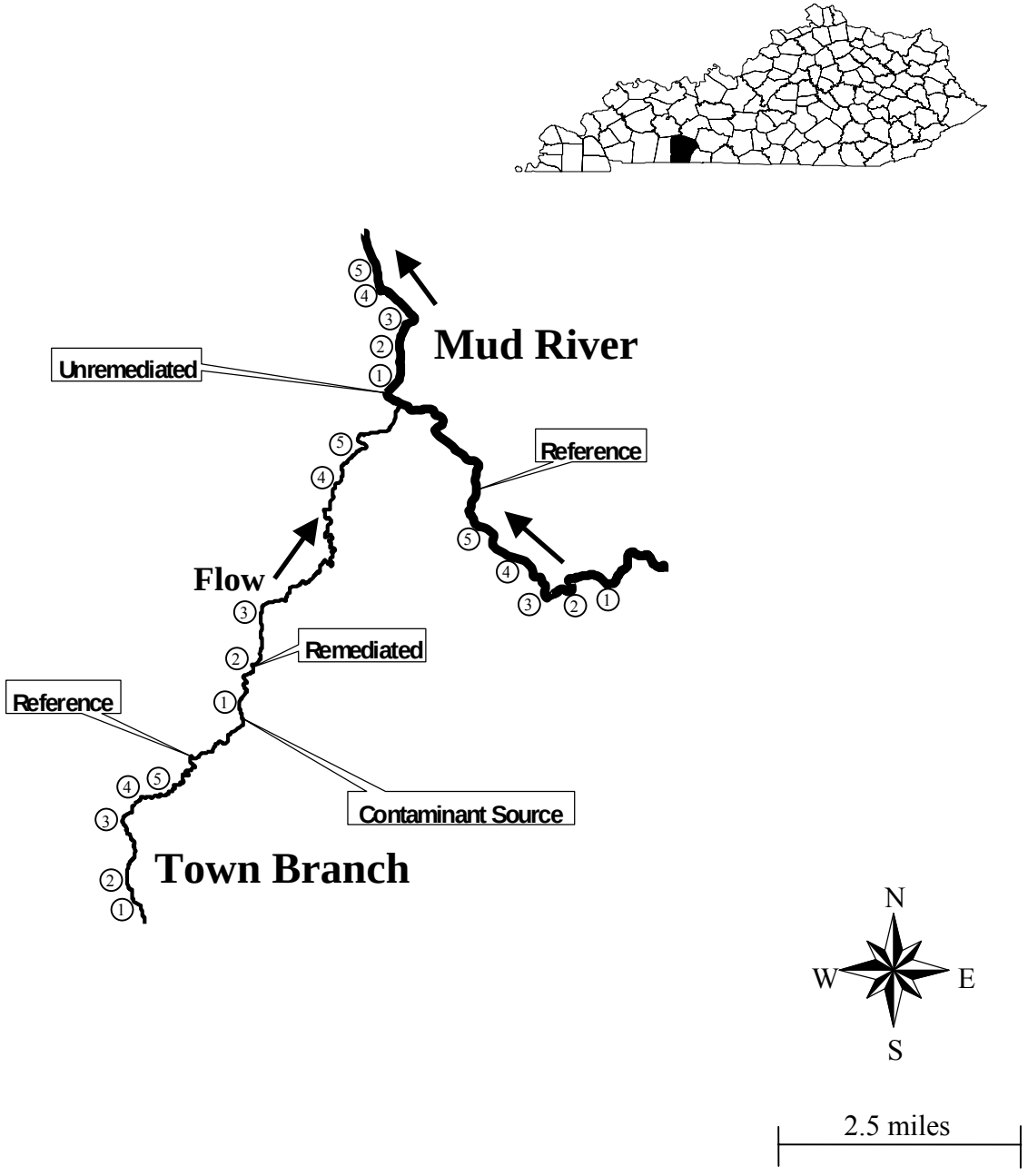
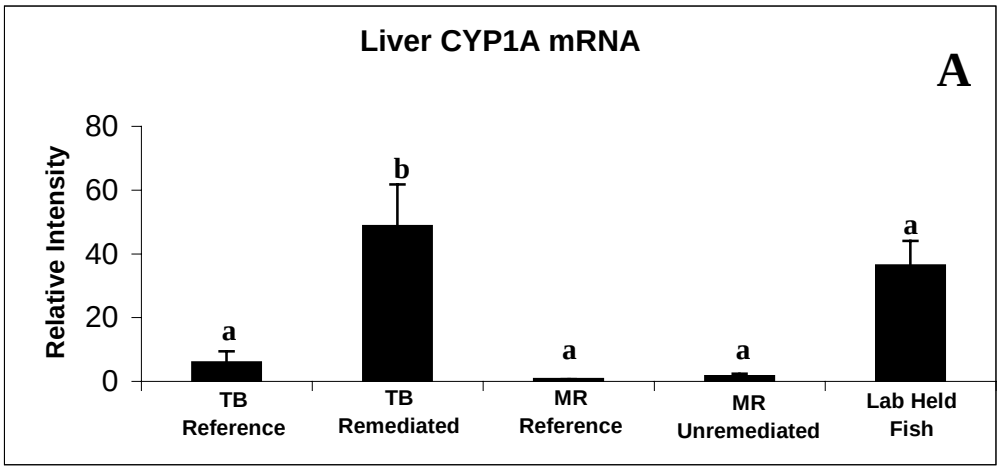


Figure 2.1. Location of study sites in the Town Branch/Mud River system, near Russellville, Kentucky.


Liver CYP1A mRNA
A
Relative Intensity
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TB Reference
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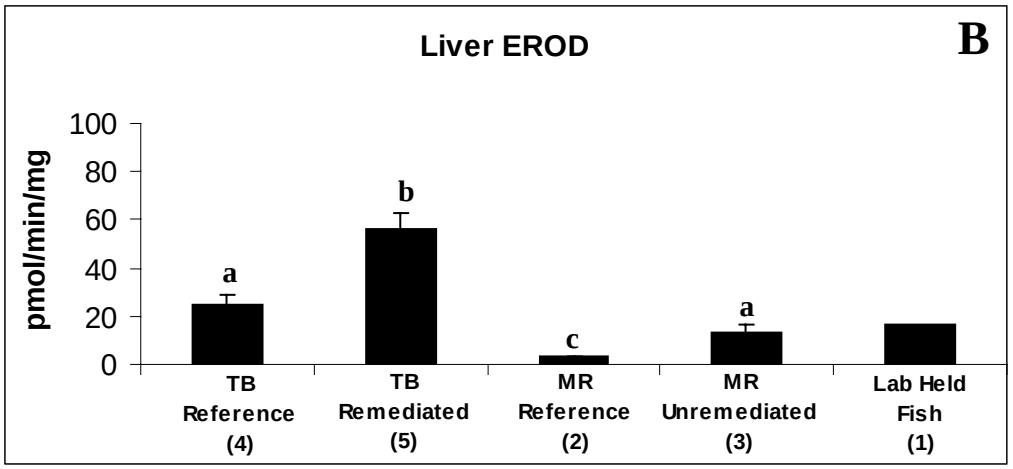
MR Reference
MR Unremediated
Lab Held Fish


Liver EROD
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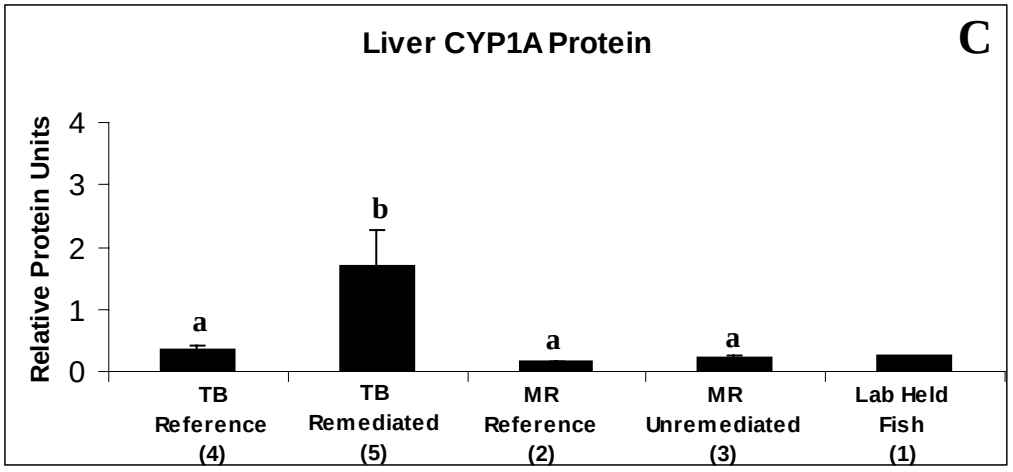
TB Remediated (5)
MR Reference (2)
MR Unremediated (3)
Lab Held Fish (1)


Liver CYP1A Protein
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Relative Protein Units
4
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TB Reference (4)
TB Remediated (5)
MR Reference (2)
MR Unremediated (3)
Lab Held Fish (1)

Figure 2.2. Relative CYP1A mRNA levels, CYP1A protein levels, and EROD activity (pmol resorufin/min/mg) in liver microsomes of rainbow trout caged in the Town Branch/Mud River system for two weeks during April/May 2002. Bars represent means ± SE. Sample size was n=5 for each treatment in A and equal to (n) for both B and C. Means with the same letter are not significantly different at $p < 0.05$.

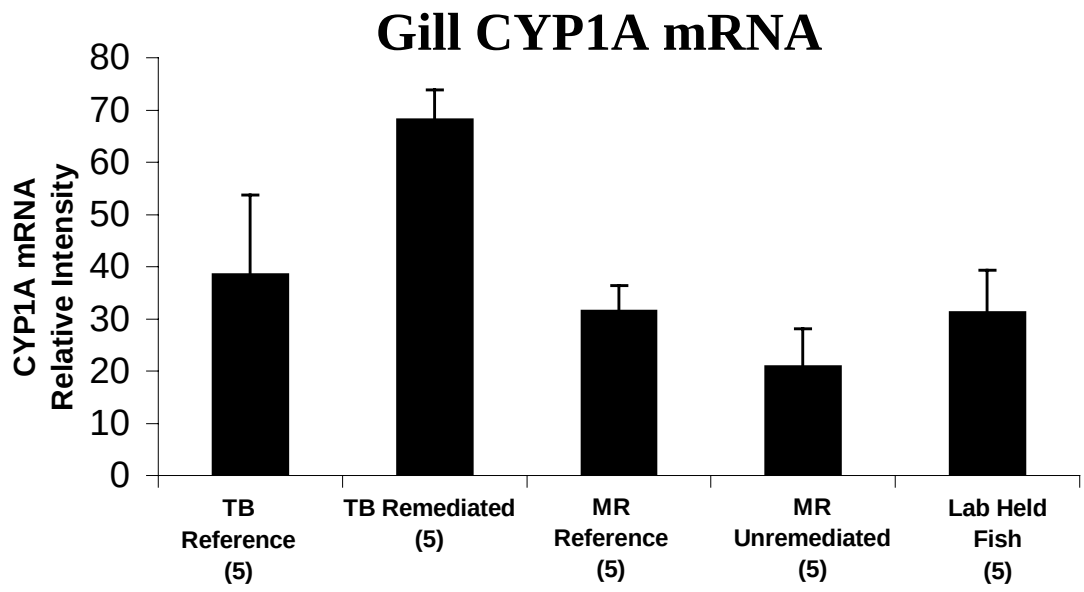


Figure 2.3. Relative CYP1A mRNA levels in gill tissue of rainbow trout caged in the Town Branch/Mud River system for two weeks during April/May 2002. Bars represent means ± SE for five individuals. All means are similar at $p < 0.05$.

Table 2.1. Water quality parameters recorded during the 2-week caging experiment at the Town Branch/Mud River study sites during April-May 2002.

Site	Temperature (°C)		pH
	Day 0	Day 14-15	
Town Branch Reference	13.0-14.0	16.5 - 17.5	7.50
Town Branch Remediated	14.0-16.0	17.0 - 18.8	7.86
Mud River Reference	15.0	18.0	7.64
Mud River Unremediated	19.0	18.5	8.23

Table 2.2. Pre- and post remediation PCB concentrations (µg/g dry weight) in sediment samples collected from Town Branch(TB)/Mud River (MR) study sites in 1987 and 2002 (Mean ± SE (n)).

	Pre-remediation (1987)a	**Post-remediation (2002)**
Site	**Mean Total PCB conc. (ppm dry weight)**	**Mean Total PCB conc. (ppm dry weight)**
TB Reference	0.08 ± 0.03 (5)	0.033 ± 0.213(2)
TB Remediated	184.3 ± 121 (5)	45.7 ± 7.44 (4)
MR Reference	0.06 ± 0.02 (3)[b]	B.D.[c](2)
MR Unremediated	2.36 ± 1.35 (5)	0.086 ± 0.047 (2)

[a] Pre-remediation data taken from Birge et al. [153].
[b] PCBs were only detected in 3 of 5 samples collected at this site
[c] Below detection (< 0.008 ppm)

Table 2.3. PCB concentrations in resident fish collected from the Town Branch/Mud River system in October 2002 (mean ± SE(n))

Study Site	Species	Mean Total PCB conc. (ppm wet weight)
Town Branch Reference	Green Sunfish	0.525 ± 0.19 (2) a
	Creek Chub	1.23 ± 0.822 (2) a
Town Branch Remediated	Green Sunfish	21.7 ± 6.8 (5)* a
	Creek Chub	75.2 ± 14.4 (4)*a
	Longear Sunfish	16.7 ± 5.05 (3) a
Mud River Unremediated	Green Sunfish	1.28 ± 0.22 (4)
	Longear Sunfish	1.63 ± 0.74 (3)
	Spotted Bass	7.42 ± 0.97 (3)
	Yellow Bullhead	4.36 ± 0.21 (4)
Mud River Reference	Longear Sunfish	0.043 ± 0.045 (4)

[a] [150]

* Significantly different from the same species collected from Town Branch reference site, $p <$ or $= 0.05$

Chapter 3. Pollutant response in species inhabiting chronically contaminated habitats: two varieties of resistance?

* PCB analysis conducted by David Price and Wesley J. Birge, Department of Biology, University of Kentucky, Lexington, KY

3.1 Abstract

Chronic exposure to organic pollutants such as polychlorinated biphenyls (PCBs) can lead to the development of resistance to these chemicals, a condition associated with reduced response of CYP1A, a pollutant-inducible biomarker. We hypothesized that wild fish in the Town Branch/Mud River system (Logan County, KY), a stream historically contaminated with PCBs, had developed resistance to these pollutants. As a first step in evaluating chemical resistance in these populations, we measured CYP1A expression and PCB body burdens in resident fish from sites previously characterized (Chapter 2) as containing biologically significant levels of CYP1A inducing compounds. Despite high PCB concentrations in muscle tissue (16.7 to 75.2 μg PCB/g wet edible flesh) most species of resident fish from the contaminated Town Branch/Mud River sites had CYP1A activity levels similar to those of reference fish, suggesting reduced sensitivity to CYP1A induction. Laboratory PCB challenge experiments demonstrated that yellow bullheads (*Ameiurus natalis*) from the Town Branch contaminated site are insensitive to CYP1A induction following PCB exposure while reference yellow bullheads responded, indicating TB bullheads have developed resistance to CYP1A mediated PCB induction. Green sunfish (*Lepomis cyanellus*) from both contaminated and reference sites were relatively insensitive to PCB treatment, consistent with results (Chapter 4) in our laboratory indicating several members of the *Lepomis* genus are relatively insensitive to PCB mediated CYP1A induction. Similar to green sunfish, spotted bass (*Micropterus punctulatus*) from both reference and contaminated sites treated with PCBs in the laboratory demonstrated no EROD response. This work provides the first evidence of pollutant resistance in a species within the family Ictaluridae and characterizes an apparent natural resistance in two members of the family Centrarchidae.

3.2 Introduction

The xenobiotic metabolizing enzyme, cytochrome P-4501A (CYP1A), is strongly induced by planar organic pollutants and is widely used as a biomarker of exposure to organic environmental contaminants such as PCBs [17, 18]. Following their uptake across the cell membrane, inducing compounds, such as planar PCBs, bind to the cytosolic aryl-hydrocarbon receptor, initiating transcription of a number of genes, including CYP1A [20]. While endogenous levels of the enzyme are relatively low, strong and rapid (within hours) induction of CYP1A mRNA and catalytically active protein occurs in response to inducing compounds [19], making CYP1A an ideal biomarker of exposure to such pollutants. Quantification of CYP1A mRNA, protein, and activity (measured as ethoxyresorufin-O-deethylase, EROD) levels are often used in biomonitoring as a measure of exposure to organic contaminants [21].

Numerous studies have demonstrated that populations living in chronically contaminated habitats can develop resistance to pollutants [100-102], a condition that may be associated with reduced response of CYP1A [103]. Fish from populations resistant to organic contaminants fail to express elevated levels of CYP1A and experience lower rates of mortality and developmental deformities relative to reference fish when exposed to inducing compounds [104], seemingly demonstrating enhanced fitness in contaminated habitats. Acquired resistance has been reported in a number of fish populations including several different species, all of which in reside in chronically contaminated habitats. Marsh minnows (*Fundulus heteroclitus*) from areas heavily contaminated with organic pollutants are reported to express lower levels of CYP1A activity in response to PCB exposure relative to reference populations [105, 106]. Acquired resistance has also been reported in Atlantic tomcod [107] and largemouth bass [108, 109] inhabiting the PCB contaminated Hudson River. Yellow perch inhabiting a lake chronically contaminated by PCBs have also been reported to display acquired resistance [110]. Acquired resistance appears to allow population survival in highly contaminated environments.

The Town Branch/Mud River system, located in southwestern Kentucky, has been heavily contaminated with PCBs from a local manufacturing plant since the 1960s [127]. The PCB levels in fish from the Town Branch contaminated section are 70 -300 times

higher than those measured in resident fish from the lower Hudson River [158], and fall at the high end of those measured in fish from New Bedford Harbor, Massachusetts, considered one of the most highly contaminated PCB Superfund sites in the U.S [143]. Furthermore, my research has demonstrated that these PCBs are bioavailable to fish residing in this system [159]. We hypothesize that resident fish in this system have developed resistance to PCB mediated CYP1A induction.

3.3 Methods

Materials

7-Ethoxyresorufin and resorufin were obtained from Molecular Probes (Eugene, OR, USA). The monoclonal antibody made against scup CYP1A protein, MAb 1-12-3, was a generous gift of Dr. John Stegeman (Woods Hole Oceanographic Institution). Cy^{TM}5-conjugated affinipure goat anti-mouse IgG was obtained from Jackson Immunoresearch Laboratories Inc. (West Grove, PA, USA) and precast polyacrylamide gradient gels were from Invitrogen (Carlsbad, CA, USA). Nitrocellulose membrane (0.45 μm) was obtained from Schleicher and Schull (Keene, NH, USA). The Bio-Dot SF Microfiltration Apparatus was obtained from Bio Rad (Hercules, CA, USA). All other reagents were obtained from Sigma, Fisher Scientific (Fair Lawn, NJ), or Invitrogen.

Study Sites

Town Branch/Mud River

The Town Branch/Mud River system (Figure 3.1) has been contaminated since the 1960s with PCBs from a local manufacturing plant. For over 20 years PCBs were released into a lagoon behind the plant that leaked waste containing high concentrations of PCBs (as high as 332,500 ppm) into Town Branch approximately 8 km (5 miles) upstream of its confluence with the Mud River [127]. Sediment PCB concentrations of 280 ppm (dry sediment, clay-silt fraction) [160] were documented in Town Branch in 1986, initiating remediation efforts on Town Branch that began in 1997. Removal of contaminated sediments from both the streambed and floodplain of Town Branch was completed in July 2001 (Michael Mills, KY Division of Water, personal comm.). Despite remediation we documented extremely high levels of PCBs (45.7 μg/g dry

weight) (Table 2.2) in sediments in the remediated Town Branch section. No remediation has been conducted in the Mud River section downstream of the Town Branch confluence, and relatively high levels of PCBs continue to be found in sediments in this area [130].

Fishing Creek

When fish were not available from the upstream reference section fish from a comparable clean stream, Fishing Creek, were utilized as an alternative. Fishing Creek is a relatively clean stream located in south central Kentucky (Pulaski, County) similar in size and habitat to the Mud River. All contaminants measured in fish tissue from this system were either very low or below detection (KY DEP 2001) making it an ideal reference stream for the contaminated Mud River section.

Experimental Design

Field Study

Fish were collected from contaminated and reference areas, sacrificed in the field or within 24 hours of returning to the laboratory, and livers flash frozen in liquid nitrogen. Several species were selected for the study based on availability and previous studies detailing pollutamt response in related species. Yellow bullheads were collected from contaminated sections of the Mud River and from Fishing Creek, green sunfish were collected from contaminated and clean sections of Town Branch, and spotted bass were collected from contaminated sections of the Mud River and from Fishing Creek.

Laboratory Study

Green sunfish and yellow bullhead were collected from Town Branch reference and contaminated sections and held in the laboratory for 14 weeks to depurate their body burden of contaminants, a length of time exceeding the half-life (eight weeks) of the predominate CYP1A inducing PCB congeners in fish tissue [161]. Fish were held at 17-20^{o}C and fed mealworms ad libetum two times per week for two months and then at a rate of approximately 3% of their body weight two times per week for the two months prior to the experiment. Spotted bass were collected from contaminated sections of the Mud River and from Fishing Creek and depurated 11 weeks prior to the experiment [162, 163]. The 11 week depuration was utilized instead of the 14 week because of difficulty

in holding these large, aggressive fish in the laboratory. Several specimens from the Mud River group perished in the lab, seemingly a result of aggression in large common tanks. Careful examination of half-lifes of CYP1A inducing congeners present in this system revealed that 11 weeks exceeded the half-lives of each congener. Fish were held at 17-20^{o}C and fed fathead minnows purchased commercially ad libetum approximately once a week. All fish were fasted for seven days prior to treatment and injected intraperitoneally with either PCB 77 at 1 mg/kg in corn oil or with a corn oil vehicle control. Fish were sacrificed on day seven following injection and livers were removed and flash frozen in liquid nitrogen.

Liver Microsomal Protein Isolation

Livers were removed from liquid nitrogen, weighed, and sub-sectioned. Aliquots for RNA analysis (0.07-0.2 g) were refrozen in liquid nitrogen. Aliquots for microsome preparation were immediately homogenized in 10 volumes (weight:volume) of ice-cold 50 mM Tris buffer (pH 7.4). Microsomal fractions were obtained by differential centrifugation as previously described [131]. The final 100,000 x g microsomal pellets were resuspended in 50 mM Tris buffer (pH 7.4) containing 1mM EDTA, 1 mM DTT and 20% glycerol at a 1:1 ratio (liver weight: resuspension buffer volume). Microsomal samples were stored in liquid nitrogen until analyzed for catalytic activity and CYP1A protein content (within three weeks).

Catalytic and Protein Assays

Ethoxyresorufin -O-deethylase (EROD) activity, a reaction catalyzed exclusively by CYP1A [4], was measured fluorometrically in 48 well CoStar™ plates using the method of Hahn et al. [133]. The reaction mixture contained 50 mM Tris, pH 7.4, 0.1 M NaCl, 2μM 7-ethoxyresorufin, and 100 to 300 ug of microsomal protein in a final volume of 200 μl. The reaction was initiated by the addition of 1.34 mM NADPH, run at room temperature (28-29^{0}C), and production of the fluorescent product, resorufin, measured for 13 minutes. The linear portion of the curve was used to evaluate the rate of the reaction. All EROD assays were run in triplicate on a PerSeptive Biosystems Cytofluor Series 4000™ multi-well plate fluorometer.

Microsomal protein was measured fluorometrically using the method described by Lorenzen and Kennedy [134] with bovine serum albumin as the standard. All protein assays were run in triplicate.

Immunoblotting Procedures

CYP1A protein was quantified by immuno-blotting using a Bio-Dot SF™ microfiltration slot-blot apparatus. Twenty micrograms of microsomal protein diluted in 200 µL buffer (20 mM Tris, 0.5 M) was loaded into each well and vacuum transferred onto a nitrocellulose membrane (0.45 µM). The membrane was incubated in TBS-5% milk at 4^{o}C overnight to block non-specific binding, followed by incubation with MAb 1-12-3 dissolved (3 µg/ml) in milk block, a monoclonal antibody which recognizes CYP1A in multiple vertebrate species [19], essentially as described by Elskus et al. 1999 [106]. CYP1A signal was detected using Cy^{TM}5-conjugated affinipure goat anti-mouse IgG as the secondary antibody (10.8 µg/ml in sodium azide) and blots were scanned at 633 nm excitation/670 nm emission using a Typhoon 8600 scanner (Molecular Dynamics) and quantified using Image Quant (Molecular Dynamics). Liver microsomes from trout treated with the CYP1A model inducer, ß-naphthoflavone, were loaded in seven concentrations ranging from 0.1 to 7 µg of protein diluted in 200 µL buffer to evaluate linearity of the CYP1A signal on each blot. All samples were run at least in triplicate.

Resident Fish Collection and PCB Analysis

Resident fish were collected from caging sites approximately five months after completion of the caging study. Fish were collected by use of a backpack shocker (Smith-Root, Inc., Model 12-B) or by a large portable floating electroshocking unit described by Price et al. 2003 [135] and were either sacrificed in the field or returned to the lab and sacrificed within 24 hours. Livers were flash frozen in liquid nitrogen. Carcasses were wrapped in aluminum foil, tagged, and stored at -20 ^{o}C until PCB extraction.

Fish were measured for length and whole body weight before fillets were taken with solvent-cleaned surgical instruments. Yellow bullhead fillets were analyzed after

removal of skin while all other fish were scaled and fillets analyzed with skin intact. PCBs in fish fillets were extracted and analyzed using standard U.S. EPA methods [136]. The fillet samples were weighed, then ground with 20g anhydrous sodium sulfate and extracted with petroleum ether in a Soxhlet apparatus for 5-h. The extracts were concentrated to near dryness in a Roto-evaporator (Buchi Model RE121). Lipid and pesticides were removed from the reconstituted samples (5.0 mL in iso-octane) as described [136] and then analyzed by gas chromatography. A 1.0 mL sub-sample was taken for lipid determinations prior to clean-up. Elemental sulfur was removed by shaking the extract with 2-propanol (2 mL) and tetrabutylammonium sulfite (2 mL), adding ultra-pure water (8 mL) and reshaking. The organic phase was removed and mixed with 2.0-mL concentrated sulfuric acid [136], SW-846 Method 3660B, sulfur cleanup]. A 4μL aliquot was then analyzed by gas chromatography.

Samples were analyzed for Aroclors 1248, 1254, and 1260 according to SW-846 Method 8082 (polychlorinated biphenyls by gas chromatography; [136]). Analysis was performed using a Hewlett-Packard (HP) Model 5890A gas chromatograph equipped with an electron capture detector and an HP Model 7673A Automatic Sampler. Samples were analyzed using a 60m X 0.53mm ID SPB-5 (0.5μm film) fused silica megabore column (Supelco, Inc.) with ultra-high purity helium and nitrogen as carrier and makeup gases, respectively. The temperature program was 160 °C (6 min); 10 °C/min-235 °C (0 min); 0.9 °C/min-260 °C (10 min); injector temperature, 280 °C; detector temperature, 300 °C. PCB peak heights were quantified using an HP Model 3396A integrator and multiple-peak linear regression analysis was performed with Lotus-123® software. Five external standards for each Aroclor were used for calibration curves and for every tenth sample, either a solvent blank or a standard was analyzed. Detection limits for tissue samples ranged from 12 to 160 μg PCB/kg tissue. Reported values are corrected for recovery.

Sediment Collection and Extraction for PCB Analysis

Sediment samples were collected from caging sites in Fall 2002, approximately five months after completion of the caging study. Samples were restricted to the upper 5-10 cm of stream sediment. All sediment samples were collected in acetone-rinsed 0.47 L glass jars with Teflon-lined lids with stainless steel spoons and scoops. Collected sediments were placed on ice for transport to the laboratory where they were refrigerated.

Wet sediment extractions of PCBs were performed following U.S EPA SW-846 Method 3540C [136]. Weighed sub-samples (61.5 ± 12.9 g wet wt.; 42.7 ± 8.9 g dry wt.) were extracted with 300 mL of acetone/methylene chloride (1:1 v:v) in a 500-mL Soxhlet extractor for 15 h. The extract was concentrated, cleaned, and analyzed as previously described [136]. Detection limits for sediment samples ranged from 8 to 17 µg PCB/kg sediment. Reported values are corrected for recovery.

Statistical Treatment of Data

Statistical analyses were performed using SYSTAT Version 10. All data were transformed (log 10) before analysis. Field EROD data were first analyzed using multivariate analysis of variance followed by two tailed t-tests to achieve mean separation. Laboratory experiment data were analyzed using a two-way ANOVA. P-value for the interaction terms were halved because of a priori assumption that the difference in treatment response would increase, not decrease, between the two sites. Differences in fish PCB body burdens were evaluated using two tailed t-tests. All differences were considered significant at $p <$ or $= 0.05$.

3.4 Results

Field Studies

Hepatic EROD activity was similar in yellow bullhead collected from contaminated areas of the Mud River and from the reference site Fishing Creek (Figure 3.2 A). Green sunfish collected from reference areas of Town Branch displayed levels of EROD activity that were significantly higher than those observed in the remediated section (Figure 3.2 B). EROD activity in spotted bass collected from the unremediated section of the Mud River was similar to that observed in fish collected from the reference

site Fishing Creek (Figure 3.2 C). Temperature is known to affect CYP1A activity and protein levels and was therefore evaluated at each site to verify uniformity among collections (Table 3.1).

PCB challenge experiments

Depurated yellow bullhead from the reference areas injected with PCB 77 displayed levels of EROD activity and CYP1A protein elevated significantly over vechicle injected animals (Figure 3.3). Yellow bullhead from the remediated sections of Town Branch failed to show significantly elevated levels of either EROD activity or CYP1A protein levels relative to control animals (Figure 3.3). The interaction term of the two-way ANOVA was significant for EROD ($p=0.035$) but not protein ($p=0.064$). A power analysis indicated that the power of this experiment to detect a difference in response to treatment between fish from the reference and remediated site was $p=0.944$ for EROD and $p= 0.852$ for protein.

Green sunfish collected from both reference and remediated areas of Town Branch failed to show significant induction of EROD activity relative to controls when treated with PCB 77 (Figure 3.4). The interaction term of the two-way ANOVA was insignificant ($p=0.250$). A power analysis indicated that the power of this experiment to detect a difference in response to treatment between fish from the clean and remediated site was $p=0.559$. EROD values were also very low in all fish, the highest levels only reaching half the lowest level observed in any other species in this study. CYP1A protein levels were below detection limits (data not shown).

Spotted bass from both the unremediated section of the Mud River and the reference site Fishing Creek failed to show induction of EROD activity in PCB treated animals relative to controls (Figure 3.5). The interaction term of the two way ANOVA was insignificant ($p=0.337$). A power analysis indicated that the power of this experiment to detect a difference in response to treatment between fish from the clean and unremediated site was $p=0.801$. EROD activities in spotted bass were greater than those observed in green sunfish. CYP1A protein levels were below detection limits (data not shown).

PCB Analysis

PCB concentrations in Mud River reference sediment were below detection limits. PCB levels in the Mud River unremediated sediment were at least ten times greater than those documented in the reference section of the Mud River (Table 3.2). PCB sediment concentrations in the Town Branch reference section were very low in stark contrast to the excessive concentrations observed in the remediated section of Town Branch (Table 3.2). PCB body burdens in fish mirrored those in sediment (Table 3.3). PCB concentrations in green sunfish (*Lepomis cyanellus*) and creek chubs (*Semotilus atromaculatus*) from the Town Branch reference site were significantly lower (41 and 58 times respectively) than PCB body burdens in fish collected in the Town Branch remediated site. Longear sunfish collected from the Mud River unremediated site had PCB body burdens approximately 38 times those observed in the Mud River reference site longear sunfish (Table 3.3).

PCB analysis was conducted on depurated yellow bullhead to determine if depuration significantly reduced PCB body burdens in this species. Yellow bullhead collected from the Town Branch remediated section and depurated for 14 weeks displayed PCB body burdens similar to those measured in yellow bullhead collected from the reference section of Town Branch and depurated 14 weeks (Table 3.4).

3.5 Discussion

The results of this study demonstrate a pollutant response previously undocumented in the species examined. The lack of CYP1A response in yellow bullhead collected from contaminated areas of the Town Branch/Mud River system in both field and laboratory studies documents for the first time the development of pollutant resistance in an Ictalurid species. The failure of green sunfish and spotted bass CYP1A to respond to PCB exposure in both field and laboratory settings suggests natural lack of sensitivity to PCBs in these species. These results provide novel information on the pollutant sensitivity of these widespread species and highlight issues that must be considered when using feral fish as biomonitoring tools.

Hepatic CYP1A activity and protein levels in field caught fish are frequently utilized as biomonitoring tools, presumably reflecting environmental pollutant levels,

although a growing body of evidence suggests inherent problems with this technique. Numerous studies report elevated hepatic CYP1A protein and/or activity in fish residing in contaminated habitats relative to fish residing in relatively clean habitats [17, 118-120]. However, recent studies have identified a number of populations residing in highly contaminated sites that fail to display elevated hepatic CYP1A activity and protein. Yellow perch captured from a lake heavily contaminated with PCBs displayed hepatic CYP1A levels similar to those observed in perch collected from a relatively clean lake, subsequent studies characterized acquired resistance in this population [110]. Likewise, resistant killifish collected from a highly contaminated PAH site displayed levels of CYP1A activity and protein similar to those observed in reference site killifish [164]. Resistant largemouth bass inhabiting highly contaminated sections of the Hudson River also displayed CYP1A activity and protein levels similar to those collected from reference sites [109]. These results are similar to those reported in this study in which all species examined showed a lack of elevated CYP1A levels in contaminated site fish and emphasize the need for caution in the use of field collected fish as biomonitoring tools.

Acquired pollutant tolerance in a population is typically characterized by exposing a population from a contaminated site and a control population from a clean site to identical pollutant treatments and characterizing their respective CYP1A responses. While some studies report complete absence of CYP1A response in resistant organisms [165], a more common scenario is reduced response in the resistant population (relative to controls). The non-significant response observed in laboratory treated yellow bullhead in this experiment is typical of fish populations exhibiting acquired resistance. For example, resistant female yellow perch from a highly PCB contaminated lake displayed EROD activity elevated two-fold (although non-significantly) over controls when treated with 0.3 mg/kg PCB 77 [110]. Arzuaga and Elskus [166] observed CYP1A mRNA (but not activity or protein) elevated (non-significantly) approximately three-fold over controls in treated versus control resistant killifish. Resistant tomcod displayed CYP1A mRNA levels elevated, although nonsignificantly, 2.1 fold over controls seven days after treatment with PCB 77 at 0.1 mg/kg [167]. In the present study, yellow bullhead from the Town Branch remediated site displayed CYP1A activity elevated only slightly over

controls and CYP1A protein elevated approximately three-fold over controls, neither difference was statistically significant (Figure 3.3).

Green sunfish pollutant response in this study was unique in that neither fish from the clean nor contaminated site responded to PCB treatment (Figure 3.4). Previous work in our laboratory has indicated several members of the *Lepomis* genus may be relatively insensitive to PCB mediated CYP1A response [168, 169]. To our knowledge there are no other studies evaluating CYP1A response following PCB exposure in *Lepomis* species. Green sunfish appear to demonstrate a natural lack of sensitivity of PCB mediated CYP1A induction, likely conveying the same adaptive traits as the afore discussed acquired resistance and possibly contributing to green sunfish's reputation as a hardy fish able to survive in highly compromised habitats. Green sunfish have long been considered highly tolerant of degraded habitats [150, 170, 171], with no mechanistic explanation.

The results of the spotted bass study provide evidence indicating that hepatic CYP1A may not be induced by PCB exposure in this species. These fish are members of the sunfish (Centrarchidae) family, to which the green sunfish and other *Lepomis* species belong, suggesting that a response similar to that exhibited by *Lepomis* would not be unexpected. Previous data on spotted bass response to PCB exposure is entirely lacking. The closely (Family Centrarchidae) related largemouth bass (*Micropterus salmoides*) has been reported to show induction of CYP1A protein and activity following PCB exposure in the laboratory [109]. Caging studies with largemouth bass demonstrated moderate induction of CYP1A protein, activity, and mRNA in fish caged in an area contaminated in part, although not exclusively, by PCBs [120]. The laboratory portion of the present study suffered from the lack of sufficient numbers of fish, a phenomenon related to the difficulty of holding these large, highly aggressive fish. However, the field study and the lack of CYP1A inducibility in reference fish (Figure 3.5) suggests spotted bass are likely to also show natural resistance to PCB-mediated CYP1A induction.

It is important to note that while PCB exposure typically leads to induction of the CYP1A enzyme, suppression of CYP1A expression has been reported following experimental treatment with high doses of CYP1A inducing congeners [172, 173]. PCB mediated suppression of CYP1A is believed to occur post transcriptionally either as a result of competitive inhibition (affecting only activity) or from oxidative inactivation of

the CYP1A enzyme [172]. Gooch et al. [174] reported that competitive inhibition played a major role in the inhibition of CYP1A by higher concentrations of PCBs consistent with the observation that increased levels of PCBs resulted in suppressed levels of CYP1A activity but not protein. Data from the present study demonstrate that both CYP1A activity and protein levels were lower in yellow bullhead collected from the Town Branch remediated site relative to reference site fish, suggesting that competitive inhibition from residual PCB levels present in fish following depuration (Table 3.3) were not responsible for the reduced CYP1A response in these fish.

The results of this study provide two lines of evidence supporting the development of resistance in yellow bullhead inhabiting contaminated areas of the Town Branch/Mud River system; the lack of elevated CYP1A in field collected fish from contaminated site relative to reference site collected fish and the failure of fish from contaminated sites to display significant EROD induction following PCB treatment in the laboratory. Previous reports documenting the development of acquired resistance in species (killifish, largemouth bass, tomcod) inhabiting chronically contaminated areas [106] make the discovery of acquired resistance in one or more species in the Town Branch/Mud River system plausible.

Further research into the consequences of pollutant resistance on fish populations in the Town Branch/ Mud River system holds great potential. Informal surveys of species abundance recently conducted revealed high numbers of low CYP1A responding species (yellow bullhead, green sunfish) in the contaminated area and low numbers of high CYP1A responding species (creek chubs) in the contaminated area [108, 115, 175] suggesting a link between CYP1A responsiveness and the ability to survive and reproduce in this highly contaminated habitat.

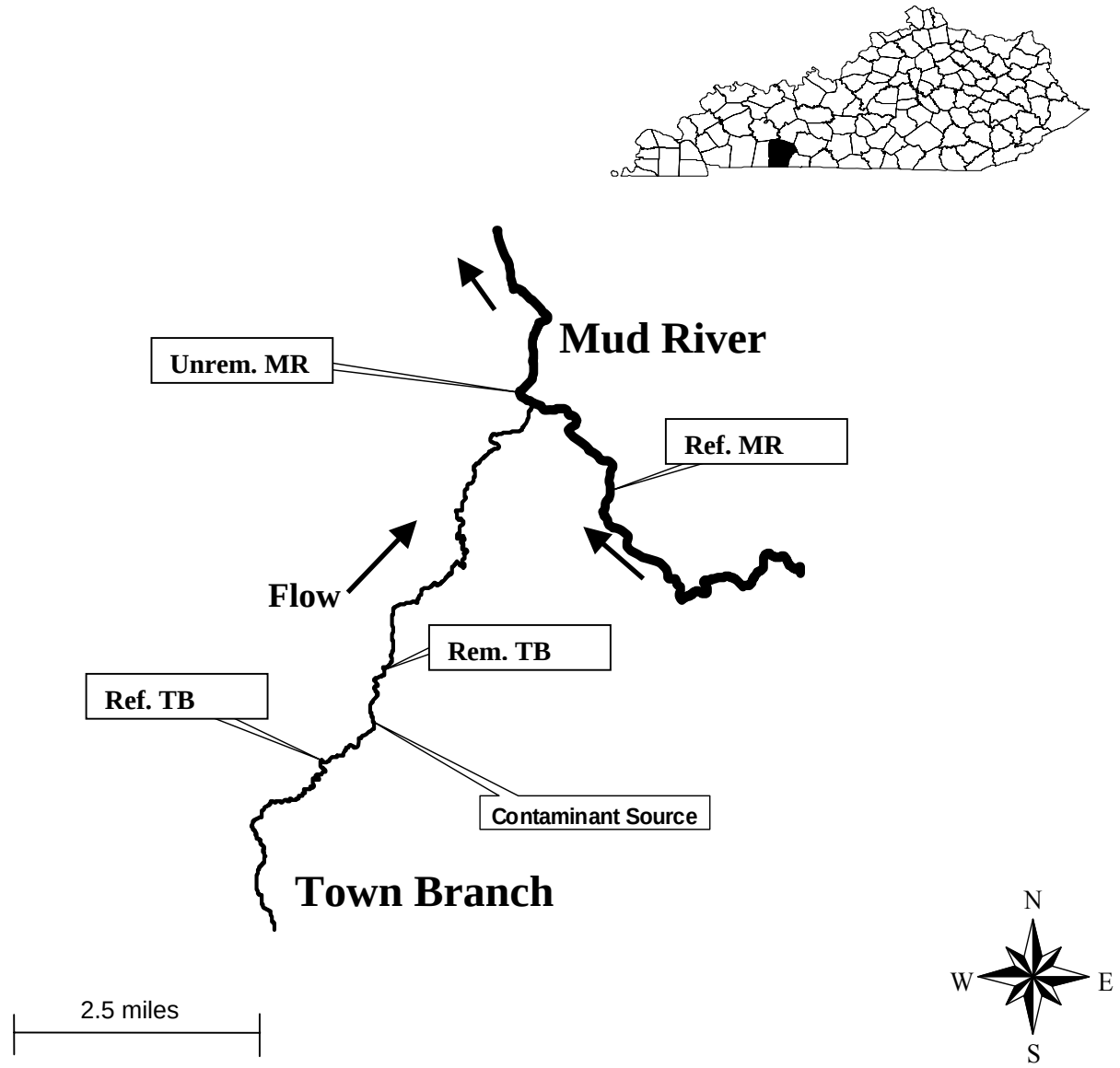


Figure 3.1. Location of study sites in the Town Branch/Mud River system (Logan County) near Russellville, Kentucky.

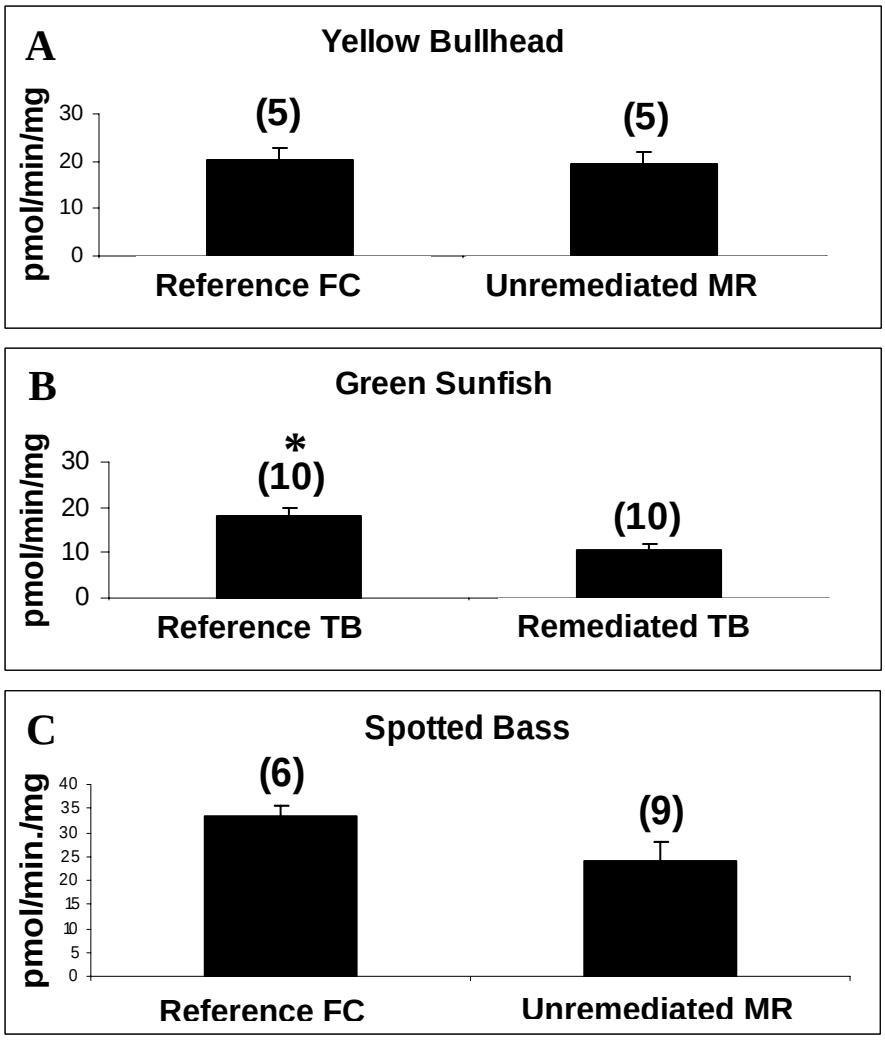
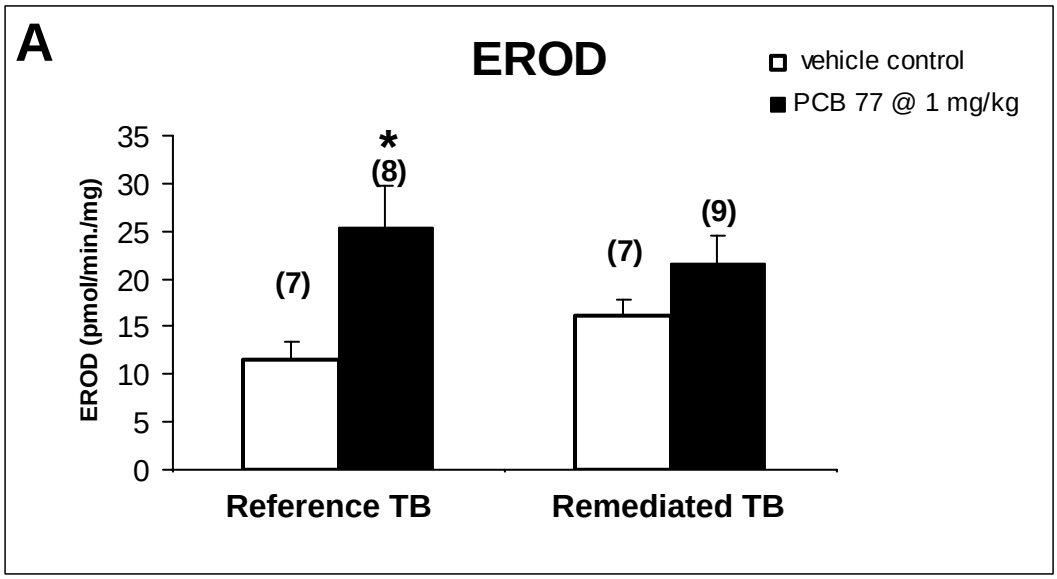


Figure 3.2. Hepatic EROD activity (in pmol resorufin/min/mg microsomal protein) in field collected (A) yellow bullhead (*Ameiurus natalis*) collected from a reference site (Fishing Creek) and unremediated areas of the Mud River, Logan County, KY (B) green sunfish (*Lepomis cyanellus*) collected from reference and remediated areas of Town Branch and (C) spotted bass (*Micropterus puntulatus*) collected from a reference site (Fishing Creek, Pulaski County, KY) and the unremediated area of the Mud River. Means +/- S.E., n numbers in parentheses, * = significantly different at $p < 0.05$.



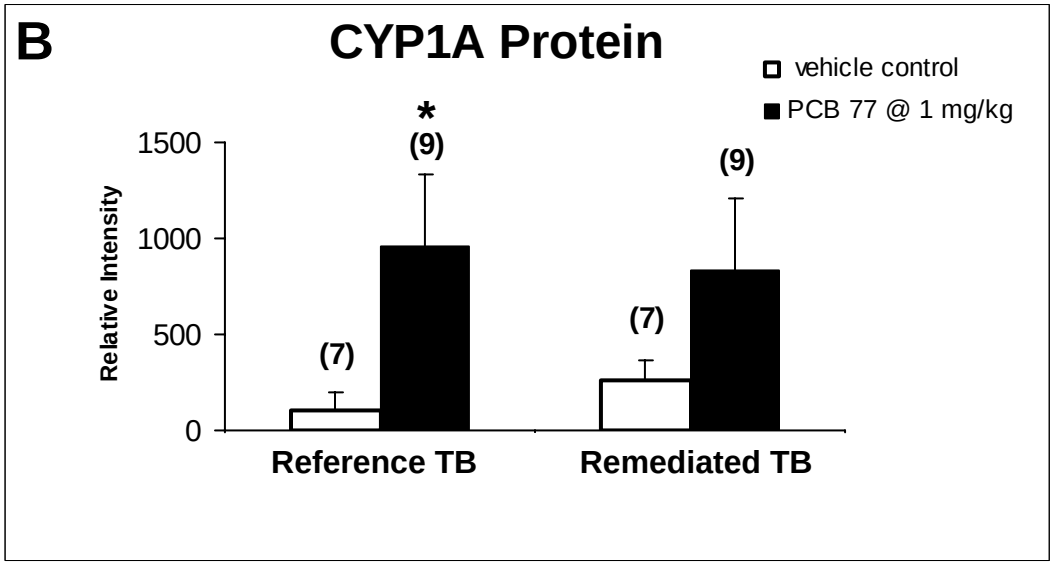


Figure 3.3. EROD activity (A) and relative CYP1A protein levels (B) in PCB–injected yellow bullhead (*Ameiurus natalis*) collected from reference and remediated areas of Town Branch, Logan County, KY. Means +/- S.E., n numbers in parentheses, * = significantly different at $p < 0.05$.

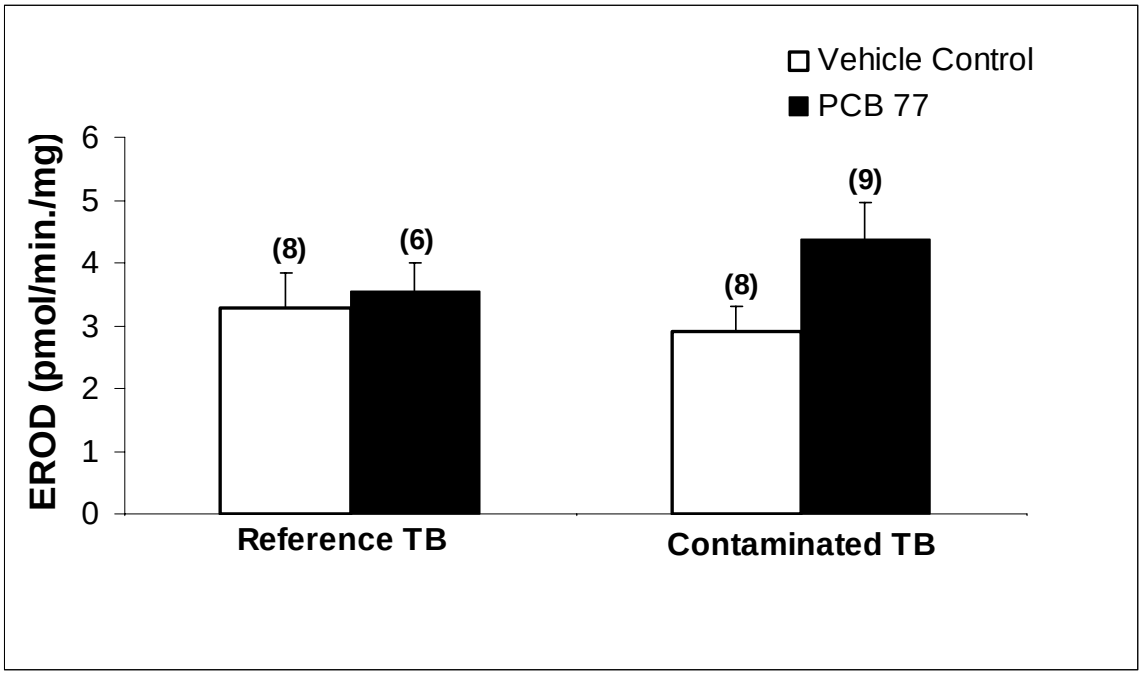


Figure 3.4. EROD activity in laboratory treated, green sunfish (*Lepomis cyanellus*) collected from reference and contaminated areas of Town Branch, Logan County, KY. Means +/- S.E., n numbers in parentheses, * = significantly differenent at $p < 0.05$.

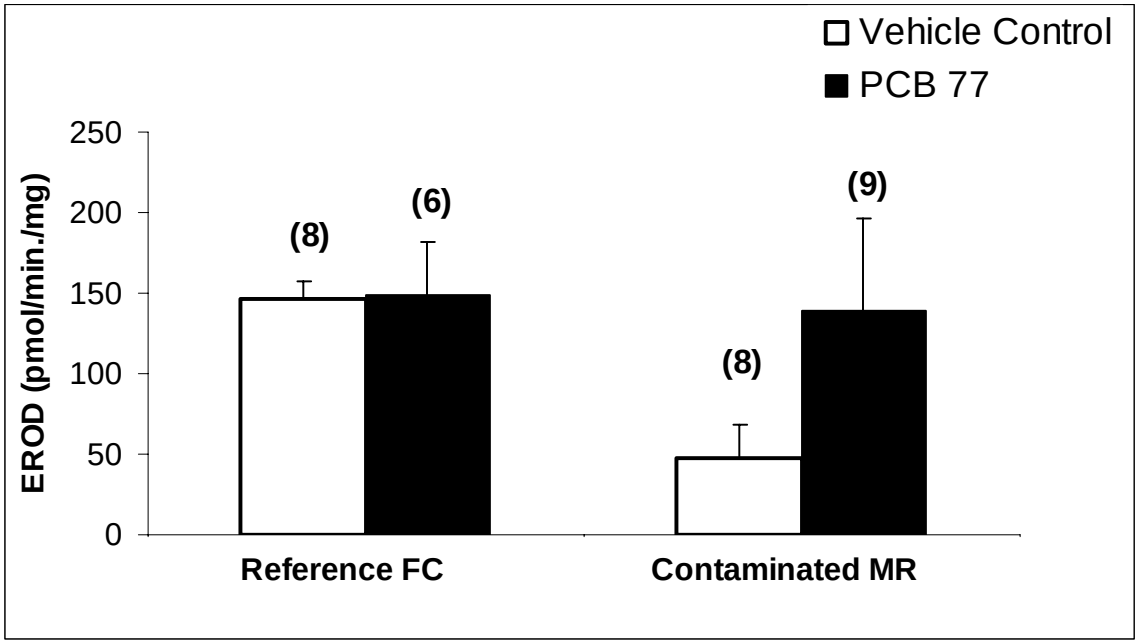


Figure 3.5. EROD activity in reference (Fishing Creek) and Mud River contaminated Spotted Bass (*Micropterus punctulatus*) following treatment with PCB 77 @ 1 mg/kg or vehicle control. Means ± S.E., n numbers in parentheses, all means were similar at p = 0.05.

Table 3.1. Collection date and water temperature for the field portion of this study.

Collection Site	Collection Date	Water Temperature (^{0}C)
Town Branch Reference	Oct. 22, 2002	18
Town Branch Remediated	Oct. 22, 2002	17
Mud River Reference	Oct. 22, 2002	14
Mud River Unremediated	Oct. 21, 2002	15
Fishing Creek	Nov. 1, 2002	12.5

Table 3.2. PCB concentrations (µg/g dry weight) in sediment from Town Branch and Mud River reference and remediated sites in 2002 (Mean ± SE (n)).

Site	Mean Total PCB conc. (ug/g dry weight)	Standard Error	n
Town Branch Reference	0.033	0.0213	2
Town Branch Remediated	45.7	7.44	4
Mud River Reference	<.009	-	2
Mud River Unremediated	0.0861	0.0469	2

Table 3.3. PCB concentrations in resident fish collected from the Town Branch/Mud River system in October 2002 (mean ± SE(n)). * = significantly different from the respective species collected from the control site.

Study Site	Species	Mean total PCB conc. (ppm wet weight)
Town Branch Ref.	Green Sunfish	0.525 ± 0.19 (2)
	Creek Chub	1.23 ± 0.822 (2) a
Town Branch Remed.	Green Sunfish	21.7 ± 6.8 (5)*
	Creek Chub	75.2 ± 14.4 (4)*a
	Longear Sunfish	16.7 ± 5.05 (3) a
Mud River Ref.	Longear Sunfish	0.043 ± 0.045 (4)
Mud River Unremed.	Green Sunfish	1.28 ± 0.22 (4)
	Longear Sunfish	1.63 ± 0.74 (3)
	Spotted Bass	7.42 ± 0.97 (3)
	Yellow Bullhead	4.36 ± 0.21 (4)

[a] [115]

* Significantly different from same species collected from Town Branch reference site, p=0.05 .

Table 3.4. PCB levels in the edible flesh of Yellow Bullheads used as vechicle controls. Means are similar at p = 0.05.

Collection Site	Mean total PCB (ppm wet weight)
TB Reference	0.371 ± 0.13 (5)
TB Remediated	3.32 ± 0.92 (5)

Mean ± S.E. (n individuals)

Chapter 4: Induction of pollutant metabolizing enzymes in *Lepomis* species following PCB and PAH exposure

* PCB analysis conducted by David Price and Wesley J. Birge, Department of Biology, University of Kentucky, Lexington, KY

4.1 Abstract

Although *Lepomis* species are abundant in a wide variety of habitats throughout North America and could be potentially valuable biomonitoring tools, few studies have examined the induction of pollutant biomarker enzymes in this genus. We hypothesized that induction of cytochrome P-450 1A, (CYP1A, Phase I) and possibly the phase II enzymes glutathione S-transferase (GST) and uridine diphosphate glucuronyltransferase (UDP-GT) would serve as effective biomarkers of pollutant exposure in *Lepomis* species. Two *Lepomis* species (longear sunfish, *Lepomis megalottis* and bluegill, *Lepomis macrochirus*) were exposed to model inducers in the laboratory representing the two major classes of CYP1A inducers, polychlorinated aromatic hydrocarbons (PAHs) and halogenated aromatic hydrocarbons (HAHs), at two doses in order to characterize the biochemical response of *Lepomis* species following pollutant exposure. Our results demonstrate differential sensitivity to the different classes of inducers in two *Lepomis* species. Longear sunfish and bluegill showed relatively low induction of CYP1A activity following exposure to 3,4,3',4'-tetrachlorobiphenyl, PCB77 (two to three fold induction over controls respectively). In sharp contrast, CYP1A activity was highly induced in longear sunfish and bluegill (15 to 37 fold induction over controls respectively) following exposure to the model polyaromatic hydrocarbon inducer benzo-a-pyrene (BaP). GST activity in bluegill and longear sunfish remained unchanged following treatment with either compound. UDPGT activity was unaffected by BaP treatment in longear sunfish, the only trial examining UDPGT response. Furthermore, longear sunfish collected from a PCB contaminated site displayed relatively low levels of CYP1A activity despite PCB body burdens that have been observed to induce CYP1A activity in other species. Our results indicate differential sensitivity to the two main classes of CYP1A inducers in

Lepomis species. These results suggest pollutant metabolizing enzymes may be poor bioindicators of PCB exposure in *Lepomis* species. However *Lepomis* species may provide a useful model system for examining the regulation and consequences of differential pollutant sensitivity.

4.2 Introduction

The use of biomarkers allows researchers to effectively evaluate the biological response to pollutants of both aquatic and terrestrial organisms [16]. Biomarkers are measurements in body cells or tissues that indicate biochemical or cellular modifications in response to the presence of a toxicant [176]. Unlike measurements of contaminants in tissues or sediments which provide no information on biological effect, alterations in biomarkers indicate that chemicals present in the system are biologically active. Biomarkers may serve as early warning systems, reflecting early adverse impacts of pollutants in ecosystems [16].

The xenobiotic metabolizing enzyme CYP1A is a phase I biotransformation enzyme widely used as a biomarker of exposure to planar organic compounds including environmental contaminants such as halogenated aromatic hydrocarbons (HAHs) and polyaromatic hydrocarbons (PAHs) [17, 18]. CYP1A is the major pollutant inducible cytochrome P-450 isoform in fish and other vertebrates [177]. While endogenous levels of the enzyme are relatively low, strong and rapid (within hours) induction of CYP1A mRNA and catalytically active protein occurs in response to inducing compounds [19], making CYP1A an ideal biomarker of exposure to such pollutants. Following their uptake across the cell membrane, inducing compounds, such as planar PCBs, bind to the cytosolic aryl-hydrocarbon receptor, initiating transcription of a number of genes including CYP1A [20]. Quantification of CYP1A mRNA, protein, and activity (measured as ethoxyresorufin-O-deethylase, EROD) levels are often used as biomonitoring tools and numerous studies report elevated levels of each hepatic CYP1A parameter in fish residing in contaminated habitats relative to fish residing in relatively clean habitats [17, 118-120].

Phase II xenobiotic metabolizing enzymes such as glutathione S-transferase (GST) and uridine diphosphate glucuronyltransferase (UDPGT)are also inducible in response to organic pollutant exposure and are used as biomarkers of exposure to these compounds [12, 113]. Phase II pollutant metabolism involves conjugation of either the parent xenobiotic compound or a metabolite with a large, water soluble endogenous co-factor thereby facilitating excretion of the compound [29, 178]. Some phase II enzymes conjugate xenobiotics directly while others require prior action by phase I

biotransformation enzymes [178]. Several phase II enzymes including both GST and UDPGT are part of the Ah gene battery and are therefore activated along with CYP1A by binding of pollutants to the Ah receptor [179, 180] making them useful biomarkers. However, relative to phase I enzymes, phase II enzyme induction is generally less pronounced [24, 26] and therefore more easily masked by natural factors affecting activity such as nutrition, season, and temperature [29]. Sensitivity of phase II enzymes to pollutant exposure also appears to vary widely by species with some species reporting induction of enzymes such as GST [28, 165, 181-183]and UDPGT [183-185] following pollutant exposure and others reporting either no response or lowered activity [12, 186, 187]. We are unaware of any studies examining the responsiveness of phase II enzymes in longear sunfish and bluegill following pollutant exposure.

Two model pollutants are utilized in this study that represent two major pollutant classes: HAHs and PAHs. Both pollutant classes are widespread environmental contaminants that induce CYP1A by binding to the aryl hydrocarbon receptor (AhR), although aspects of induction often differ. Halogenated contaminants such as PCBs generally lead to a more sustained induction, resulting in elevated levels of biotransformation enzymes that persist for two weeks or longer while PAH mediated induction typically dissipates after five days [147-149]. Resistance of HAHs to metabolism is believed to account for the observed sustained induction relative to rapidly metabolized PAHs [121]. In addition, the potency of induction by different pollutant classes is known to vary among fish species and even within different populations within a species [110, 167, 188, 189]. Few studies exist characterizing the response of *Lepomis* species to either class of inducers.

The genus *Lepomis* includes thirteen species commonly known as sunfish, representatives of which are found in nearly every body of water in North America [190]. In addition to their abundance, *Lepomis* species are often sexually dimorphic and typically have restricted home ranges [191], both characteristics that make them ideal biomonitoring candidates. In this study two of the most widespread members of this genus, longear sunfish (*Lepomis megalottis*) and bluegill (*Lepomis macrochirus*) were utilized as potential biomonitoring organisms. Both species have great potential as

biological sentinels because of their nearly ubiquitous distribution throughout much of North America.

The objectives of this study were to evaluate the suitability of hepatic pollutant metabolizing enzymes in these two species as bioindicators of pollutant exposure. Laboratory studies were conducted examining the effects of two pollutant classes on hepatic enzyme induction in longear sunfish and bluegill in order to evaluate pollutant response in a controlled environment. Field studies were conducted to determine if the response observed in laboratory studies was indicative of response in feral organisms. Longear sunfish were collected at two time points from a PCB contaminated area and CYP1A activity and PCB concentrations assessed. I hypothesized that hepatic enzyme activity and/or protein levels would be an effective biomarker of pollutant exposure in *Lepomis* species in both laboratory and field conditions.

4.3 Methods

Materials

7-Ethoxyresorufin and resorufin were obtained from Molecular Probes (Eugene, OR, USA). The monoclonal antibody made against scup CYP1A protein, MAb 1-12-3, was a generous gift of Dr. John Stegeman (Woods Hole Oceanographic Institution). Cy^{TM}5-conjugated affinipure goat anti-mouse IgG was obtained from Jackson Immunoresearch Laboratories Inc. (West Grove, PA, USA) and precast polyacrylamide gradient gels were from Invitrogen (Carlsbad, CA, USA). Nitrocellulose membrane (0.45 μm) was obtained from Schleicher and Schull (Keene, NH, USA). The Bio-Dot SF Microfiltration Apparatus was obtained from Bio Rad (Hercules, CA, USA). All other reagents were obtained from Sigma, Fisher Scientific (Fair Lawn, NJ), or Invitrogen.

Animals

Laboratory Studies

- Bluegill Study

Adult fish were donated by the a Kentucky Fish and Wildlife hatchery and held in the laboratory for two years before the experiment. During this time fish were fed a diet

of Purina Gamefish Chow approximately every other day and held in circular 250 – gallon flow through tanks.

- Longear and Bluegill Study

Longear sunfish were collected from the South Elkhorn, a relatively clean stream in Central Kentucky. Bluegill were obtained from Jones Fish Farm in Cincinnati, Ohio. All fish were returned to the lab and held for at least 30 days prior to the experiment. Fish were housed in 37 liter re-circulating tanks at approximately 17-20^{0}C and fed a mixture of mealworms and Tetramin®.

Experimental Design

Laboratory Studies

- Bluegill Study

Fish were divided into treatment groups, held in experimental 10 gallon tanks equipped with whisper filters for 7 days, then injected intraperitoneally with PCB 77 (0, 0.1, 0.5, 1.0 mg/kg; 0, 0.343, 1.71, 3.43 μM) in corn oil and sacrificed seven (PCB) days following treatment. Doses and time-points were selected based on the dosage of contaminants required for CYP1A induction in related studies.

- Longear and Bluegill Study

Fish were divided into treatment groups, held in experimental tanks for 7 days, then injected intraperitoneally with PCB 77 (0, 0.1, 1.0 mg/kg; 0, 1.71, 3.43 μM) or BaP (0,10,50 mg/kg; 0, 40, 198 μM) in corn oil and sacrificed either two (BaP) or seven (PCB) days following treatment. Livers of laboratory exposed fish were removed, flash frozen in liquid nitrogen, and microsomes prepared within four days, as described.

- Field Study

Longear sunfish were collected during August of 2001 and June of 2002 from the Bayou Creek System in McCracken County, Kentucky. This system is contaminated with PCBs and other pollutants originating from the Paducah Gaseous Diffusion Plant. Fish were collected using backpack electrofishing, sacrificed in the field, and livers removed and flash frozen in liquid nitrogen. Carcasses were returned to the lab on ice for PCB analysis.

Liver Microsomal Protein Isolation

Livers were removed from liquid nitrogen, weighed, and immediately homogenized in 10 volumes (weight:volume) of ice-cold 50 mM Tris buffer (pH 7.4). Microsomal fractions were obtained by differential centrifugation as previously described [131]. The final 100,000 x g microsomal pellets were resuspended in 50 mM Tris buffer (pH 7.4) containing 1mM EDTA, 1 mM DTT and 20% glycerol at a 1:1 ratio (liver weight: resuspension buffer volume). Microsomal samples were stored in liquid nitrogen until analyzed for catalytic activity and CYP1A protein content.

Catalytic and Protein Assays

Ethoxyresorufin -O-deethylase (EROD) activity, a reaction catalyzed exclusively by CYP1A [4], was measured fluorometrically in 48 well CoStar™ plates using the method of Hahn et al. [133]. The reaction mixture contained 50 mM Tris, pH 7.4, 0.1 M NaCl, 2μM 7-ethoxyresorufin, and 100 to 300 ug of microsomal protein in a final volume of 200 μl. The reaction was initiated by the addition of 1.34 mM NADPH, run at room temperature (28-29^{0}C), and production of the fluorescent product, resorufin, measured for 13 minutes. The linear portion of the curve was used to evaluate the rate of the reaction. All EROD assays were run in triplicate on a PerSeptive Biosystems Cytofluor Series 4000™ multi-well plate fluorometer.

Microsomal protein was measured fluorometrically using the method described by Lorenzen and Kennedy [134] with bovine serum albumin as the standard. All protein assays were run in triplicate.

CYP1A Catalytic Assay

Ethoxyresorufin -O-deethylase (EROD) activity, a reaction catalyzed exclusively by CYP1A [4], was measured fluorometrically in 48 well CoStar™ plates using the method of Hahn et al. [133]. The reaction mixture contained 50 mM Tris, pH 7.4, 0.1 M NaCl, 2μM 7-ethoxyresorufin, and 100 to 300 ug of microsomal protein in a final volume of 200 μl. The reaction was initiated by the addition of 1.34 mM NADPH, run at room temperature (28-29^{0}C), and production of the fluorescent product, resorufin, measured for 13 minutes. The linear portion of the curve was used to evaluate the rate of the reaction.

All EROD assays were run in triplicate on a PerSeptive Biosystems Cytofluor Series 4000™ multi-well plate fluorometer.

Western Blots (Bluegill Study)

Microsomal proteins (15 μg/lane) were resolved on 12% SDS-PAGE gels and electrophoretically transferred to nitrocellulose membrane (0.45 μM). The membrane was incubated in TBS-5% milk at 4°C overnight to block non-specific binding, followed by incubation with MAb 1-12-3 (3 μg/ml in 5% milk block), a monoclonal antibody which recognizes CYP1A in multiple vertebrate species [166], essentially as described [106]. CYP1A signal was detected using ECL Plus anti-mouse IgG as the secondary antibody (1:40 dilution) and blots were scanned using a Storm gel and blot imaging system (Molecular Dynamics) and quantified using Image Quant (Molecular Dynamics).

Slot Blots (Longear and Bluegill Study)

CYP1A protein was quantified by immuno-blotting using a Bio-Dot SF™ microfiltration slot-blot apparatus. Twenty micrograms of microsomal protein diluted in 200 μL buffer (20 mM Tris, 0.5 M) was loaded into each well and vacuum transferred onto a nitrocellulose membrane (0.45 μM). The membrane was incubated in TBS-5% milk at 4°C overnight to block non-specific binding, followed by incubation with MAb 1-12-3 dissolved (3 μg/ml) in milk block, a monoclonal antibody which recognizes CYP1A in multiple vertebrate species [19], essentially as described by Elskus et al. 1999 [106]. CYP1A signal was detected using Cy™5-conjugated affinipure goat anti-mouse IgG as the secondary antibody (10.8 μg/ml in sodium azide) and blots were scanned at 633 nm excitation/670 nm emission using a Typhoon 8600 scanner (Molecular Dynamics) and quantified using Image Quant (Molecular Dynamics). Liver microsomes from trout treated with the CYP1A model inducer, ß-naphthoflavone, were loaded in seven concentrations ranging from 0.1 to 7 μg of protein diluted in 200 μL buffer to evaluate linearity of the CYP1A signal on each blot. All samples were run at least in triplicate.

GST Catalytic Assay

Cytosolic GST activity was measured using the nonspecific substrate 1-chloro-2,4-dinitrobenzene (CDNB) as described in [192]. 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.

UDPGT Catalytic Assay

Uridine diphosphate glucuronyl transferase (UDPGT) activity was measured as described [193], 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 [194]. All samples assays were performed in duplicate.

PCB Analysis of Field Fish

Fish were measured for length and whole body weight before fillets were taken with solvent-cleaned surgical instruments. Yellow bullhead fillets were analyzed after removal of skin while all other fish were scaled and fillets analyzed with skin intact. PCBs in fish fillets were extracted and analyzed using standard U.S. EPA methods [136]. The fillet samples were weighed, then ground with 20g anhydrous sodium sulfate and

extracted with petroleum ether in a Soxhlet apparatus for 5-h. The extracts were concentrated to near dryness in a Roto-evaporator (Buchi Model RE121). Lipid and pesticides were removed from the reconstituted samples (5.0 mL in iso-octane) as described [136] and then analyzed by gas chromatography. A 1.0 mL sub-sample was taken for lipid determinations prior to clean-up. Elemental sulfur was removed by shaking the extract with 2-propanol (2 mL) and tetrabutylammonium sulfite (2 mL), adding ultra-pure water (8 mL) and reshaking. The organic phase was removed and mixed with 2.0-mL concentrated sulfuric acid [136], SW-846 Method 3660B, sulfur cleanup]. A 4µL aliquot was then analyzed by gas chromatography.

Samples were analyzed for Aroclors 1248, 1254, and 1260 according to SW-846 Method 8082 (polychlorinated biphenyls by gas chromatography; [136]). Analysis was performed using a Hewlett-Packard (HP) Model 5890A gas chromatograph equipped with an electron capture detector and an HP Model 7673A Automatic Sampler. Samples were analyzed using a 60m X 0.53mm ID SPB-5 (0.5µm film) fused silica megabore column (Supelco, Inc.) with ultra-high purity helium and nitrogen as carrier and makeup gases, respectively. The temperature program was 160 °C (6 min); 10 °C/min-235 °C (0 min); 0.9 °C/min-260 °C (10 min); injector temperature, 280 °C; detector temperature, 300 °C. PCB peak heights were quantified using an HP Model 3396A integrator and multiple-peak linear regression analysis was performed with Lotus-123® software. Five external standards for each Aroclor were used for calibration curves and for every tenth sample, either a solvent blank or a standard was analyzed. Detection limits for tissue samples ranged from 12 to 160 µg PCB/kg tissue. Reported values are corrected for recovery.

Statistical Treatment of Data

Statistical analyses were performed using SYSTAT Version 10. All data were transformed (log 10) before analysis. EROD and protein data were first analyzed using multivariate analysis of variance followed by 1-way analysis of variance to achieve mean separation if significance differences were detected. In addition UDPGT and GST activity in longear sunfish were analyzed using multivariate analysis of variance. Differences in means were tested using the Bonferroni test. CYP1A protein levels in

PCB treated bluegill sunfish were tested using a two-tailed t-test as only two treatment groups were available. All differences were considered significant at $p <$ or $= 0.05$.

4.4 Results

Laboratory Studies

- Bluegill Study

CYP1A activity (EROD) was induced weakly but significantly (3-4) over controls in each PCB treatment (Figure 4.1A). EROD levels were relatively low relative to those typically observed in other fish species ranging from 5-26 pmol/min/mg. CYP1A protein levels did not differ between treatments (Figure 4.1B).

Bluegill and Longear Laboratory Study: PCB and BaP treatments

Bluegill treated with BaP displayed CYP1A activity elevated significantly at both doses (9-38 fold) over controls (Figure 4.2A). CYP1A protein levels in BaP treated fish were elevated slightly although significantly in the case of the 50 mg/kg treatment (0.8 - 2 fold) over controls (Figure 4.2B). In striking contrast bluegill treated with the CYP1A inducing PCB congener 77 at 1 mg/kg exhibited CYP1A activity elevated weakly but significantly (3 fold) over controls (Figure 4.2C) and a complete lack of elevated CYP1A protein in the 0.1 mg/kg treatment group (Figure 4.2D). CYP1A protein data from the 0.1 mg/kg PCB treatment were not available as a result of a shortage in available tissue. GST activity in bluegill sunfish did not vary regardless of pollutant treatment or dose (Figure 4.3).

Longear sunfish treated with BaP exhibited CYP1A activity elevated greatly over controls (4-15 fold) (Figure 4.4A). CYP1A protein appeared was significantly elevated over controls at the highest dose only (Figure 4.4B). PCB treated longear sunfish did not differ in CYP1A activity from controls (Figure 4.4C). No differences were apparent in CYP1A protein levels observed in PCB treated longear sunfish (Fig. 4.2D). GST activity in longear sunfish remained constant regardless of inducer or dose (Figure 4.5). In an identical manner, UDPGT activity following BaP treatment also remained constant

following BaP exposure (Figure 4.6) at doses which resulted in highly induced EROD activity. UDPGT was not measured in PCB treated fish because of limited tissue availability.

Field Study

CYP1A activity in longear sunfish collected from a PCB contaminated site ranged from a mean of 22.9 to 98.4 pmol/min/mg (Table 4.1). EROD levels were somewhat higher (5-10 fold) than those observed in the laboratory PCB treated longear sunfish (Fig. 4.4C) but lower (up to three fold) than those observed in the longear sunfish treated with BaP in the laboratory (Fig. 4.4A). PCB concentrations in fish ranged from approximately 0.183 to 0.291 μg/g (Table 4.1).

4.5 Discussion

The results of these experiments present a previously undocumented response to pollutant exposure in the widespread genus *Lepomis*. Species of the genus *Lepomis* utilized in this study were highly sensitive to polycyclic aromatic hydrocarbon (BaP) exposure as demonstrated by dramatic increases in both CYP1A activity and protein levels in laboratory studies. However, these same species exhibited a remarkable lack of sensitivity to chlorinated aromatic hydrocarbons (PCBs) when exposed to doses that typically induce high levels of pollutant metabolizing enzymes in fish. Unlike the phase I enzyme CYP1A, phase II enzymes examined (GST and UDPGT) were unaffected by treatment with any class or concentration of pollutant in both species. Field studies utilizing longear sunfish collected from areas highly contaminated with PCBs provide supporting evidence that CYP1A in this species is relatively insensitive to pollutants in this class. The results presented in this study have implications for the use of *Lepomis* species as a biomonitoring tool, suggesting CYP1A induction in this genus may be an effective biomonitoring tool for one class of pollutants but not another.

Although data concerning the response of pollutant metabolizing enzymes to PAH exposure is scarce for the genus *Lepomis*, our results are generally consistent with the few existing studies. Jimenez and Burtis [195] reported significant induction of hepatic CYP1A activity (30 fold) and protein (3 fold) in bluegill following exposure to

BaP. Green sunfish – bluegill hybrids treated with the model PAH inducers BaP and β-naphthoflovone (BNF) demonstrated CYP1A activity induced 15 and 23 fold respectively over controls [196]. The elevation in CYP1A activity (38 fold in bluegill and 15 fold in longear sunfish) observed in the laboratory portion of this study following treatment with the PAH BaP is similar to these previously reported laboratory studies. Elevation of EROD activity relative to reference animals has been reported in both bluegill and redbreast sunfish (*Lepomis auritus*) collected from streams contaminated with a number of pollutants including both PAHs and PCBs [197, 198]. In a manner similar to previous studies my data demonstrates that inducers of the PAH class are powerful and effective inducers of CYP1A in *Lepomis* species.

Studies examining the effects of HAHs on pollutant metabolizing enzymes in *Lepomis* are equally rare. The only laboratory study I am aware of that examined the effects of PCBs on pollutant metabolizing enzymes was conducted by Zielinski who reported an approximate 3 fold induction of CYP1A activity in pumpkinseed sunfish (*Lepomis gibbosus*) injected with 1 mg/kg PCB 77 [108, 109]. These results are similar to the 3-fold induction observed in bluegill and the 1.7 fold induction in longear sunfish exposed to 1 mg/kg PCB 77 in this study. Treatment with equal or lesser doses of PCB 77 typically results in greater induction in other fish species. Melancon and Lech [117] reported CYP1A activity induced fifty-five fold over controls in rainbow trout (*Oncorhynchus mykiss)* and twenty fold over controls in common carp (*Cyprinus carpio*) treated with 0.2 mg/kg PCB 77. White et. al [121] reported CYP1A activity induced nine fold over controls in scup treated with 0.1 mg/kg PCB 77. The results of this experiment are consistent with previous research examining effects of PCBs on *Lepomis* biotransformation enzymes indicating the genus as a whole demonstrates a relative lack of sensitivity to PCB mediated CYP1A induction.

Few studies provide a comparison of an organism's sensitivity to multiple pollutants. Certain populations of killifish (*Fundulus heteroclitus*), yellow perch (*Perca fluviatilitis*), and tomcod (*Microgadus tomcod*) from highly contaminated environments and resistant to the harmful effects of PCBs demonstrate low sensitivity to CYP1A induction via HAHs but remain relatively sensitive to induction via PAHs [167, 199, 200]. The mechanism of this differential response is not known. The only other study I

am aware of that contrasts pollutant response in organisms from an uncontaminated site compares the response of American eel (*Anguilla rostrata*) to PAH and HAH exposure. Schlezinger and Stegeman [188]observed an approximate four-fold induction over controls in eels treated with 1 mg/kg PCB 77 in contrast to the approximate 21-fold induction over controls in eels treated with 10 mg/kg BaP. When eels were treated with 10 mg/kg of either PCB 77 or BaP CYP1A activities of 600 and 1700 pmol/min/mg were observed, respectively [188]. While the mechanism underlying these results remains unknown, they are strikingly similar to the observations of this study and strongly suggest certain species from non-contaminated environments exhibit differential sensitivity to halogenated and non-halogenated inducers.

Glutathione *S*-transferase (GST) is a phase II biotransformation enzyme that transfers a tripeptide glutathione to electrophilic centers of a wide variety of xenobiotic compounds providing protection against oxidative damage [23]. GSTs are divided into four classes, all of which are located primarily in the cytosolic fraction of the liver [24]. Many studies, including this one, utilize an artificial substrate that is conjugated by three of the four GST classes thereby providing a measurement of near total GST activity. A number of studies demonstrate elevated GST activity following exposure to both PAHs and PCBs although this response appears to be highly species specific and the majority (67%) of fish studies to date do not demonstrate a significant increase in GST exposure following pollutant exposure [29]. One factor that may confound such studies is the observation that GST elevation is generally very slight relative to CYP1A elevation [26], meaning differences would be difficult to detect. GST induction in rainbow trout hepatic tissue following PCB exposure has been reported [26-28] although this induction is typically far less than the concurrent CYP1A induction [185]. The only data concerning the response of *Lepomis* species GST to pollutant exposure we are aware of is reported by Oikari and Jimenez [196] who reported a significant decrease in GST activity in bluegill-green sunfish hybrids following BaP exposure. Although statistical significance was not achieved a similar trend is evident in the BaP exposed bluegill in this study (Figure 4.3A). Our results could also be explained by the time course of GST induction. Andersson [26] reported a maximum GST induction (48% over controls) three to four weeks after exposure. Our measurements seven days after PCB exposure and two days

after PAH exposure would likely have failed to capture a similar induction. GST does not appear to be a reliable indicator of either PCB or PAH exposure in members of the genus *Lepomis*.

UDPGT is another phase II enzyme that comprises a major pathway for the inactivation and excretion of a number of both endogenous and exogenous organic compounds [24, 30]. Uridine 5′- diphosphoglucuronic acid is formed and attached to a wide variety of substrates by UDPGT, increasing their hydrophilicity thereby enabling excretion [30]. Unlike GST, UDPGT activity is reported to increase following pollutant exposure in the majority of fish studies (52%) and is considered the phase II enzyme most responsive to pollutant exposure [29]. UDPGT activity is reported to increase following exposure to both PCBs and PAHs in a number of, but not all, fish species studied [29]. Our results are somewhat surprising in that a distinct increase in EROD activity in longear sunfish following exposure to BaP was observed (Figure 4.4A) with no concomitant increase in UDPGT activity (Figure 4.6). Several laboratory studies report exposure to PAH contaminants with no change in UDPGT activity in species such as common carp (*Cyprinus carpio*) [201], Dab (*Limanda limanda*) [12], and sea bass (*Dicentrarchus labrax*) [12]. However, similar to GST measurement, the time course of induction may have prevented detection of an induction in this enzyme. Celander et al. [28] found rainbow trout (*Oncorhynchus mykiss*) treated with the PAH BNF exhibited maximally induced UDPGT activity 72 hours following exposure and activity only slightly and non-significantly elevated over controls 48 hours following exposure. Therefore the 48 hour kill time for PAH exposure in this experiment may have failed to detect a similar induction.

CYP1A activity observed in male and immature field collected longear sunfish was lower than expected given the contaminant body burden observed, another observation indicating lack of PCB sensitivity in *Lepomis* species. Jimenez and Burtis [202] reported mean CYP1A activity levels of 75 pmol/min/mg in bluegill collected from a reference stream while fish collected concurrently from a nearby contaminated site displayed levels ranging from 190-400 pmol/min/mg. In addition bluegill collected from a clean site and held in the laboratory for at least a month showed levels of CYP1A activity similar to those observed in the contaminated longear sunfish of the present study

[195]. Studies with a number of fish species have demonstrated elevated levels of CYP1A in fish containing PCB concentrations similar to or greater than those observed in this study. Orn et al. [203] observed hepatic CYP1A activity induced significantly over controls in zebrafish (*Danio rerio*) which had mean total muscle tissue PCB concentrations of 0.11 μg/g wet weight. Jorgeson et al. [204] observed hepatic CYP1A induction in arctic charr (*Salvelinus alpinus*) at liver PCB concentrations above a threshold 1 μg/g. Although variation is found among species, liver PCB concentrations often exceed muscle PCB concentrations by 10–fold or more [205] as a result of the high lipid content of this organ, easily placing the field PCB measurements in this study within the 1 μg/g range.

Mature female longear sunfish exhibited lower levels of CYP1A activity relative to male and immature fish, likely a result of the suppressive effects of estrogen on hepatic CYP1A expression. Numerous studies report reduced levels of CYP1A activity or protein in estrogen-treated and sexually mature female fish [206-208]. One suggested mechanism for this suppression is a possible link between CYP1A and estrogen responsive genes leading to decreased enzymatic activity during peak reproductive periods preventing excessive metabolism of estrogen [209]. The results observed in the field portion of this study are consistent with previous studies and demonstrate the necessity of considering sex and reproductive status when selecting a biomonitoring candidate.

The results of this study provide novel information concerning the effects of pollutant exposure on biotransformation enzymes in *Lepomis* species. These results indicate that the phase I biotransformation enzyme CYP1A is an effective biomarker of PAH but not HAH exposure in at least two members of the genus *Lepomis*. Longear sunfish and bluegill, like American eels and several populations of fish residing in highly contaminated areas, appear to exhibit differential sensitivity to chlorinated and non-chlorinated contaminants. Phase II enzymes in this genus, at least at the time-points utilized in this study, appear to be inadequate biomarkers of exposure to either pollutant class. In addition to providing essential biomonitoring data this study provides a useful vertebrate model in which to examine the mechanism underlying the differences in response to pollutant classes.

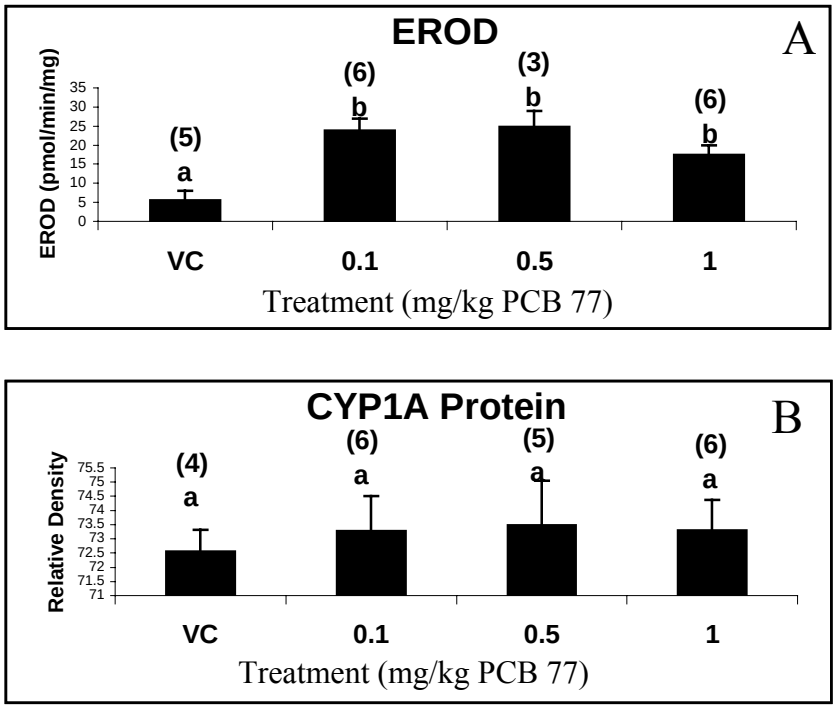


Figure 4.1. EROD activity (A) and CYP1A protein levels (B) in liver microsomes of bluegill sunfish treated with either corn oil (VC) or PCB 77 (0.1, 0.5, and 1.0 mg/kg). Bars represent Means ± SE for (n) individuals. Means with the same letters are not significantly different at $P < 0.05$, all protein levels were similar ($P < 0.05$) among treatments.

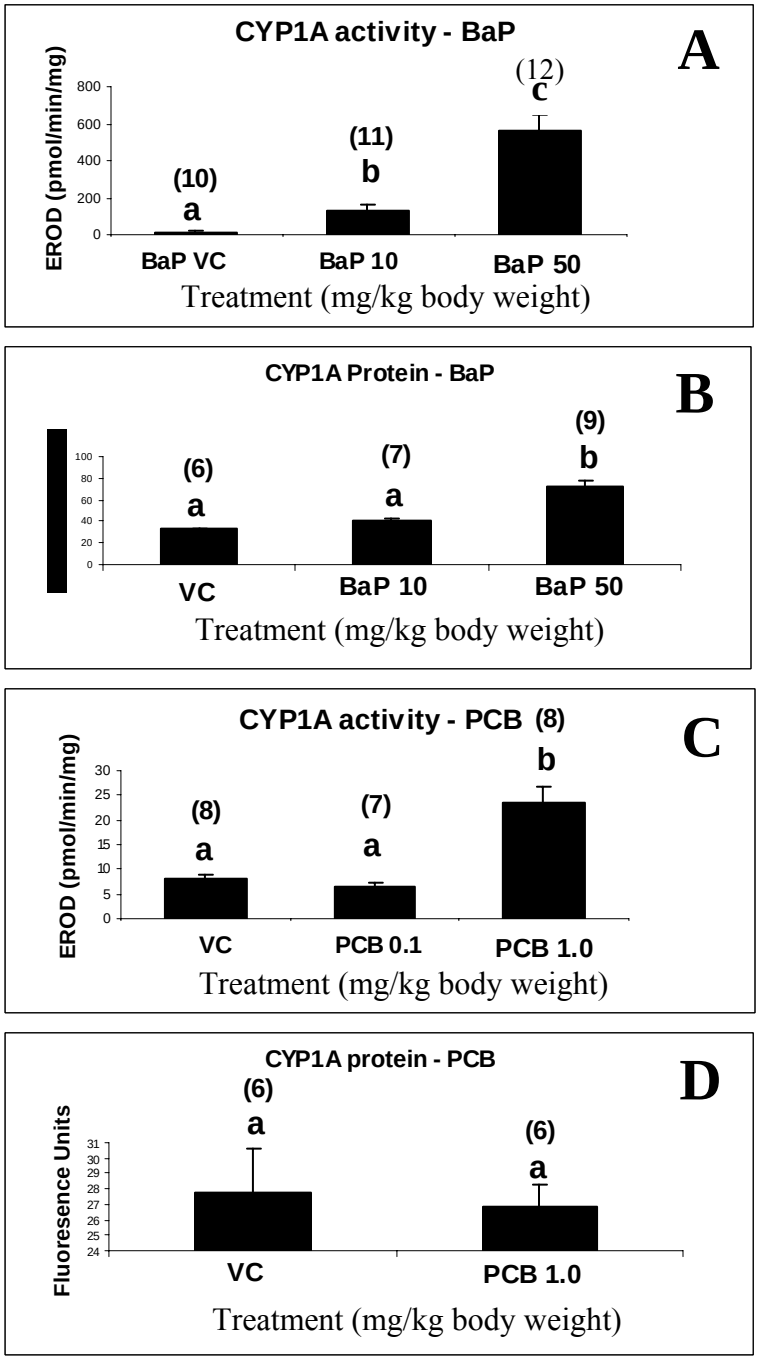


Figure 4.2. EROD activity and CYP1A protein levels in liver microsomes of bluegill sunfish treated with either corn oil (VC), BaP (0, 10, and 50 mg/kg) (A, B), or PCB 77 (0, 0.1, and 1.0 mg/kg) (C, D). Bars represent Means ± SE for (n) individuals. Means with the same letters are not significantly different at $P < 0.05$.

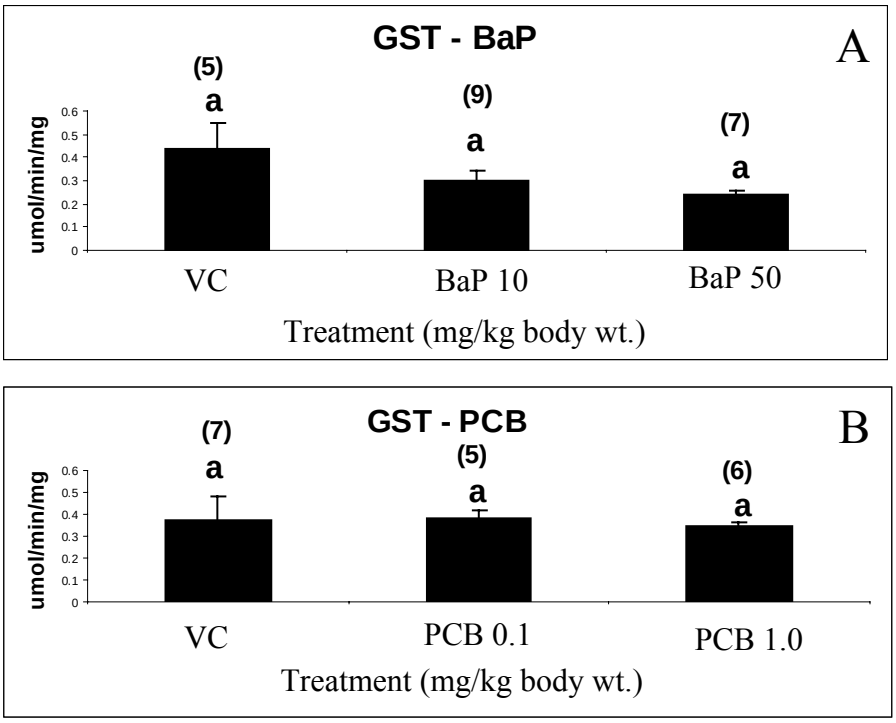


Figure 4.3. GST activity in liver microsomes of bluegill sunfish treated with either corn oil (VC), BaP (10 and 50 mg/kg) or PCB 77 (0.1 and 1.0 mg/kg). Bars represent Means ± SE for (n) individuals. Means with the same letter are not significantly different at P <0.05.

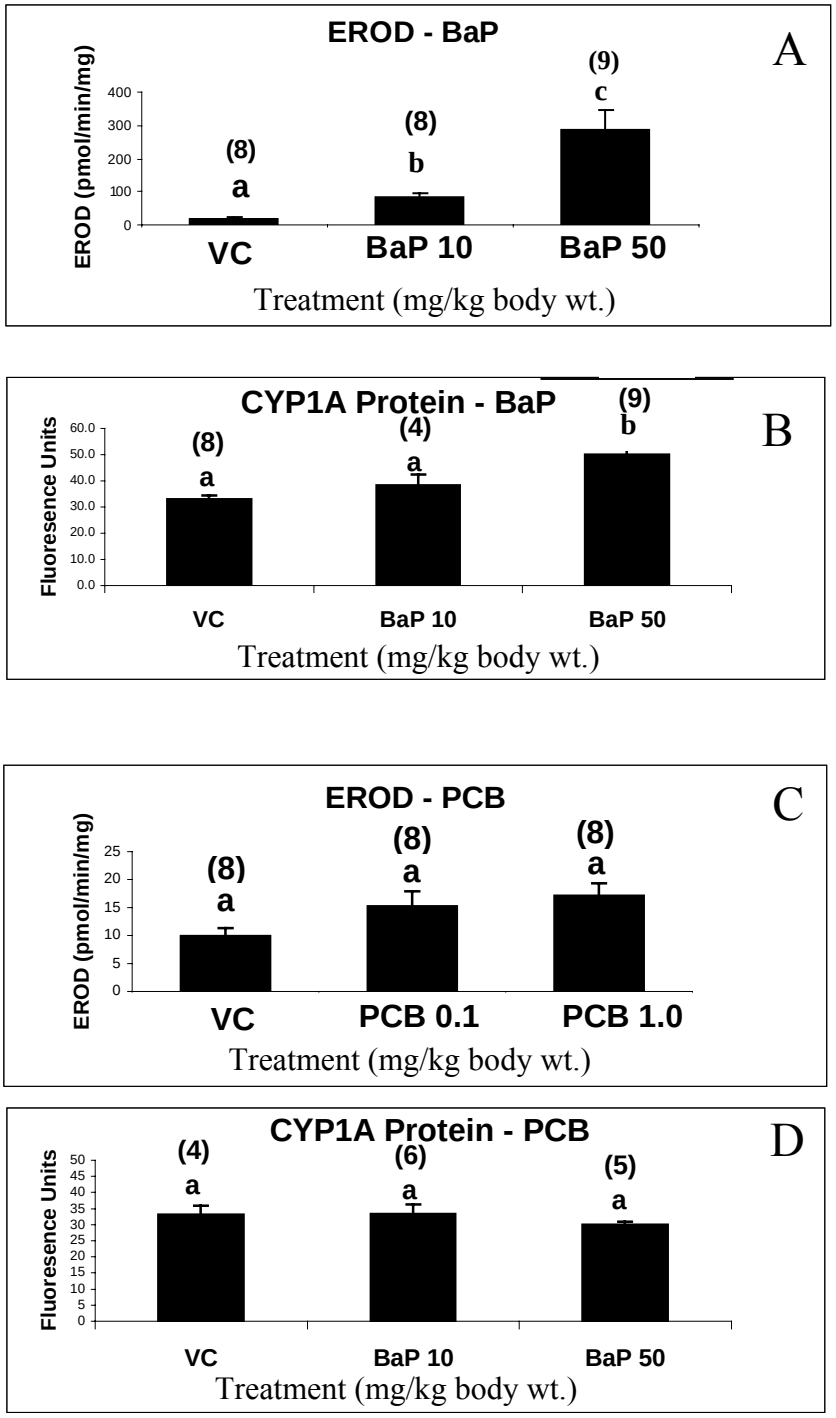


Figure 4.4. EROD activity in liver microsomes of longear sunfish treated with either corn oil (VC), BaP (10 and 50 mg/kg) (A) or PCB 77 (0.1 and 1.0 mg/kg) (B). Bars represent Means ± SE for (n) individuals. Means with the same letter are not significantly different at $P < 0.05$.

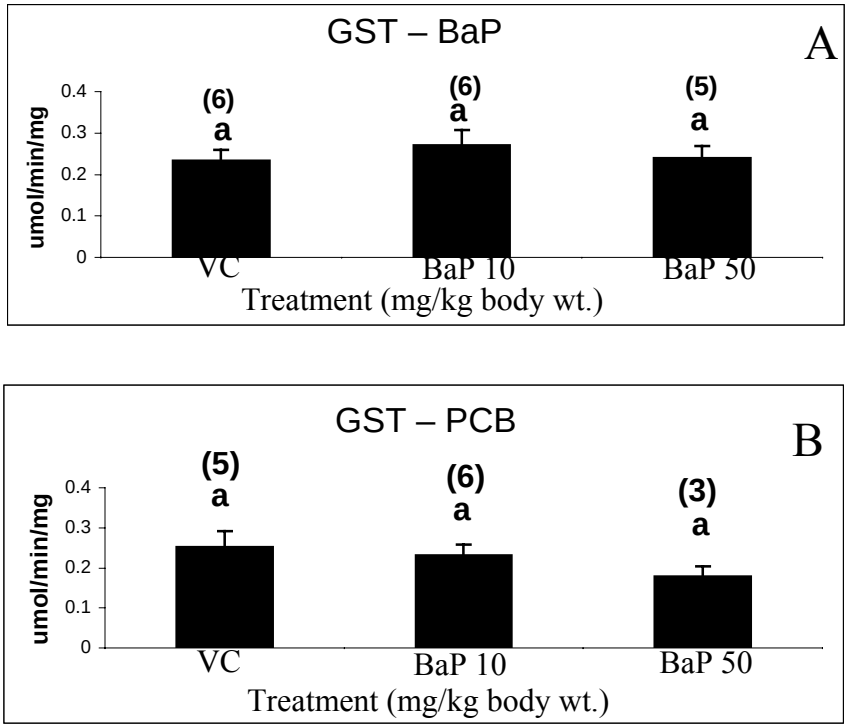


Figure 4.5. GST activity in liver microsomes of longear sunfish treated with either corn oil (VC), BaP (10, and 50 mg/kg) or PCB 77 (0.1 and 1.0 mg/kg). Bars represent Means ± SE for (n) individuals. Means with the same letter are not significantly different at P <0.05.

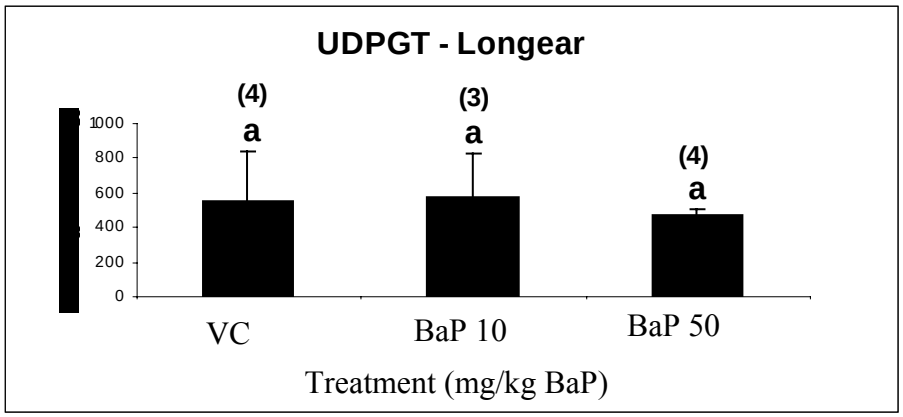


Figure 4.6. UDPGT activity in liver microsomes of longear sunfish treated with corn oil (VC), or BaP at 0, 10 and 50 mg/kg. Bars represent Means ± SE for (n) individuals, means with the same letter are not significantly different at $P < 0.05$.

Table 4.1. Hepatic EROD activity and muscle tissue PCB concentrations in longear sunfish collected from the Bayou Creek system in western Kentucky during August 2001 and June 2002. Means ± (SE (n)).

Collection	Species	Sex	Mean EROD (pmol/min/mg)	PCB conc. (ppm wet weight)
Aug. 2001	Longear Sunfish	Male and Immature	97.5 ± 16.3(13)	0.183 ± 0.0436(13)
		Female	33.5 ± 11.2(2)	0.240 ± 0.00488(2)
June 2002	Longear Sunfish	Male	98.4 ± 19.2(5)	0.159 ± 0.0542(5)
		Female	22.9 ± 4.37(6)	0.291 ± 0.0844(6)

Chapter 5: Effects of Polybrominated Diphenyl Ethers on Fish Physiology and Development

* Thyroid hormone analysis conducted by Dr. Stephen D. McCormick and Amy Moeckel, USGS, Conte Anadromous Fish Research Center, Turners Falls, MA

5.1 Abstract

Polybrominated diphenyl ethers (PBDEs) are widely produced in industry as flame retardants in plastics and textile coatings and enter the environment through a number of routes. Relatively high levels of PBDEs have been detected in organisms near the top of food chains such as fish, fish eating birds, marine mammals, and humans. I hypothesize that PBDEs will induce biological effects similar to those induced by structurally similar PCBs; inducing Cytochrome P450 1A (CYP1A), altering normal development, and generating oxygen radicals that cause oxidative stress. Two fish models were used to test this hypothesis, juvenile catfish (*Ictalurus puntatus*) and embryonic killifish (*Fundulus heteroclitus*). Juvenile channel catfish were exposed to two concentrations of the PBDE technical mixture PBDE -71 or the PCB mixture Aroclor 1254. CYP1A activity was induced in PCB but not PBDE treated channel catfish. Uridinediphosphate-glucuronosyltransferase (UDPGT) activity was unchanged in channel catfish regardless of dose. Plasma thyroxine (T4) levels were quantified but found to be below detection limits. Certain populations residing in PCB contaminated areas display resistance to PCBs; undergoing PCB exposure without exhibiting the typically observed toxic effects of these compounds. Developing killifish embryos from both PCB resistant and responsive populations were exposed to a range of concentrations of PBDE -71 and co-exposed to Aroclor 1254 and PBDE-71. CYP1A was induced in the PBDE + PCB exposures only. Time to hatch and length at hatch were both decreased significantly by PDBE doses in the responsive fish only. Responsive killifish embryos were exposed to low or high doses of PBDE – 71 alone or in combination with PCB 126. Reactive oxygen species (ROS) production was similar to controls in all treatment groups. The results of these studies indicate that PBDEs have no effect on hepatic enzymes in adult teleosts but do lead to alterations in normal developmental processes.

Furthermore these results suggest that resistance to PCBs may confer resistance to the harmful effects of PBDEs.

5.2 Introduction

PBDEs are manufactured for use as flame-retardants and have become widespread environmental contaminants with relatively high concentrations found in biological samples from different locations around the globe. PBDEs are included as additive flame retardants in products such as plastics, electrical components, building materials, and synthetic textiles [81]. Unlike other halogenated compounds such as DDT and PCBs, which have been banned from use in many countries, restrictive regulations regarding PBDEs have not been enacted [82]. Global production of PBDEs was 40,000 tons per year in 1990 and has remained relatively constant since [81]. PBDEs enter the environment through a variety of routes including disposal of PBDE containing products, leachate from landfills, emissions from production plants, and volatilization [81, 82]. Due to their lipophillic nature, PBDEs bioaccumulate in tissue and relatively high levels of PBDEs have been detected in biota [81], including fish [210], fish eating birds [84], marine mammals [85], and even humans [86]. The distribution of PBDEs in the environment coupled with their tendency to accumulate in biota has led to widespread PBDE exposure.

Studies examining temporal trends of organohalogenated contaminants reveal that while most other organohalogen contaminant levels are decreasing, PBDE levels are either remaining constant or increasing. The total concentrations of several PBDE congeners in guillemot eggs from the Baltic Sea increased from 1970 to 1989 [84]. Data from a Swedish Lake indicates the concentrations of PBDEs in pike muscle increased from 1974 to 1991 [87]. PBDE concentrations in eel from the River Roer increased steadily during the 1980s [211]. Perhaps of most concern are data from Sweden that indicate an exponential increase in PBDE levels in human breast milk between 1972 and 1996 [89]. Clearly, PBDEs are a growing threat to wildlife and humans. Studies detailing the effects of PBDE exposure are greatly needed.

Many environmental contaminants are known to interfere with regulation of the endocrine system thereby disrupting normal development and reproduction. A striking

example is the reproductive dysfunction observed in populations of American alligators in Lake Apopka [212]. These alligators displayed low clutch viability, decreased juvenile population densities, and adult mortality following a pesticide spill in the Lake [47]. Additional studies discovered alligators exhibited abnormal circulating hormone levels as well as gonadal abnormalities, symptoms consistent with endocrine disruption [46, 48].

Fish are also known to be sensitive to endocrine disrupting compounds in the environment. Numerous studies in Europe have reported induction of vitellogenin, the egg yolk precursor protein, in male fish and the development of intersex gonads in fish exposed to synthetic or natural estrogens released into waterways via municipal sewage [49-51]. Other studies have reported symptoms of endocrine disruption in fish including depressed levels of sex hormones [52], lower gonadosomatic index, altered thyroid hormone levels (increased T_3, decreased T_4), and increased plasma testosterone concentrations in males [53]. Several recent studies have correlated reproductive dysfunction in wild fish with halogenated aromatic hydrocarbons found in the environment, possibly the result of endocrine disruption [54, 55]. These data indicate that fish are sensitive to endocrine disruption and that wild fish populations currently exhibit symptoms of endocrine disruption, the consequences of which are not known.

The thyroid system is known to be sensitive to endocrine disrupting compounds and regulates a number of parameters in fish including osmoregulation [213], growth [214], and sexual maturation [215] as well as playing a role in embryonic development [216]. Thyroid hormone is transferred from female fish to eggs [217] and promotes survival of eggs and larvae [216, 218]. The mechanisms of this enhancement of egg quality remain unclear, although maternal T_3 treatment prior to spawning is a promising technique in aquaculture [219]. Endocrine disrupting compounds commonly interfere with thyroid hormone levels through direct effects on the thyroid gland [220], competition for thyroid hormone translocating proteins [221] or through alteration of thyroid hormone metabolizing enzymes such as uridinediphosphate-glucuronosyltransferase (UDPGT) [222]. Depressed maternal levels of thyroid hormone prior to spawning could result in compromised spawning success through physiological interference with spawning or through lowered egg and fry survival.

PBDEs are structurally similar to polychlorinated biphenyls (PCBs), globally ubiquitous contaminants known to bioaccumulate and have a variety of harmful effects on organisms [57]. The detrimental effects of PCBs on fish reproduction are well documented [75-77, 223-226]. Although the mechanisms are largely unknown, they may include alteration of thyroid hormone levels [71, 72] as well as estrogenic activity [73, 74]. Due to their structural similarity to PCBs, PBDEs may affect fish development and reproduction through similar mechanisms.

The most sensitive endpoints of PBDE toxicity examined to this point are thyroid effects in rats and mice, primarily the alteration of thyroid hormone levels. Fowles et al. [90] reported that mice orally dosed with the commercial PBDE mixture DE-71 displayed decreased plasma T_4 levels in a dose dependent manner. Similarly Hallgren and Darnerud [91] reported rats administered PBDEs through gastric intubation exhibited a significant reduction in T_4 levels. Weanling rats exposed to the commercial PBDE mixtures DE-71 and DE-79 demonstrated dose dependent reduction of T_4 levels and three to four fold induction of UDGPT [92]. Although thyroid hormone levels in fish are known to be sensitive to endocrine disrupting compounds [53, 227], the effect of PBDEs on the fish thyroid hormone system has not been examined.

Initial studies indicate PBDEs affect fish reproduction. Holm et al. [226] reported a 60% lower rate of spawning success in female three-spined sticklebacks fed a diet spiked with PBDEs compared to controls. Female sticklebacks fed a diet spiked with PBDEs in combination with other halogenated hydrocarbons had significantly higher levels of progesterone 6β-hydroxlase, a key enzyme in the biosynthesis of reproductive hormones in the gonads [228]. These findings indicate PBDEs may have a significant effect on fish reproduction and/or development and indicate further investigation is necessary.

Oxidative stress is caused by increased levels of oxygen radicals (superoxide, hydrogen peroxide, and hydroxyl radicals) and is known to adversely affect cell health and function [229, 230]. Exposure to environmental contaminants such as coplanar PCBs and TCDD is known to induce oxidative stress in vertebrates including human cell lines [231], rodents [232-235] and fish [236]. Oxidative stress resulting from PCB or TCDD

exposure is believed to result from uncoupling of the CYP1A enzyme [237, 238]. No reports on the effects of PBDEs on oxidative stress currently exist.

Although PBDEs are structurally similar to PCBs, the similarity of effects on aquatic organisms remains undocumented. Numerous fish populations living in chronically contaminated habitats can develop resistance to pollutants [100, 102, 103]. Fish from populations resistant to organic contaminants fail to express elevated levels of CYP1A and experience lower rates of mortality and developmental deformities relative to reference fish when exposed to inducing compounds [104]. Certain populations of resistant fish demonstrate differential sensitivity to different classes of contaminants. Populations of tomcod (*Microgadus tomcod*) from highly contaminated environments demonstrate low sensitivity to CYP1A induction via HAHs but remain relatively sensitive to induction via PAHs [167, 199, 200]. In addition mosquitofish chronically exposed to toxaphene developed heritable resistance to toxaphene but not pyrethoid insecticides [239]. The mechanism of these differential responses currently remain unclear. Killifish (Fundulus heteroclitus) from Newark Bay (New Jersey) reside in a habitat contaminated by a mixture of PBCs, PAHs, TCDD, and heavy metals and exhibit resistance to both PCBs and PAHs [106]. The sensitivity of resistant organisms to PBDEs has not been examined.

I hypothesize that PBDEs will induce biological effects similar to those induced by structurally similar PCBs; inducing CYP1A, altering normal development, and generating toxic oxygen radicals. To address this, I used juvenile channel catfish to investigate the effects of pollutants such as PCBs on hepatic enzyme induction [240]. Killifish embryos were utilized to examine CYP1A induction [241], developmental abnormalities [242], and ROS production [243] following exposure to a variety of pollutants.

5.3 Methods

Materials

PBDE-71 and Aroclor 1254 were generously provided by Dr. Larry Robertson (Univ. of KY). 7-Ethoxyresorufin and resorufin were obtained from Molecular Probes

(Eugene, OR, USA). 3',4,4',5-Pentachlorobiphenyl (PCB 126) was purchased from AccuStandard (New Haven, CT). Nylon screen and plastic mesh were obtained from Aquatic Ecosystems (Apopka, FL). Optimal Cutting Temperature compound was purchased from Tissue-Tek (Torrance, CA). Artificial seawater salt mixture (Bio-crystals) for preparing 30ppt seawater was purchased from Marine Enterprises International (Baltimore, MD). Purina Gamefish Chow was purchased from Southern States Feed. All other reagents were obtained from Sigma, Fisher Scientific (Fair Lawn, NJ), or Invitrogen. Tetramin Flakes were purchased from a local pet supply store.

Adult Catfish CYP1A

Experimental Design

Adult channel catfish were obtained from the Kentucky Department of Fish and Wildlife Frankfort Fish Hatchery and held in flow through tanks receiving carbon filtered municipal water. During this time fish were fed a diet of Purina Gamefish Chow approximately every other day until one week prior to injection. At this time fish were transferred to 10-gallon tanks equipped with continuous flow through water and vigorous aeration but no feed. Based on the results of my pilot study (data not shown), these experiments were conducted at 18-20°C to optimize conditions for CYP1A induction by halogenated compounds. All fish used in the experiment weighed less than 40 grams (sexually immature) and were housed 10-15 per tank.

Catfish (10-15 per treatment for each time point) were injected intraperitoneally with Aroclor 1254 (10 or 100 mg/kg), PBDE-71 (10 ug/kg or 10 mg/kg), or vehicle (corn oil) and held in flow-through tanks at 19 - 20°C. The pentabrominated mixture, PBDE-71, was chosen as the test compound as it was the only commercial mixture to demonstrate significant effects on thyroid hormone and CYP1A in rodents [92]. We chose the PBDE-71 doses based on recent studies of PBDE effects on salmonids by Boon et al. [244]. Groups of 10-15 fish from each treatment were euthanized on Days 3 and 10. Blood was collected using heparinized 1 CC syringes and placed in microfuge tubes on ice immediately. Upon completion of processing of all experimental groups, blood was spun at 5,000 x g for 10 minutes to separate plasma. The plasma was divided into two aliquots and stored at –80 ^{0}C in separate microfuge tubes. Two aliquots were used to

facilitate thyroid hormone analysis, if necessary providing a second sample to analyze. Livers were removed and immediately placed in liquid nitrogen where they were stored until microsomes were made approximately two weeks later.

Liver Microsomal Protein Isolation

Livers were removed from liquid nitrogen, weighed, and sub-sectioned. Aliquots for RNA analysis (0.07-0.2 g) were refrozen in liquid nitrogen. Aliquots for microsome preparation were immediately homogenized in 10 volumes (weight:volume) of ice-cold 50 mM Tris buffer (pH 7.4). Microsomal fractions were obtained by differential centrifugation as previously described [131]. The final 100,000 x g microsomal pellets were resuspended in 50 mM Tris buffer (pH 7.4) containing 1mM EDTA, 1 mM DTT and 20% glycerol at a 1:1 ratio (liver weight: resuspension buffer volume). Microsomal samples were stored in liquid nitrogen until analyzed for catalytic activity and CYP1A protein content (within three weeks).

Catalytic and Protein Assays

Ethoxyresorufin -O-deethylase (EROD) activity, a reaction catalyzed exclusively by CYP1A [4], was measured fluorometrically in 48 well CoStar™ plates using the method of Hahn et al. [133]. The reaction mixture contained 50 mM Tris, pH 7.4, 0.1 M NaCl, 2μM 7-ethoxyresorufin, and 100 to 300 ug of microsomal protein in a final volume of 200 μl. The reaction was initiated by the addition of 1.34 mM NADPH, run at room temperature (28-29^{0}C), and production of the fluorescent product, resorufin, measured for 13 minutes. The linear portion of the curve was used to evaluate the rate of the reaction. All EROD assays were run in triplicate on a PerSeptive Biosystems Cytofluor Series 4000™ multi-well plate fluorometer.

Microsomal protein was measured fluorometrically using the method described by Lorenzen and Kennedy [134] with bovine serum albumin as the standard. All protein assays were run in triplicate.

UDPGT Catalytic Assay

Uridine diphosphate glucuronyl transferase (UDPGT) activity was measured as described [193], 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 [194]. All samples assays were performed in duplicate.

Thyroid Hormone Analysis

Thyroxine (T4) and 3,5,3'-triiodo-L-thyronine (T3) concentrations were measured by a direct radioimmunoassay (RIA). The lower detection limits were 0.5 ng/ml (T4) and 0.2 ng/ml (T3). Intra- and interassay coefficients of variation for these assays were 4.3-11% and 3.2-5%, respectively. 10 µl of plasma was added to 12 x 75 borosillicate glass tubes (in duplicate) and was incubated for 30 minutes at 37°C with assay mixture containing: 50 µl (5000 cpm) of ^{125} I labeled T3 or T4 (Perkin Elmer Life Sciences) diluted in 11 mM sodium barbital buffer pH 7.8, 0.1% sodium azide, 100 µl of T3 or T4 polyclonal antibody (Accurate Chemical & Scientific Corporation) diluted to approximately 1:6000 in 11 mM sodium barbital buffer pH 7.8, 0.1% sodium azide, 100 µl gamma globulins (Sigma) dissolved in diluted in 11 mM sodium barbital buffer pH 7.8, 0.1% sodium azide plus 0.5 mg/mL 8 aninlino 1 napthalene sulfonic acid (Sigma)

Samples were incubated overnight at 4°C. Proteins were precipitated with 300 µl of 25% polyethylene glycol (Sigma) in 11mM sodium barbital pH 7.8, sodium azide 0.1%. Tubes were centrifuged in at 3,000 x g for 20 minutes at 4°C. The supernatant was aspirated and the pellets counted in Beckman 5500 gamma counter.

Embryo CYP1A and Developmental Parameters

Experimental Design

Reproductively mature but not actively spawning *Fundulus heteroclitus* were collected from Succotash Salt Marsh (Wakefield, RI) and Newark Bay (New Jersy) by D. Nacci (US EPA, ERLN) and shipped to our laboratory overnight. Newark Bay is contaminated by PCBs and other contaminants and *F. heterclitous* from this site exhibit chemical resistance to PCBs [106]. Succotash Salt Marsh is a relatively clean site, *F. heterclitous* collected from this site are known to exhibit sensitivity to PCB toxicity [169]. Approximately 20 adults of each sex were placed into 25 gallon tanks containing 30 ppt artificial SW and held under static, recirculating (Whisper filters) conditions on a 14/10 L/D cycle. Fish were fed daily with an ad libitum diet of Tetramin fish flakes and freeze-dried brine shrimp.

In ovo CYP1A activity

The effects of chemical exposure on CYP1A activity and embryo development were evaluated using a modification of the method of Nacci et al. [241]. Briefly, embryos were collected for two days before and two days following the full and new moons and placed in 20 mL scintillation vials, one embryo per vial, in 2 mL of 30 ppt artificial SW (Day 1). Embryos were held overnight at 25°C under 14/10 L/D cycle in an environmental chamber. The following morning (Day 2) embryos were examined for evidence of successful fertilization. Water in the vials of fertilized embryos was replaced with 10 mL of exposure SW containing 2 uM 7-ethoxyresorufin (7ER, a CYP1A substrate) and the test chemical and maintained in the environmental chamber. Exposure solutions were vehicle control, PBDE-71 (10, 100 or 1000 ug/L), CB126 (2 ug/L, a dose which induces both CYP1A and developmental abnormalities in embryos [241], a combination of CB126 (2.0 ug/L) + 1000 ug PBDE-71/L. CB126 served as a positive control for CYP1A induction and PCB-induced abnormalities in fish embryos [241]. Doses of PBDE-71 ranged from environmentally realistic levels (10 ug/L, [244] to high concentrations (1000 ug/L) intended to determine upper toxicity limits.

After 6 days of exposure (i.e. Day 7), test water was replaced with clean SW. On Day 9, CYP1A activity was measured in embryos using a UV/fluorescence microscope (Eclipse E800) as described [241]. Conversion of the substrate, 7ER, to the fluorescent metabolite, resorufin (RR), is specifically catalyzed by CYP1A. Thus bladder RR fluorescence signal provides a relative measure of CYP1A induction. To normalize for differences in bladder area among embryos, UV signals were also measured for bladder of each embryo. For each bladder, UV and fluorescence signals were quantified using NIH- Image software and CYP1A activity expressed as a ratio of bladder fluorescence density/UV density (RR/UV).

Developmental endpoints

Embryos were screened daily for hatching and after hatching were euthanized in a concentrated solution of Tricaine Methane Sulfonate (MS-222). Fry length was then measured to the nearest millimeter.

Embryo Reactive Oxygen Species

Experimental Design

Spawning adult killifish were collected from Beaufort, NC 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 embryos were collected on July 3, 2004. During evening hours spawning baskets were placed in the bottom of the tank housing the parental fish [245]. 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.

Embryos were exposed to either a vehicle control (acetone), PBDE – 71 at 1000 µg/L, PCB 126 at 0.01 µg/L, PBDE – 71 at 1000 µg/L + PCB 126 at 0.01 µg/L, PBDE 71 at 10 µg/L, or PBDE 71 at 10 µg/L + PCB 126 at 0.01 µg/L. The PCB 126 doses are

based on studies reporting maximal ROS production at 0.01 μg/L in F. heteroclitus embryos [245].

In ovo superoxide detection assay (SoDA)

Superoxide dismutase was measured according to the method described by Arzuaga [242, 245]. For this superoxide detection assay (SoDA), killifish embryos were exposed to the pro-oxidant 3,4,3',4'-tetrachlorobiphenyl (PCB126) 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 different concentrations of the AHR ligand PCB126 (see figure legends for concentrations). Embryos were exposed from two to seven dpf to 10 mL of exposure medium 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 post fertilization. Two hours prior to ROS measurement, the exposure medium was replaced with 2 mL of fresh medium (vehicle or ligand) containing DHE dissolved in seawater. DHE reacts with ROS to form ethidium, which is fluorescent and intercalates with DNA [246]. The DHE medium was prepared by dissolving DHE in DMSO (158mg/ml), then adding an aliquot to artificial seawater to obtain the desired DHE concentration (see figure legends for concentrations), and sonicating for 20 minutes in the dark. The final DMSO concentration in the DHE-water medium was 0.01%. Embryos were placed into 2 ml of the DHE-water medium and kept in the dark on a plate shaker (~70 rpm) from 0.5 to 1.5 hours, after which the DHE medium was replaced with 10ml of clean artificial seawater. DHE staining of embryo tissues was visualized as described above for *in ovo* CYP1A activity. 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). The pixel intensity (integrated density) of the ethidium fluorescence observed in liver images was analyzed using Scion Image (Frederick, MD) to semi-quantitatively integrate 'boxed portions' of the liver, as described above for CYP1A activity. Production of ROS was measured as the intensity of the ROS signal (integrated density) in the livers of PCB126 treated animals relative to vehicle controls.

Statistical Treatment of Data

Statistical analyses were performed using either SYSTAT Version 10 or SPSS. All data were transformed (log 10) before analysis. EROD and UDPGT activity from whole animal experiments were evaluate using one-way ANOVA and Tukey's test to achieve mean separation. Embryo EROD, length at hatch, and time until hatch data were evaluated using two-way ANOVA. Embryo percent hatch data was evaluated using Wald's. Embryo reactive oxygen species data was evaluated using one-way ANOVA. All differences were considered significant at $p < \text{or} = 0.05$.

5.4 Results

Adult Fish Physiology

Channel catfish CYP1A activity was similar among all treatments three days after treatment but elevated significantly over controls in the Aroclor 1254 @ 100 mg/kg group ten days after treatment (Figure 5.1). UDPGT activity in channel catfish did not differ from controls regardless of treatment or kill time (Figure 5.2). A slight depression (although not significant) in UDPGT activity was evident in the high PBDE treatment group at both the three and ten day time point. Plasma thyroxine levels (T_4) were below the range of reliable quantification and are therefore not displayed.

Embryo Experiment #1

CYP1A

PBDE-71 alone had no significant effect, at any dose, on CYP1A activity (Figure 5.3) in both responsive and resistant populations. In addition the interaction term describing the difference in EROD response between sites was likewise insignificant ($p=0.655$). In the combined exposure to PBDE/PCB, CYP1A was strongly induced over controls in the responsive population (off scale-data not shown). This induction demonstrates embryos were responsive to CYP1A inducers therefore confirming the lack of CYP1A inducing activity of the PBDE mixture. The response to the combined PCB/PBDE dose in the resistant population suggested an increase in response to contaminant treatment although the increase was not significant (Figure 5.3B). In addition, the induction in this combined treatment (PCB+PBDE) seems to indicate a lack

of antagonistic action between the two contaminants. Parallel studies with responsive killifish embryos in our laboratory found significant CYP1A induction, similar to that observed in responsive fish treated with the combined PCB/PBDE dose, in responsive fish treated with PCB alone [242].

Developmental endpoints

Neither the PBDE alone nor the PBDE/PCB mixture had any effect on hatching success (>81% percent hatch in all groups) (Figure 6.4). In contrast, exposure to the low and medium PBDE doses slightly delayed hatching in responsive embryos, from a mean of 12 days (controls) to a mean of 15 days (100 µg PBDE/L) and 16 days (10 µg PBDE/L) post-fertilization (Figure 5.5A). No differences across treatment groups were visible in resistant embryos (Figure 5.5B). The interaction term describing the difference in days to hatch by treatment among sites was significant ($p=0.0221$) indicating differential response among populations.

Length at hatch was significantly suppressed in responsive embryos by the PBDE/CB126 combination treatment (5.79 ± 0.24 mm) relative to controls (6.12 ± 0.4 mm) (Figure 5.6). No differences in length at hatch across treatments were observed for resistant embryos (Figure 5.6). The interaction term describing the difference in length at hatch by treatment among sites was insignificant ($p=0.622$).

Reactive Oxygen Species Endpoint

Measurements of reactive oxygen species (ROS) in embryos were significantly higher in the PCB 126 and PCB 126 + PBDE treated groups relative to vehicle controls and PBDE treated embryos (Fig. 5.7). Embryos treated with only PBDE appeared similar to controls (Fig. 5.7).

5.5 Discussion

The results of the present studies indicate that PBDEs display relatively low levels of toxicity but may alter development in teleosts. Furthermore, the observation of significantly altered developmental endpoints in non-resistant fish embryos (SSM) but

not resistant fish embryos (Newark) suggests resistance to PCB type inducers confers resistance to PBDEs. Contrary to previous reports, we observed no evidence of PBDE suppression of PCB mediated CYP1A induction. PBDEs appear to exhibit low toxicity in teleosts but may lead to developmental abnormalities (smaller fry size at hatching, increased time to hatch) that could compromise the survival of offspring.

The results of the adult channel catfish study suggest that, as in the killifish study, PBDEs have no discernable effect on CYP1A activity. Reports on the effects of PBDEs on CYP1A activity are varied although the majority are consistent with the result of these studies. Boon et al. [244] reported no change in CYP1A activity following oral exposure of Atlantic salmon (*Salmo salar*) to penta- and octabrominated PBDEs while Holm et al. [226, 244] detected no change in CYP1A activity in three-spine stickleback (*Gasterosteus aculeatus*) following exposure to tetra- and pentabrominated PBDEs. Likewise, Tomy et al. [247] reported no effect on CYP1A activity in juvenile lake trout (*Salvelinus namaycush*) exposed to dietary PBDEs for 56 days. Chen et al. [248] examined the induction of CYP1A activity using 12 different PBDE congeners in six different cell types, including two from fish, and reported that PBDEs were two to five times less potent than TCDD in inducing EROD activity. However, two other studies report suppressive effects including Tjarnlund et al. [249] who reported levels of CYP1A activity suppressed below controls in rainbow trout fed a single PBDE congener for either six or twenty-two days. Kuiper et al. [250] also reported suppression of 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) induced CYP1A activity when primary carp hepatocytes were co-treated with TCDD and DE-71. While reports of CYP1A induction following exposure to technical mixtures of PBDEs do exist [251, 252] there is some speculation that contamination from known CYP1A inducers contribute to this observed induction [81, 248]. In conclusion, the results of the present study are consistent with the majority of existing literature indicating that PBDEs are poor inducers of CYP1A.

Aroclor 1254, a well established inducer of CYP1A, induced EROD in this experiment although with less potency than anticipated. Although the induction observed was slightly lower than expected it is within the range of previous reports examining the effects of Aroclor 1254 on channel catfish hepatic CYP1A induction [186, 240]. Temperature is known to have a significant impact on CYP1A levels, low temperatures

are associated with lowered induction and increased time periods before the observation of induction [195]. Channel catfish are warm water fish [253] and the temperature at which this experiment was conducted (19 - 20°C) may have contributed to the relatively low level of induction observed. However, the observation of significant induction, albeit low, is an indication that the channel catfish in this study do have an inducible CYP1A system.

PBDEs also failed to significantly alter levels of hepatic UDPGT activity regardless of dose or time point. The effects of PBDEs on hepatic UDPGT activity in fish have not been previously examined although several studies have examined these effects in mammals. Zhou [92, 254] reported PBDE exposure in weanling rats induced UDPGT levels 3-5 fold, a response accompanied by a decrease in plasma T_4 levels. Chahoud et al. [255] reported UDPGT was increased at post natal day one but not twenty-two in rat offspring treated with two PBDE congeners in utero. Halgren and Darerud [256] reported only moderate UDPGT elevation in seven week old rats treated with PBDE-47 daily for seven days. UDPGT is part of the Ah gene battery and is activated along with CYP1A by binding of substrates to the Ah receptor [179, 180], therefore the lack of induction in both enzymes would not be unexpected.

Channel catfish thyroxine (T_4) levels in this study were very low, below the limits of reliable quantification. The catfish utilized in the study were fasted for seven days prior to and during the course of the experiment, possibly contributing to the observed low T_4 levels. Depressed levels of circulating thyroid hormone (both T_3 and T_4) are a commonly observed endocrine response in feed deprived fish and are thought to enhance survival by limiting the mobilization of energy reserves [37, 257, 258]. Gaylord et al. [259] observed significant decreases in circulating T_3 and T_4 within 72 hours of food deprivation in fingerling channel catfish held at 28^0C. Similarly, declines in T_4 were noted within 24 hours of cessation of feeding in rainbow trout (*Oncorhynchus mykiss*) [260] and tilapia (*Oreochromis niloticus*) [261]. In conclusion, any alteration in thyroid hormone levels attributable to toxicant exposure in the present experiment appears to have been masked by suppression resulting from lack of feeding during our experiment.

The exposure of both responsive and resistant killifish (*Fundulus heteroclitus*) to PBDEs failed to significantly alter CYP1A activity at all concentrations tested. These

results are consistent with both our adult catfish study and, as previously discussed, with the majority of literature reports indicating PBDEs are poor inducers of CYP1A in fish and other vertebrates. The observation of significant (off the scale) CYP1A induction in SSM embryos and suggestion of moderate induction in Newark embryos following treatment with a combination of PCBs and PBDEs indicated the assay was working properly. In summary, like adult fish in this study, CYP1A activity in fish embryos was unaffected by PBDE exposure.

Percent hatch appeared to be unaffected by all PBDE treatments and the combined PCB + PBDE treatment in embryos from both resistant and responsive populations. While percent hatch is a developmental endpoint frequently impacted by pollutant exposure, not all studies report significant impacts. Billison et al. [262] reported high embryo and larval mortality following exposure to 2,3,4,4' - tetrachlorobiphenyl (PCB #60) and 2,2',4,6,6' – pentachlorobiphenyl (#104) in zebrafish (*Brachydanio rerio*). However, other studies report results similar to my findings. Fonds et al. [263] reported no impact on either percent hatch or time to hatch in dab (*Limanda limanda*) exposed to the technical PCB mixture Clophon A40. Interestingly Monosson et al. [264] reported no significant impact on hatching success but found significantly decreased larval survival in white perch (*Morone americana*) embryos produced by females exposed to 0.2 to 5.0 mg/kg PCB 77. Likewise, Von Westernhagen et al. [265] reported mortality of marine fish eggs was more dependent on total pesticide concentrations than PCB concentration and Hansen et al. [266] found PCBs only had a minor effect on the hatching success of herring. While percent hatch was unaffected by PBDE treatment either alone or in combination with PCBs in this experiment, previous research indicates percent hatch may not be the most sensitive indicator of toxicant impact.

Time to hatch was significantly lengthened by exposure to the lower PBDE doses in SSM (responsive) but not Newark (resistant) embryos. A number of studies on the impact of various toxicants on fish development report alterations in hatch time correlated with toxicant exposure. In an experiment similar to the present study Timme-Laragy et al. [267] exposed *F. heteroclitus* embryos to PBDE- 71 at concentrations (0.001-100 μg/L) encompassed by the concentrations utilized in this study (10 – 1000

μg/L) and observed hatching delayed up to 4.5 days at the 100 μg/L dose but not lower doses. Hatching delays were observed in this study at doses of 10 and 100 μg/L. Gormley and Teather [268] report significantly extended hatching time in Japanese medaka (*Oryzias latipes*) exposed to endosulfan. Gauthier et al. [269] reported significantly lengthened hatching time in fathead minnows (*Pimephales promelas*) embryos exposed to water from metal contaminated lakes. Billison et al. [270] reported delayed hatching following exposure to PCB #60 and #104 in zebrafish embryos. Hatching in teleost eggs is initiated by release of the hatching enzyme chorionase, an enzyme that breaks down the chorion surrounding the embryo [268]. It has been proposed that certain chemicals either inhibit the release of or interfere with the action of this enzyme, therefore delaying hatching [271]. Another proposed mechanism of delayed hatching is a reduction in embryo vigor resulting in lengthened attempts to break free of the chorion [272], an effect observed in rainbow trout embryos exposed to high concentrations of petroleum hydrocarbons [273]. PBDE treatment may have lengthened time until hatch in responsive (SSM) embryos through one of the mechanisms discussed above, possibly in combination with effects on thyroid hormone.

Length at hatch in the present experiment was significantly suppressed relative to controls by the intermediate PBDE dose and the PBDE + PCB 126 dose in SSM (responsive) but not Newark (resistant) embryos. Reduction in development rate has been reported in other experiments testing the effects of pollutants on fish development. Gormley and Teather [268] reported Japanese medaka (*Oryzias latipes*) exposed to endosulfan were significantly smaller than controls one week following hatch. Billsson et al. [262] reported reduced growth in zebrafish embryos exposed to several PCB congeners. Bentivegna and Piatkowski [274] reported dose dependent reduction in the development of Japanese medaka exposed to tributyltin (TBT). While length at hatch should certainly not be considered independent of other developmental endpoints, the observation of both reduced length at hatch and extended time to hatch suggests PBDE induced toxicity in developing embryos.

Thyroid hormone is known to play a crucial role in the development of a number of vertebrates, including fish [216], and may be linked to PBDE induced abnormal development. Thyroid hormone is transferred from female fish to eggs [217] and

promotes survival of eggs [216, 218] as well as regulating the rate of development [37]. The mechanisms of this TH action remain unclear [275, 276]. While the TH present in teleost embryos appears to result primarily from maternal transfer, there is evidence of active regulation of TH levels in early salmonid embryos [275]. PBDEs are well established thyroid hormone disruptors in mammalian models, generally resulting in depressed levels relative to controls [90, 92]. Although embryo TH hormone levels were not quantified in this experiment, PBDE impact on TH in exposed embryos could have affected the previously discussed developmental endpoints.

Alterations in developmental endpoints were observed exclusively in embryos from the non-resistant populations, suggesting PCB resistant fish may be resistant to PBDEs as well. Previous research has indicated the killifish inhabiting Newark Bay are resistant to both PAHs and PCBs [106, 166], the results of this study suggest they are resistant to PBDEs as well. Interestingly, previous studies with Newark Bay killifish and reference (Flax Pond, NY) killifish failed to detect any site or dose related impact of PCB exposure on percent hatch or size at hatch [106], seemingly a result of the high variability in these parameters. The presence of developmental alterations in this study in non-resistant but not resistant fish is particularly interesting in light of the fact that these alterations occurred despite lack of CYP1A induction, suggesting CYP1A may play a minimal role in development.

Staining of embryos with the superoxide sensitive fluorescent dye dihydroethidium revealed increases in superoxide production in PCB but not PBDE treated embryos, an observation likely linked to the absence of CYP1A induction following PBDE exposure. PCBs are thought to generate reactive oxygen species via uncoupling of the CYP1A enzyme [80, 237]. In fact, only PCB congeners that are able to bind the Ah receptor induce oxidative stress [277-279]. Although some reports have indicated that certain PBDEs have a limited capacity to bind the Ah receptor [280], studies with other congeners report a complete inability of PBDEs to activate the AhR pathway [281] and induce CYP1A activity [248]. The inability of PBDE exposure to induce either CYP1A or UDPGT in this study suggests that PBDEs are not binding to and activating the fish AhR. Failure of PBDEs to induce biotransformation enzymes via the AhR pathway likely explains the lack of increased ROS in PBDE treated fish.

The results of these studies provide novel data concerning the response of channel catfish to PBDE exposure, the response of resistant and responsive fish embryos to PBDE exposure, and the induction of oxidative stress in responsive fish embryos. Our data coincide with a growing body of literature indicating PBDEs are poor AhR ligands and therefore weak inducers of both CYP1A activity and oxidative stress. Alteration of thyroid hormone levels, the primary response to PBDEs observed in rat and mice models, was undetectable in this study, likely a result of food deprivation. Alteration in certain developmental endpoints were noted among embryos from responsive populations but not resistant populations, suggesting organisms resistant to the toxic effects of PCBs may also be resistant to PBDEs. The results of these studies add credence to numerous literature reports indicating PBDEs do not affect many of the toxicological endpoints frequently impacted by environmental pollutants. However, PBDEs may affect organisms through alteration of embryonic growth and development involving currently unknown mechanisms.

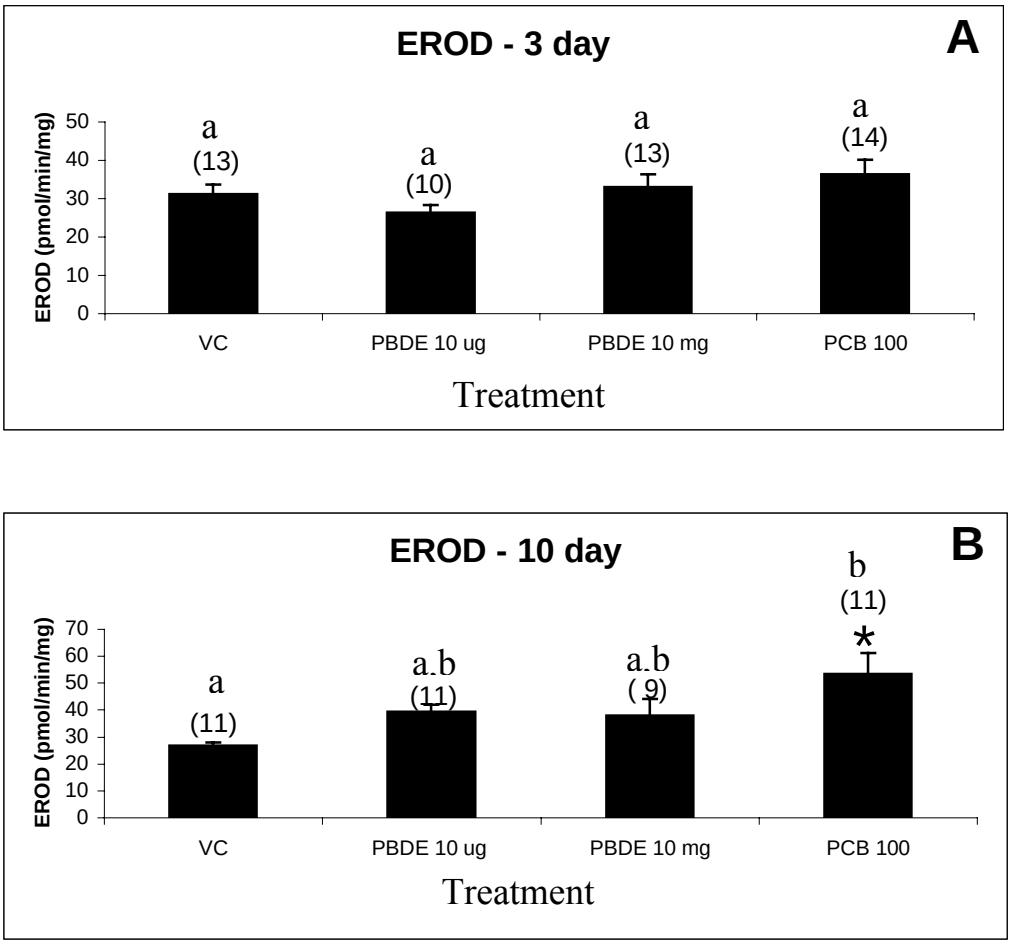


Figure 5.1. Effects of injection of 0, 10µg/g, or 10 mg/kg PBDE-71 or PCB 126 at 100 mg/kg on hepatic CYP1A activity (measured as EROD) in channel catfish 3 (A) and 10 (B) days following treatment. Values represent means ± SE. Means with the same letter are not significantly different at $p \leq 0.05$.

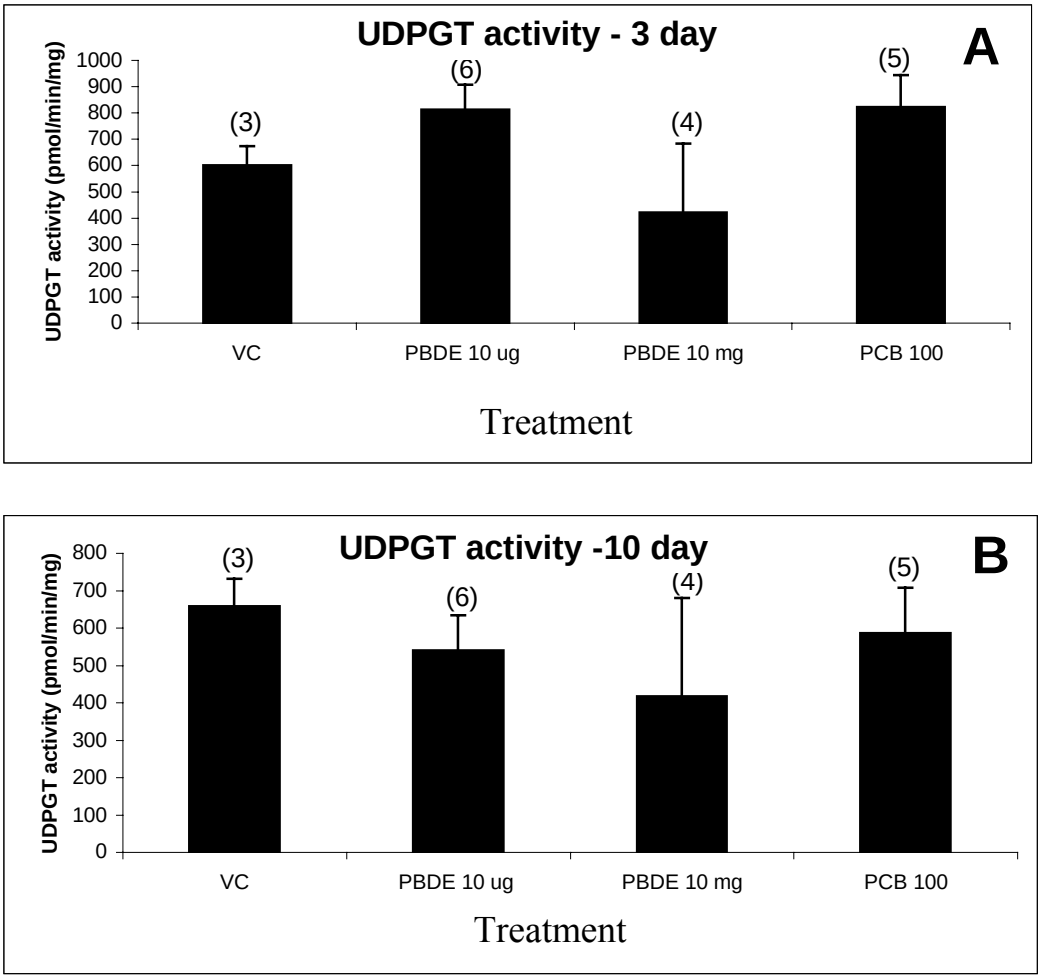
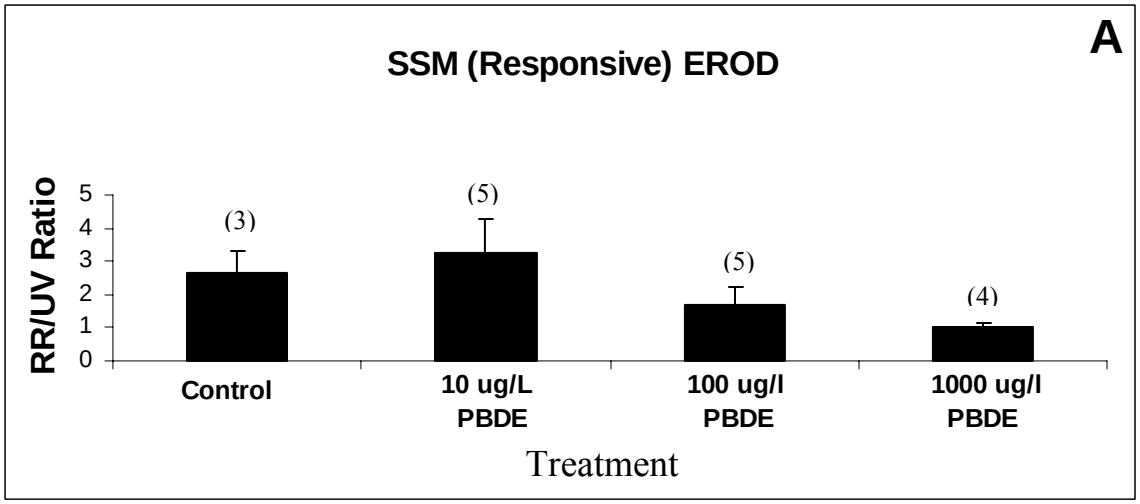


Figure 5.2. Effects of injection with 0, 10µg/g, or 10 mg/kg PBDE-71 or PCB 126 at 100 mg/kg on hepatic UDPGT activity in channel catfish 3 (A) and 10 (B) days following treatment. Values represent means ± SE (n). All means are similar at $P \leq 0.05$.



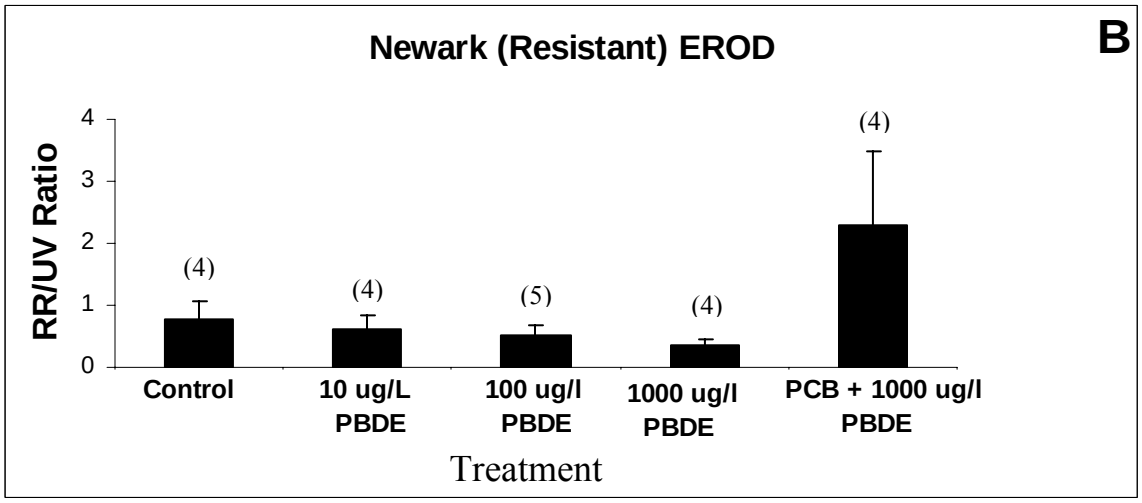


Figure 5.3. Effects of treatment with 0, 10µg/L, 100 µg/L, or 1000 µg/L PBDE-71 and 1000 µg/L PBDE-71 + PCB 126 at 1000 µg/L on CYP1A activity (RR/UV ratio) in embryos exposed to test compounds from post-fertilization day 2 through 7. Values represent means ± SE for (n) individuals. All means are similar at $p \leq 0.05$. CYP1A values for the PBDE-71 + CB126 combined dose for SSM fish (A) were off-scale (high) relative to the other treatment groups and are not shown.

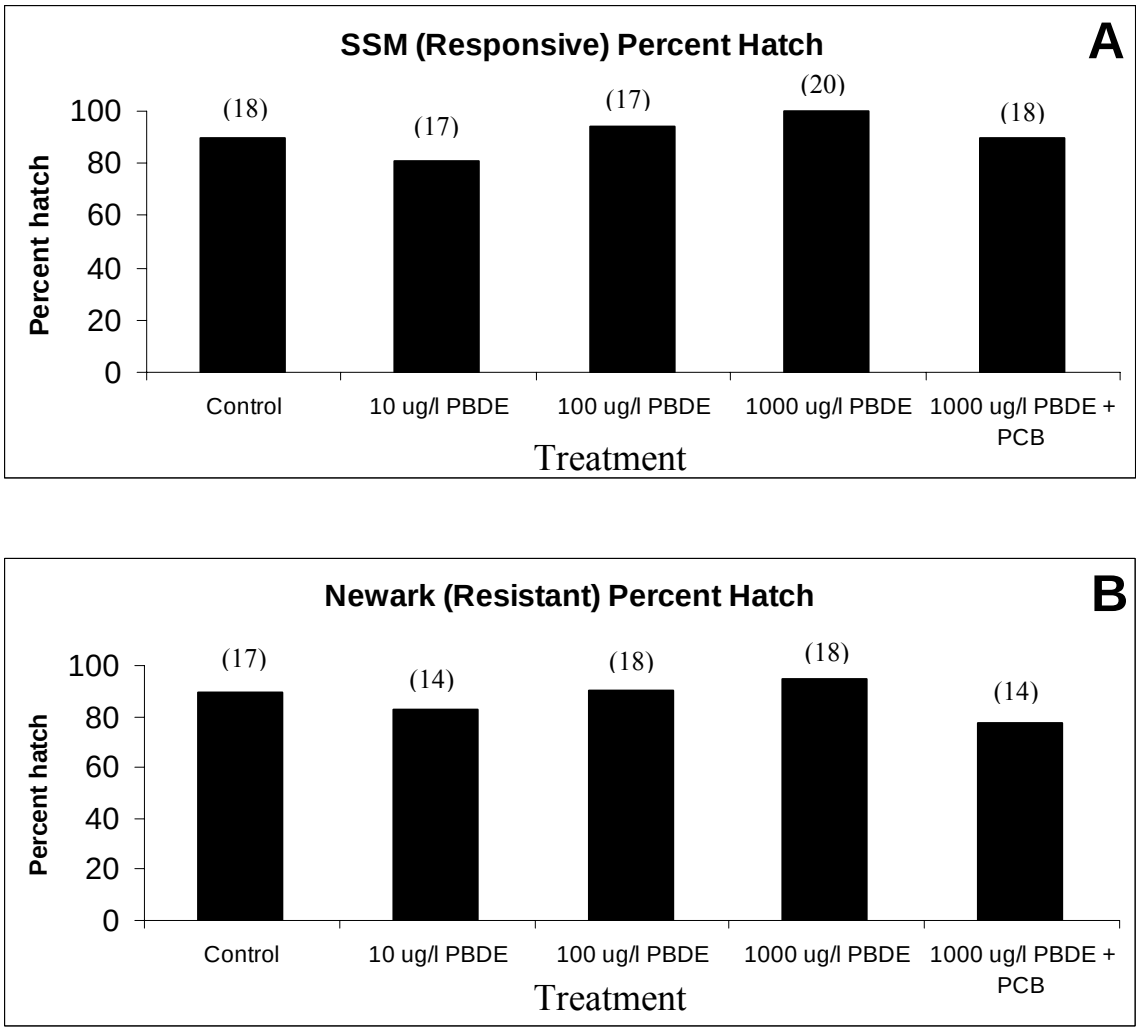
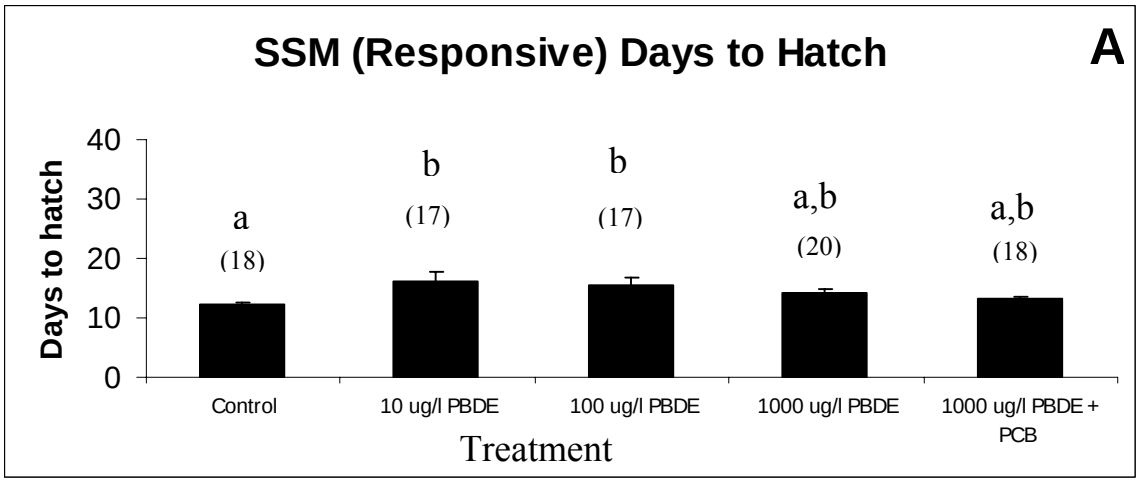


Figure 5.4. Effects of treatment with 0, 10µg/L, 100 µg/L, or 1000 µg/L PBDE-71 or 1000 µg/L PBDE-71 + PCB 126 at 1000 µg/L on on percent hatch in embryos exposed to test compounds from post-fertilization day 2 through 7. All means are similar at $p \leq 0.05$.



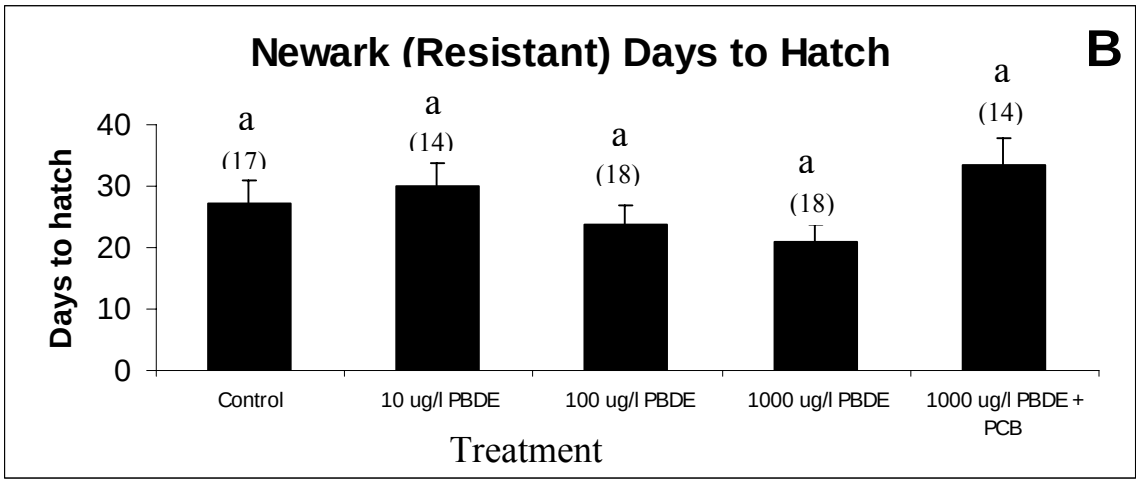
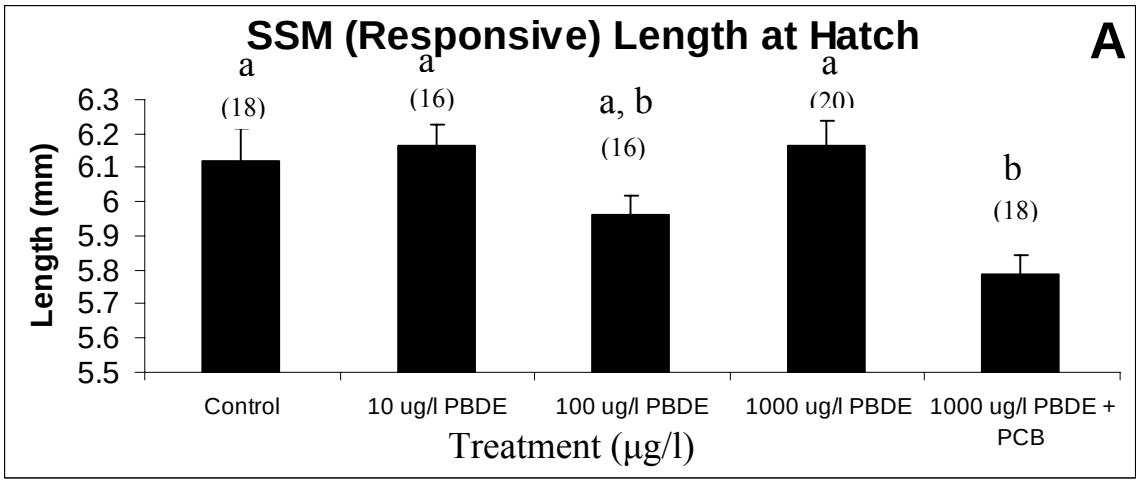


Figure 5.5. Effects of treatment with 0, 10µg/L, 100 µg/L, or 1000 µg/L PBDE-71 or 1000 µg/L PBDE-71 + PCB 126 at 1000 µg/L on days until hatch in killifish embryos exposed to test compounds from post-fertilization day 2 through 7. Values represent means ± SE (n) individuals. Means with the same letter are not significantly different at $p \leq 0.05$.



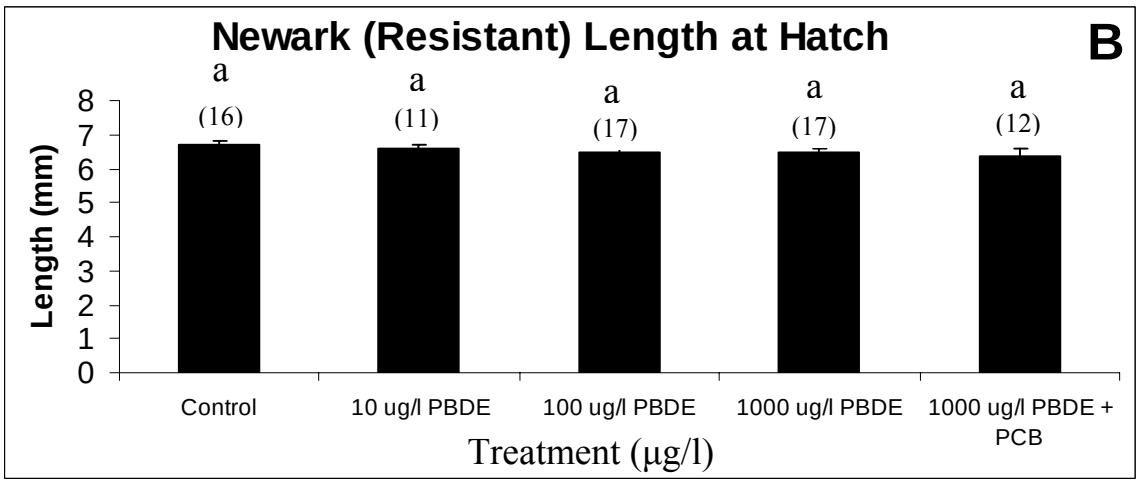


Figure 5.6. Effects of treatment with 0, 10µg/L, 100 µg/L, or 1000 µg/L PBDE-71 and 1000 µg/L PBDE-71 + PCB 126 at 1000 µg/L on length at hatch in killifish embryos exposed to test compounds from post-fertilization day 2 through 7. Means with the same letter are not significantly different at $p \leq 0.05$. Values are mean ± SE (n).

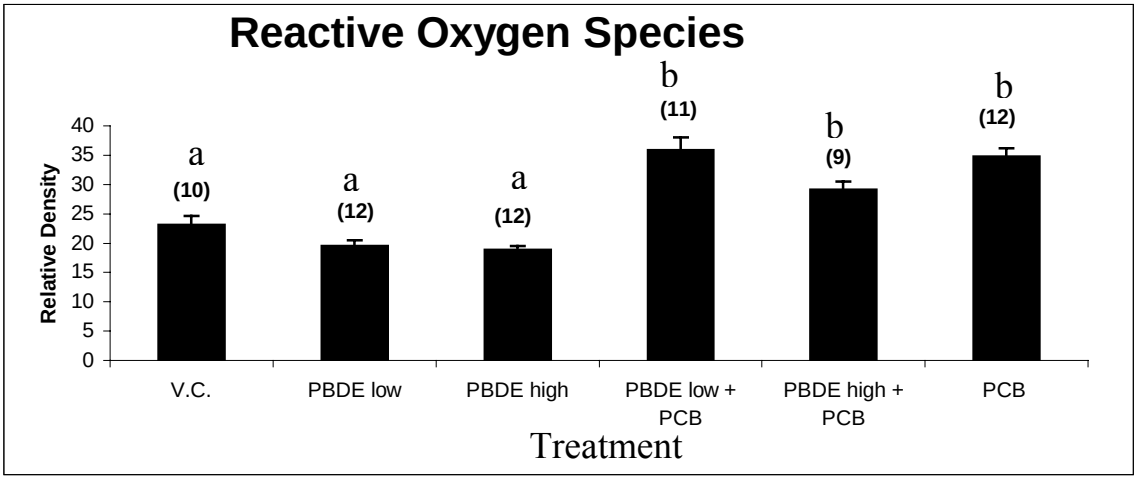


Figure 5.7. Effects of treatment with vehicle control (acetone), PBDE – 71 at 10 µg/L (PBDE low), PBDE – 71 at 1000 µg/L (PBDE high), PCB 126 at 0.01 µg/L + PBDE – 71 at 10 µg/L, PCB 126 at 2.0 µg/L + PBDE 71 at 1000 µg/L, or PCB 126 at 0.01 µg/L on oxidative stress in embryos exposed to test compounds from post-fertilization day 2 through 7. Values represent means ± SE for n. Means with the same letter are not significantly different at $p \leq 0.05$.

Chapter 6: Effects of Pollution Resistance on Polychlorinated Biphenyl Mediated Thyroid Hormone Disruption

* Thyroid hormone analysis conducted by Dr. Stephen D. McCormick and Amy Moeckel, USGS, Conte Anadromous Fish Research Center, Turners Falls, MA

6.1 Abstract

While the overt toxic effects of environmental pollutants are often well characterized, less obvious effects such as endocrine disruption have only recently been recognized and are incompletely understood. Polychlorinated biphenyls (PCBs) are ubiquitous environmental contaminants well established as disruptors of normal endocrine function in a number of vertebrates including fish. Certain organisms have been characterized as having developed resistance to contaminants such as PCBs, a condition typically characterized by reduced expression of cytochrome P-4501A (CYP1A), a phase I biotransformation enzyme frequently involved in detoxification pathways. The consequences of this resistance on the endocrine disrupting effects of environmental contaminants are entirely unknown. We hypothesized that acquired resistance to polychlorinated biphenyls (PCBs), as measured by altered expression of CYP1A, results in resistance to the endocrine disrupting effects of PCBs. Adult *Fundulus heteroclitus* from PCB resistant and non-resistant populations were treated with PCB 77 at 0, 0.1 and 1.0 mg/kg and sacrificed at days ten and thirty. CYP1A activity was unchanged in fish from both populations following PCB treatment. Although CYP1A protein levels were significantly elevated in responsive fish thirty days following PCB treatment relative to controls interaction terms indicated no difference in response between populations. Uridine diphosphate glucuronyltransferase (UDPGT) activity was unchanged in both populations. Plasma levels of triiodothyronine (T3) and thyroxine (T4) were highly variable, although treated fish were similar to controls in both populations at all time points. These results indicate that PCBs do not alter the thyroid endpoints examined in this study in either responsive or resistant killifish populations.

6.2 Introduction

Although the overt toxic effects of environmental pollutants are often well established it has not been until recently that new, less obvious, effects of pollution have been recognized. One of the most important of these is the capacity of some chemicals to alter the normal hormonal processes in the body, termed 'endocrine disruption'. In some cases even low concentrations of environmental pollutants are capable of affecting normal hormonal function in both humans and wildlife [282], resulting in altered hormone levels [52, 283], reduced gonad size [48], and reproductive failure [284]. Endocrine disruption has been widely reported in wild fish populations [54, 55, 282, 285]. The role of hormones in regulating both reproduction and development means alterations in hormone function may ultimately threaten the survival of affected populations [212, 286].

Although pollution exposure most often leads to deleterious effects, an alternate response recently recognized in a number of populations is genetic adaptation. Fish populations may develop resistance to pollutants, a condition in which exposure to pollution fails to induce levels of CYP1A, a xenobiotic metabolizing enzyme normally strongly induced by exposure to organic pollutants, including PCBs [103]. This phenomenon has been observed in numerous fish populations [104, 287].

There is evidence that in addition to altering levels of the enzyme CYP1A, PCBs also alter hormone balance in vertebrates, including fish. PCBs are known to alter thyroid hormones through induction of the conjugating enzyme, uridine diphosphate-glucuronosyltransferase (UDPGT). UDPGT lowers plasma levels of thyroxine (T4) by metabolizing T4 to a conjugated, water soluble metabolite that is rapidly excreted [222]. The thyroid system regulates critical physiological processes in fish, and alterations of normal thyroid function by potent thyroid hormone disruptors such as PCBs could severely compromise population fitness. Thyroid hormones regulate osmoregulation [213], growth and development [214, 288], and sexual maturation [215]. Further, maternally-derived thyroid hormone [217] influences embryonic development [216] and promotes larval survival [216, 218, 219].

PCBs are well-established thyroid hormone (TH) disrupters in fish [289] altering TH levels under both field and laboratory conditions. Ingestion of PCBs through the diet

suppressed blood levels of TH in coho salmon and rainbow trout [290-292], and muscle TH levels in rainbow trout [289]. Although most studies report lower TH levels in PCB exposed fish, some researchers have also reported increased TH levels in response to dietary [293] and waterborne [294] PCB exposures. Munkittrick et al. [295] found increased serum TH levels in white suckers collected below a bleached kraft pulp-mill effluent outfall contaminated with PCBs and dioxins. Differences among studies may reflect differences in life stage, reproductive condition, diet, or temperature, all of which affect thyroid hormone titers in teleosts [296]. Regardless of the direction of the effect, these studies clearly demonstrate the disruptive actions of PCBs on thyroid homeostasis in fish.

PCB regulation of CYP1A and regulation of thyroid hormone levels are mechanistically linked by the aryl hydrocarbon receptor (AhR) [297]. The induction of both CYP1A and the TH metabolizing enzyme UDPGT are regulated via the AhR [297]. Binding of planar PCBs to the AhR initiates transcription of genes within the AhR gene battery, including both CYP1A and, although to a lesser degree, UDPGT [57, 297]. Induction of hepatic UDPGT can lower plasma TH levels [298, 299].

The goal of this study is to determine whether acquired resistance to polychlorinated biphenyls (PCBs), as measured by altered expression of cytochrome P450 1A (CYP1A), results in resistance to the endocrine disrupting effects of PCBs. I hypothesize that acquired PCB resistance confers additional resistance to the hormone disrupting actions of these compounds. Acquired resistance to PCBs has been reported in a number of fish populations but the consequences of this resistance on endocrine function are unknown. This study is the first to my knowledge to investigate the hormonal consequences of chemical resistance.

6.3 Methods

Materials

7-Ethoxyresorufin and resorufin were obtained from Molecular Probes (Eugene, OR, USA). The monoclonal antibody made against scup CYP1A protein, MAb 1-12-3, was a generous gift of Dr. John Stegeman (Woods Hole Oceanographic Institution). Cy^{TM}5-conjugated affinipure goat anti-mouse IgG was obtained from Jackson

Immunoresearch Laboratories Inc. (West Grove, PA, USA). Nitrocellulose membrane (0.45 μm) was obtained from Schleicher and Schull (Keene, NH, USA). The Bio-Dot SF Microfiltration Apparatus was obtained from Bio Rad (Hercules, CA, USA). All other reagents were obtained from Sigma, Fisher Scientific (Fair Lawn, NJ), or Invitrogen.

Experimental Design

Adult killifish (*Fundulus heteroclitus*) were collected from either PCB resistant (New Bedford Harbor, MA) [106] or from the reference site West Island (RI) and held in the laboratory for at least four months (the halflife life of PCBs in fish tissue [300]) to depurate contaminant body burdens prior to the experiment. Fish were in post-spawn condition at the onset of the experiment. Fish were maintained during the depuration in 10 or 20-gallon recirculating tanks equipped with charcoal Whisper^{TM} filters in 30 ppt artifical seawater at 24-25 ^{0}C on a 16/8 L/D cycle. Fifty percent of the tank water was replaced every other week. Fish were Tetramin ad libitim approximately every other day.

Fish from each population were then divided into four treatment groups and given intraperitoneal injections of PCB 77 in corn oil at 0.1 or 1.0 mg/kg or vehicle alone. One treatment group from each site was sacrificed on day 10 post-injection and one on day 30 post-injection. Ten and thirty days were chosen as time points based on data from previous studies examining thyroid hormone response in teleosts. Some studies report alterations in a relatively short period of time (7-15 days) while others report alterations at a later time point(greater than 20 days). [301].

Liver Microsomal Protein Isolation

Livers were removed from liquid nitrogen, weighed, and homogenized in 10 volumes (weight:volume) of ice-cold 50 mM Tris buffer (pH 7.4). Microsomal fractions were obtained by differential centrifugation as previously described [131]. The final 100,000 x g microsomal pellets were resuspended in 50 mM Tris buffer (pH 7.4) containing 1mM EDTA, 1 mM DTT and 20% glycerol at a 1:1 ratio (liver weight: resuspension buffer volume). Microsomal samples were stored in liquid nitrogen until analyzed for catalytic activity and CYP1A protein content (within three weeks).

Catalytic and Protein Assays

Ethoxyresorufin -O-deethylase (EROD) activity, a reaction catalyzed exclusively by CYP1A [4], was measured fluorometrically in 48 well CoStar™ plates using the method of Hahn et al. [133]. The reaction mixture contained 50 mM Tris, pH 7.4, 0.1 M NaCl, 2μM 7-ethoxyresorufin, and 100 to 300 ug of microsomal protein in a final volume of 200 μl. The reaction was initiated by the addition of 1.34 mM NADPH, run at room temperature (28-29^{0}C), and production of the fluorescent product, resorufin, measured for 13 minutes. The linear portion of the curve was used to evaluate the rate of the reaction. All EROD assays were run in triplicate on a PerSeptive Biosystems Cytofluor Series 4000™ multi-well plate fluorometer.

Microsomal protein was measured fluorometrically using the method described by Lorenzen and Kennedy [134] with bovine serum albumin as the standard. All protein assays were run in triplicate.

UDPGT Catalytic Assay

Uridine diphosphate glucuronyl transferase (UDPGT) activity was measured as described [193], 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 [194]. All samples assays were performed in duplicate.

Immunoblotting Procedures

CYP1A protein was quantified by immuno-blotting using a Bio-Dot SF™ microfiltration slot-blot apparatus. Twenty micrograms of microsomal protein diluted in 200 µL buffer (20 mM Tris, 0.5 M) was loaded into each well and vacuum transferred onto a nitrocellulose membrane (0.45 µM). The membrane was incubated in TBS-5% milk at 4^{o}C overnight to block non-specific binding, followed by incubation with MAb 1-12-3 dissolved (3 µg/ml) in milk block, a monoclonal antibody which recognizes CYP1A in multiple vertebrate species [19], essentially as described by Elskus et al. 1999 [106]. CYP1A signal was detected using Cy^{TM}5-conjugated affinipure goat anti-mouse IgG as the secondary antibody (10.8 µg/ml in sodium azide) and blots were scanned at 633 nm excitation/670 nm emission using a Typhoon 8600 scanner (Molecular Dynamics) and quantified using Image Quant (Molecular Dynamics). Liver microsomes from trout treated with the CYP1A model inducer, ß-naphthoflavone, were loaded in seven concentrations ranging from 0.1 to 7 µg of protein diluted in 200 µL buffer to evaluate linearity of the CYP1A signal on each blot. All samples were run at least in triplicate.

Thyroid Hormone Analysis

Thyroxine (T4) and 3,5,3'-triiodo-L-thyronine (T3) concentrations were measured by a direct radioimmunoassay. The lower detection limits were 0.5 ng/ml (T4) and 0.2 ng/ml (T3). Intra- and interassay coefficients of variation for these assays were 4.3-11% and 3.2-5%, respectively. 10 µl of plasma was added to 12 x 75 borosillicate glass tubes (in duplicate) and was incubated for 30 minutes at 37°C with assay mixture containing: 50 µl (5000 cpm) of ^{125} I labeled T3 or T4 (Perkin Elmer Life Sciences) diluted in 11 mM sodium barbital buffer pH 7.8, 0.1% sodium azide, 100 µl of T3 or T4 polyclonal antibody (Accurate Chemical & Scientific Corporation) diluted to approximately 1:6000 in 11 mM sodium barbital buffer pH 7.8, 0.1% sodium azide, 100 µl gamma globulins (Sigma) dissolved in diluted in 11 mM sodium barbital buffer pH 7.8, 0.1% sodium azide plus 0.5 mg/mL 8 aninlino 1 napthalene sulfonic acid (Sigma)

Samples were incubated overnight at 4°C. Proteins were precipitated with 300 µl of 25% polyethylene glycol (Sigma) in 11mM sodium barbital pH 7.8, sodium azide 0.1%. Tubes were centrifuged in at 3,000 x g for 20 minutes at 4°C. The supernatant was aspirated and the pellets counted in Beckman 5500 gamma counter.

Statistical Treatment of Data

Statistical analyses were performed using SYSTAT Version 10. All data were transformed (log 10) before analysis. Data were analyzed using both a two and three-way ANOVA. All differences were considered significant at $p <$ or $= 0.05$.

6.4 Results

The interaction term comparing treatment response between sites (two-way ANOVA) was significant for the 10-day (p=0.00403) but not the 30-day (p=0.729) time point. The interaction term of a three way ANOVA indicated significant impact of time point (ten or thirty day) on EROD activity (p=0.0245). The highest PCB dose (1 mg/kg) induced CYP1A activity in West Island killifish (responsive) ten days following exposure (Figure 6.1A). In contrast New Bedford Harbor killifish (resistant) at this same time-point displayed a suppression of CYP1A activity (Figure 6.1A). No apparent dose related effects were visible in CYP1A activity in either population thirty days following exposure (Figure 6.1B).

CYP1A protein levels in West Island fish treated with 0.1 mg/kg PCB 77 were suppressed significantly below controls at the ten-day time point (Figure 6.2A). No other differences were evident in CYP1A protein levels in fish from either site (Figure 6.2A). CYP1A protein levels 30 days following treatment were significantly elevated over controls in West Island fish treated with 0.1 and 1.0 mg/kg PCB 77 (Figure 7.2B). In sharp contrast CYP1A protein levels in PCB treated NBH fish were unchanged relative to controls at this time point (Figure 6.2B). However, the interaction terms comparing treatment response among sites (two–way ANOVA) for both the 10-day (p=0.241) and 30-day (p=0.334) time points were insignificant. However, the interaction term examing the impact of time point on CYP1A protein levels was significant (p=0.00703).

No differences were observed in UDPGT levels regardless of site or treatment (Figure 6.3). The interaction terms comparing treatment response between sites (two-way ANOVA) for both the 10-day (p=0.410) and 30-day (p=0.711) time points were insignificant. In a similar manner the interaction term of a three-way ANOVA indicated a lack of significant effect of time point on UDPGT levels (p=0.901).

Plasma triiodothyronine (T3) concentrations were relatively homogenous among treatments in NBH fish at the ten-day time point and no significant differences were observed among treatments in fish from either site (Figure 6.4A). At the 30 day time point T3 levels in NBH fish in the 0.1 mg/kg treatment group were suppressed relative to controls (Figure 6.4B). The interaction terms comparing treatment response between sites for both the 10-day ($p=0.145$) and 30-day ($p=0.690$) time points were insignificant. Likewise, analysis by three-way ANOVA revealed lack of significant impact of time point on plama T3 levels ($p=0.221$).

Plasma thyroxine (T4) concentrations were statistically similar regardless of treatment or site at the ten-day time point. Thirty days following treatment the WI 0.1 mg/kg treatment group was suppressed relative to controls (Figure 6.5B) while no differences were observed in the NBH fish. The interaction terms comparing treatment response between sites for both the 10-day ($p=0.163$) and 30-day ($p=0.705$) time points were insignificant. Time point also failed to significantly impact plasma T4 levels ($p=0.152$).

6.5 Discussion

The results of this study provide valuable and novel information on physiologically relevant endpoints for organisms residing in contaminated areas. Chemical resistance in fish is a recently recognized phenomenon and little is known regarding the consequences in affected populations. These results suggest populations that have developed resistance to PCBs experience alterations in thyroid hormone homeostasis that do not differ from those observed in non-resistant populations following PCB exposure. The data presented in this study is the first, to my knowledge, to report on the effects of chemical resistance on endocrine disruption.

Induced CYP1A activity and protein in WI fish confirmed the presence of a PCB induced response in non-resistant fish. The significant interaction term comparing treatment EROD response among sites at the 10-day time point confirms a differential pollutant response in these two populations. Similar to the present study, previous studies with reference populations of *F. heteroclitus* typically demonstrate induction of both CYP1A protein and activity following exposure to inducing compounds [106, 302].

The significant elevation of CYP1A protein and activity in WI fish and lack of response in NBH fish illustrates, as expected, elevated CYP1A levels in responsive but not resistant fish.

The lack of induction of UDPGT in WI fish and similarity of UDPGT treatment response between populations is consistent with the homogenous thyroid hormone response observed between populations. Glucuronidation of thyroid hormones is a major pathway for hepatic metabolism and excretion of thyroid hormones in rainbow trout [303] and presumably other teleosts. In addition, increased hepatic elimination of T4-glucuronide has been recognized as the most important mechanism facilitating impact of TCDD [304] and co-planar PCB [305] exposure on thyroid homeostasis in mammals. Fish studies frequently report alterations in UDPGT activity accompanied by alterations in thyroid hormone levels. Brown et al. [306] reported a dose related increase in T4–glucuronidation in lake trout (*Salvelinus namaycush*) following exposure to PCB 126 accompanied by T4 levels first elevated and then depressed relative to controls. While UDPGT is not the only physiological mechanism by which thyroid hormone levels are regulated, the similarity in UDPGT response between populations is consistent with the observed similarity in thyroid hormone response.

The heterogeneity in TH levels observed in PCB treated fish from both sites is consistent with previously reported research. Several studies report decreases in T3 levels following PCB exposure. Adams et. al. [301] observed decreased levels in T3 but not T4 seven days after treatment with PCB 77. Leatherland and Sonstergard [307] reported depressed levels of both T3 and T4 in rainbow trout (*Oncorhynchus mykiss*) following exposure to technical mixtures of PCBs. However, other studies report increases [294, 301] or no effects [308, 309] on levels of plasma thyroid hormone following PCB exposure. In some cases elevation and then suppression of thyroid hormone has been observed in a single experiment [306]. Clearly PCB exposure does not elicit a consistent response in plasma levels of thyroid hormones in teleosts. Thyroid hormone levels likely fluctuate in most cases in response to pollutant exposure leaving the observed response dependent on the time point at which measurements are taken. The heterogeneity of thyroid hormone levels observed in this experiment likely provides snapshots of thyroid hormone levels during this readjustment process.

The wide variety of responses reported in teleost thyroid hormone levels following PCB exposure is likely a result of both the complexity of mechanisms by which thyroid homeostasis is maintained and the sheer number of factors affecting thyroid hormone levels. Thyroid hormone is produced primarily as the prohormone T4 and then converted to T3, the primary active form of the hormone, in extrathyroidal tissues by T4 outer ring deiodination (T4-ORD). Adams et al. [301] reported increased T4 outer ring deiodination in American plaice (*Hippoglossoides platessoides*) in response to PCB exposure. However, a decrease in T3 levels was observed in synchrony with this increase in T4-ORD leading the authors to speculate that the increased T4-ORD activity was a compensatory mechanism to ameliorate lowered T3 levels resulting from increased UDPGT facilitated excretion [301]. Zhou et al. [227] speculated that the goiterous condition and elevated plasma T4 levels he observed in F. *heteroclitus* collected from a highly contaminated site were a result of contaminant blocking tissue receptors preventing negative feedback from reaching the hypothalamic-pituitary-thyroid axis. A number of factors can affect thyroid hormone levels including temperature [310], feeding [259], growth [311], and stress [312]. While the homeostatic mechanisms regulating thyroid hormone levels are normally capable of maintaining appropriate levels of thyroid hormone during periods when the above factors are not critical, pollutant exposure during a time when thyroid hormone requirements are high could lead to serious consequences [306].

In conclusion the results of this study indicate that the thyroid hormone endpoints examined in this study are not impacted by PCB exposure in either responsive or resistant killifish. The relative uniformity in thyroid hormone response between treatments and populations supports this premise. Further studies into this topic should examine TH levels at a wider variety of time points as well as examining other aspects of thyroid hormone regulation such deiodination activity. In addition, future studies might examine the effects of pollutant exposure on thyroid hormone regulation in concert with other thyroid affecting factors such as stress, reproduction, fasting, or metamorphosis.

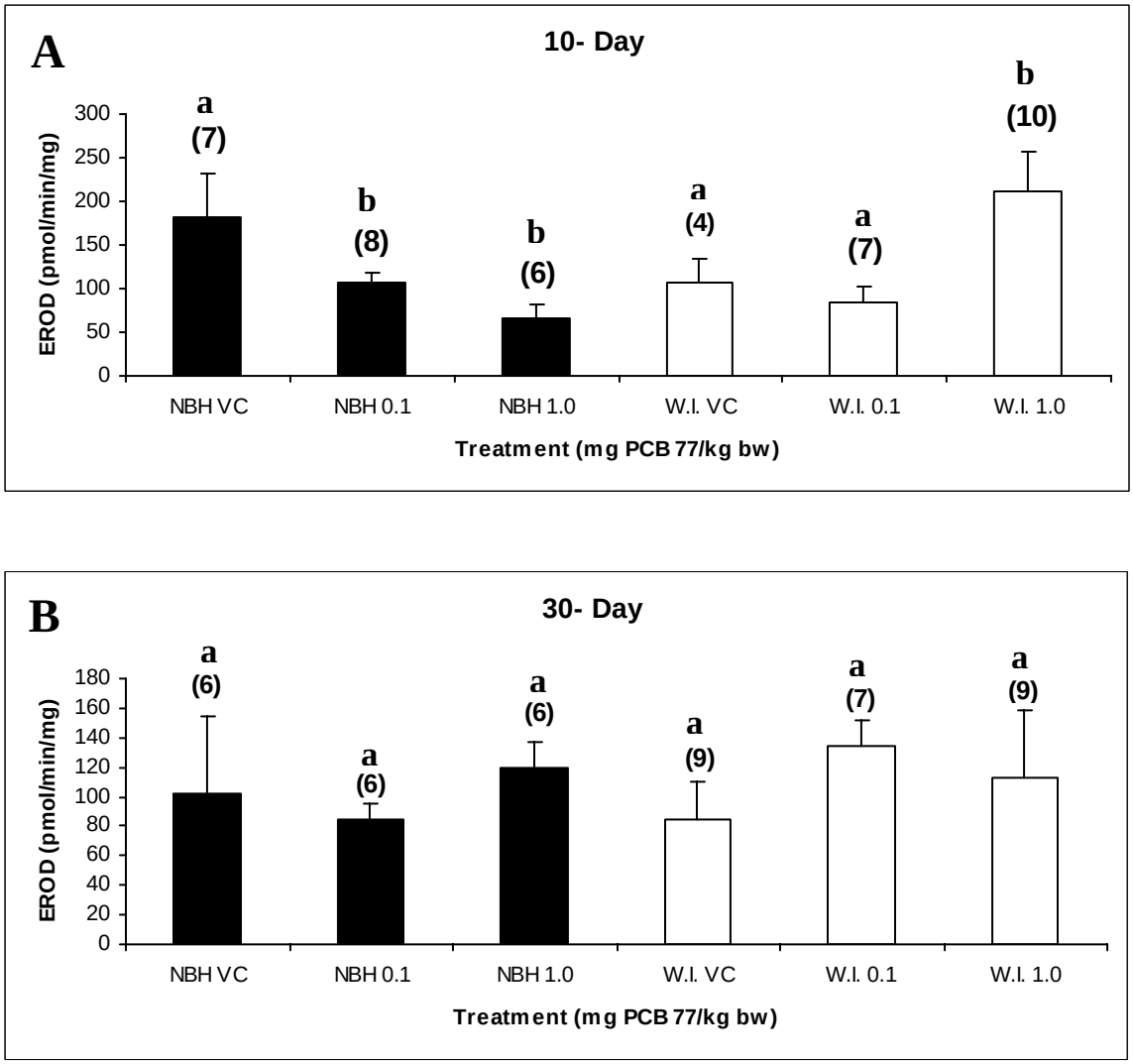


Figure 6.1. Hepatic microsomal EROD activity (pmol resorufin/min/mg) in *F. heteroclitus* treated with either a vehicle control (VC) or PCB 77 at 0.1 or 1.0 mg/kg and sacrificed on day 10 (A) or day 30 (B). Bars represent means ± SE for (n) individuals. Similar letters indicate treatment means do not differ from vehicle control within populations (NBH or WI) at $p < 0.05$.

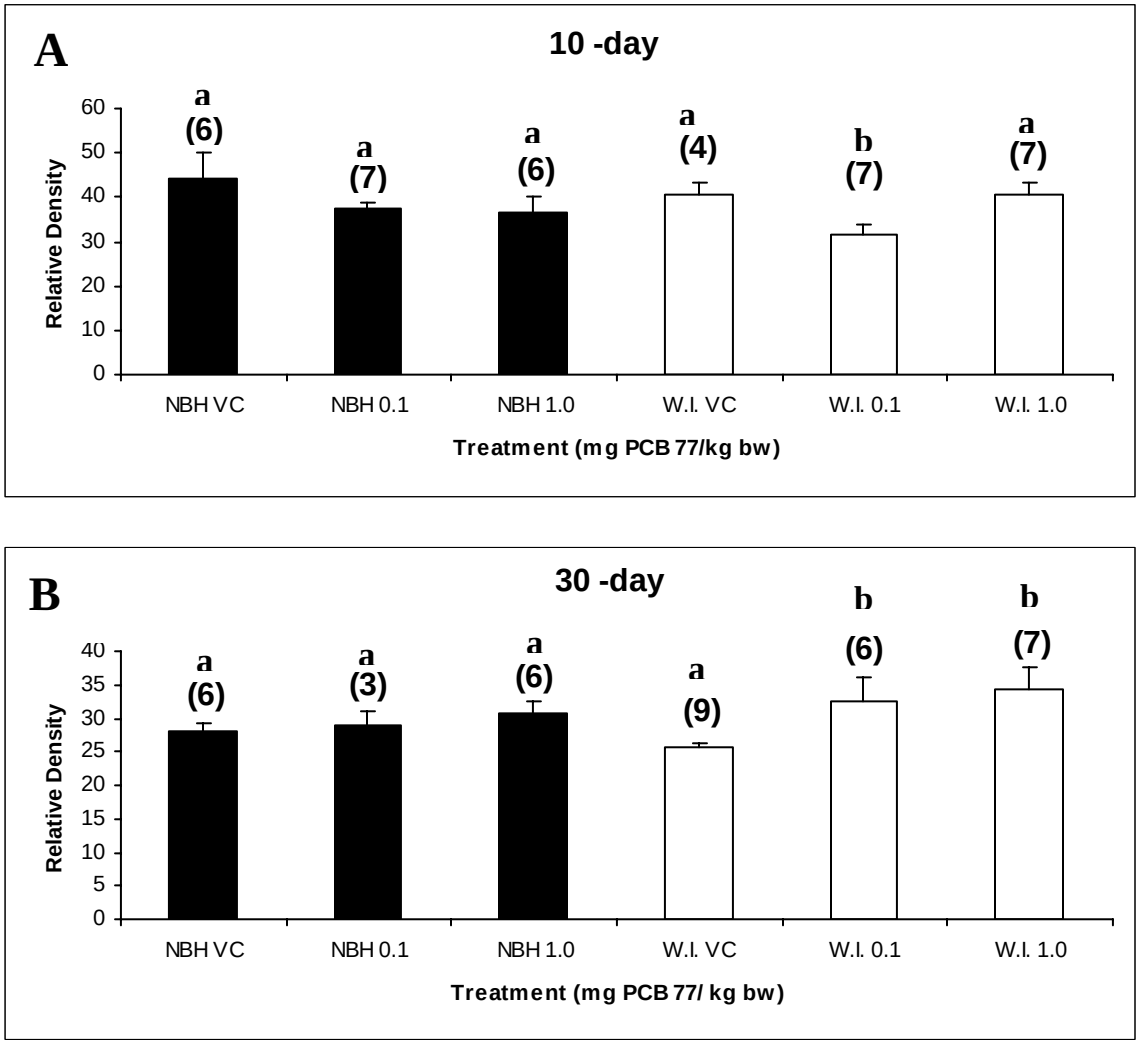


Figure 6.2. Hepatic microsomal CYP1A protein levels in *F. heteroclitus* treated with either a vehicle control (VC) or PCB 77 at 0.1 or 1.0 mg/kg and sacrificed on day 10 (A) or day 30 (B). Bars represent means ± SE for (n) individuals. * = significantly different from VC of the same population (NBH or WI) at $p < 0.05$.

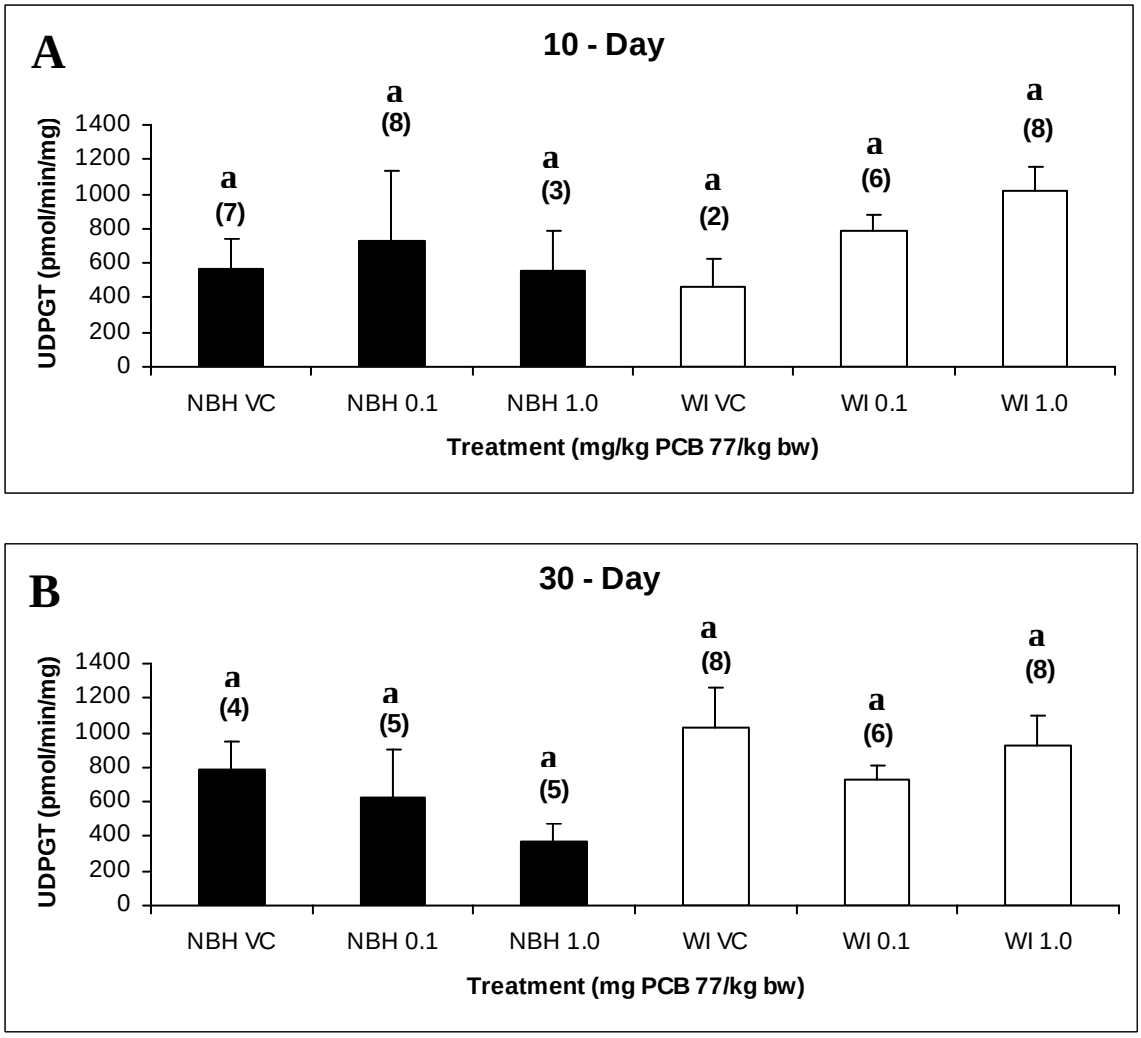


Figure 6.3. Hepatic UDPGT activity (pmol//min/mg) in *F. heteroclitus* treated with either a vehicle control (VC) or PCB 77 at 0.1 or 1.0 mg/kg and sacrificed on day 10 (A) or day 30 (B). Bars represent means ± SE for (n) individuals. Similar letters indicate treatment means do not differ from vehicle control within populations (NBH or WI) at $p < 0.05$.

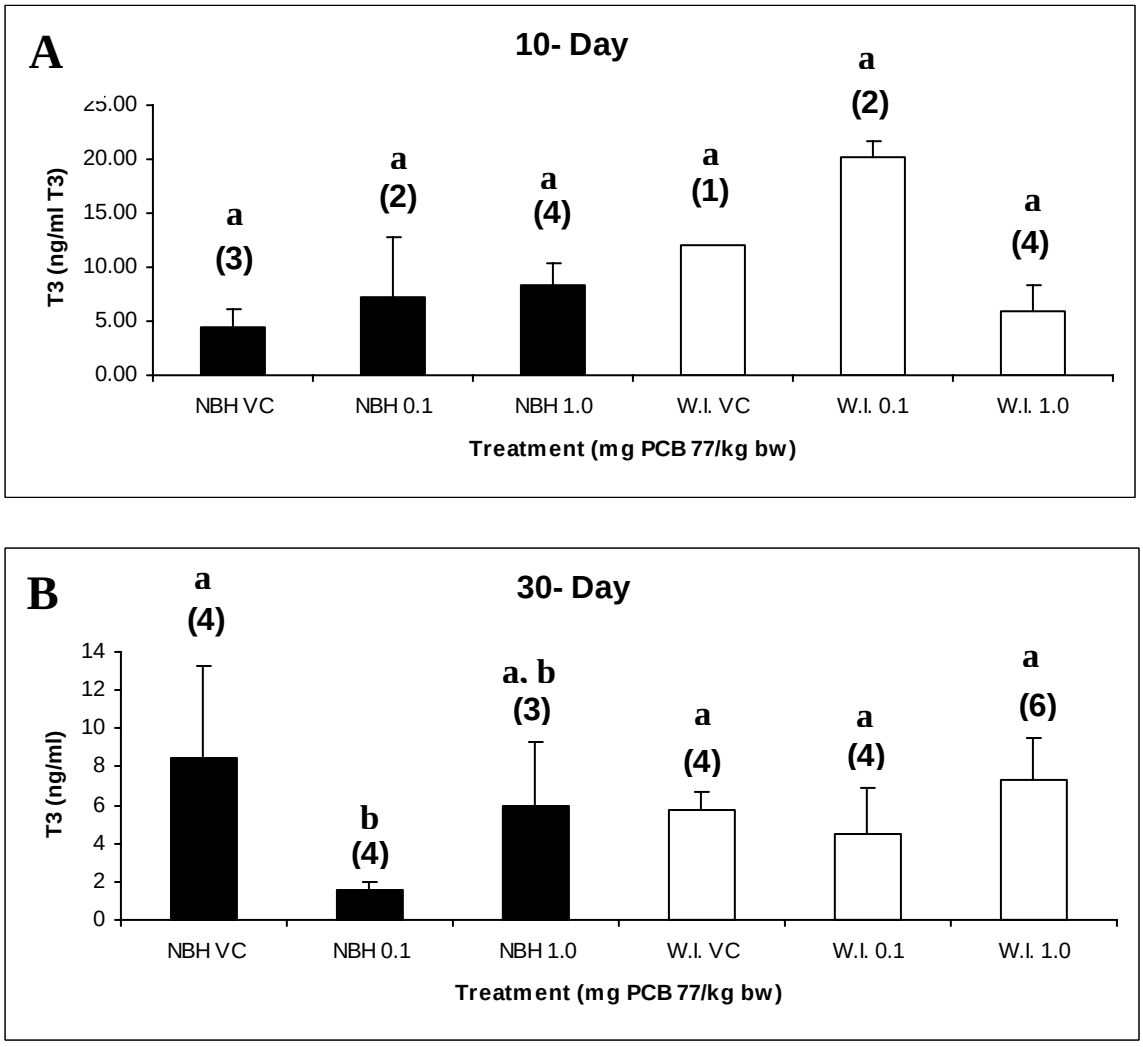


Figure 6.4. Plasma triiodothyronine (T3) levels in *F. heteroclitus* treated with either a vehicle control (VC) or PCB 77 at 0.1 or 1.0 mg/kg and sacrificed on day 10 (A) or day 30 (B). Bars represent means ± SE for (n) individuals. Similar letters indicate treatment means do not differ from vehicle control within populations (NBH or WI) at $p < 0.05$.

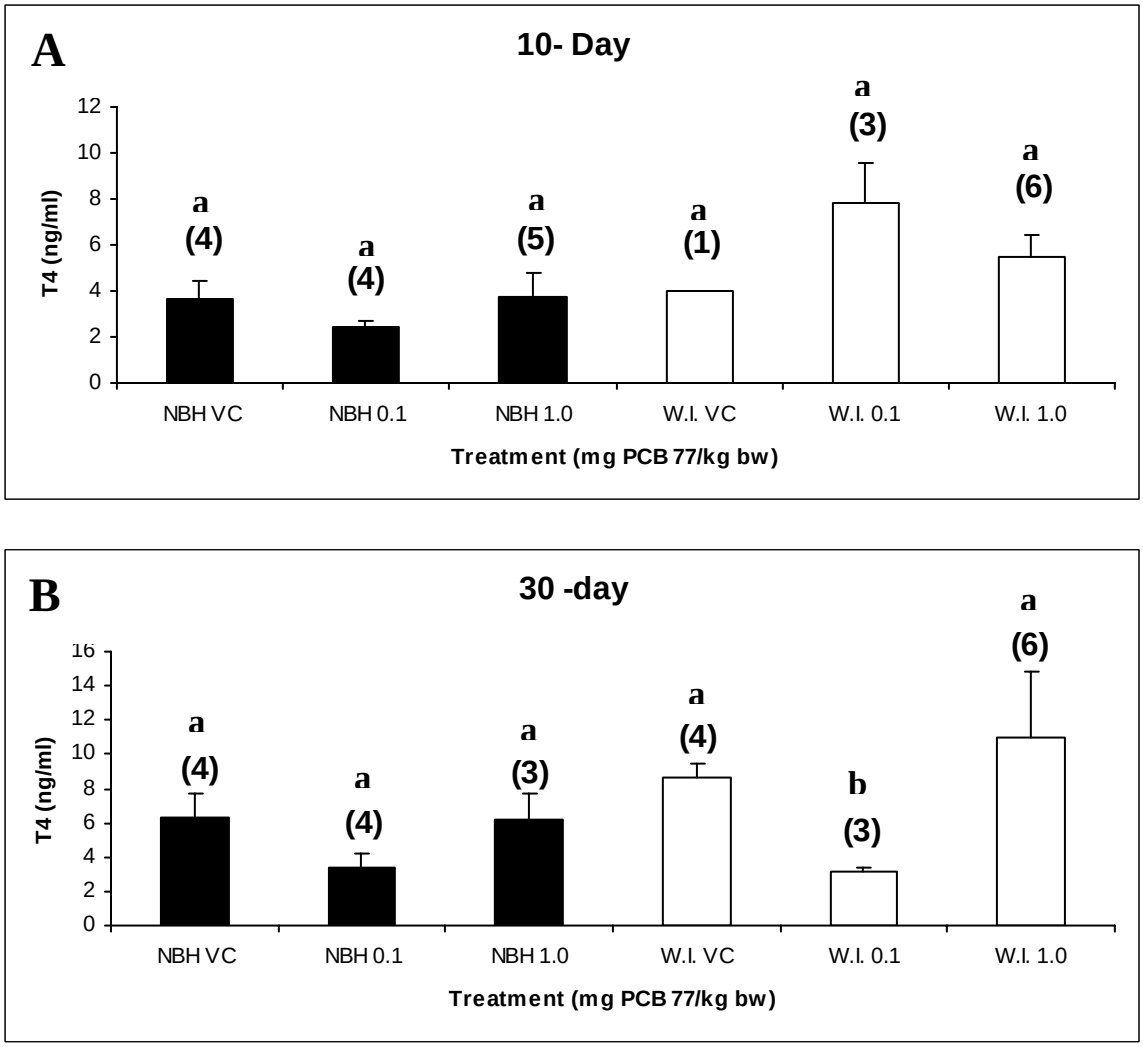


Figure 6.5. Plasma thyroxine (T4) levels in *F. heteroclitus* treated with either a vehicle control (VC) or PCB 77 at 0.1 or 1.0 mg/kg and sacrificed on day 10 (A) or day 30 (B). Bars represent means ± SE for (n) individuals. Similar letters indicate treatment means do not differ from vehicle control within populations (NBH or WI) at $p < 0.05$.

Chapter 7: Linking chemical tolerance to reproductive fitness in fish from contaminated environments.

* Vitellogenin analysis conducted by Kevin J Kroll, Center for Environmental & Human Toxicology, University of Florida, Gainesville, FL

7.1 Abstract

The disruptive effects of polychlorinated biphenyls (PCBs) on reproduction in a number of vertebrates including fish, are well established and include altered hormone levels, reduced fecundity, decreased hatching success, and lower offspring survival. PCB resistant fish display lowered levels of the pollutant metabolizing enzyme cytochrome P4501A (CYP1A) in response to PCB exposure relative to non-resistant fish. The present study examines the consequences of natural resistance on the antiestrogenic effects of PCBs *in vitro*. Isolated primary hepatocytes of PCB responsive fish (rainbow trout, *Oncorhynchus mykiss*) and a PCB resistant fish (green sunfish, *Lepomis cyanellus*) were treated with estradiol in the presence and absence of two concentrations of the potent CYP1A agonist PCB 126. We hypothesized that the effects of PCB exposure would be evident in rainbow trout but not green sunfish hepatocytes; CYP1A activity would be elevated in rainbow trout but not green sunfish hepatocytes and vitellogenin levels would be suppressed in rainbow trout but not green sunfish hepatocytes. Activity of CYP1A and production of the egg yolk protein vitellogenin (VTG) were quantified. As anticipated CYP1A activity was significantly elevated over controls in PCB treated rainbow trout but not green sunfish cells. PCB exposure suppressed VTG production in vehicle but not estradiol treated rainbow trout hepatocytes. Green sunfish cells exhibited no response to estradiol treatment, either in the presence or absence of PCBs. The results of this study demonstrate PCB mediated suppression of VTG synthesis in a PCB sensitive fish and provide data useful to future research utilizing *Lepomis* species as *in vitro* models examining the impact of differential CYP1A regulation.

7.2 Introduction

PCBs are globally ubiquitous contaminants known to bioaccumulate and exert a variety of harmful effects on organisms [57]. The detrimental effects of PCBs on reproduction are well documented in a number of vertebrates [313-315] including fish [75, 77, 78, 225, 316-318]. Effects of PCBs on fish reproduction include altered hormone levels [319], reduced fecundity [320], decreased hatching success [319], decreased gonad size [203, 321], and lower offspring survival [264, 318, 319]. PCB exposure has frequently been associated with anti-estrogenic effects [322], effects in fish have been observed in laboratory studies both *in vivo* [323] and *in vitro* [324]. Field studies have corroborated these results, providing evidence of reduced levels of the egg yolk precursor protein, vitellogenin [55] and the female sex steroid hormone, estradiol [325] in recrudescing fish. Several recent studies have provided compelling evidence linking reproductive dysfunction in wild fish with environmental pollution [285], particularly with halogenated aromatic hydrocarbons [55, 325, 326]. The well established effects of halogenated compounds on fish reproduction coupled with the widespread nature of this contamination illustrate the need for a better understanding of the nature and scope of these effects.

The most widely used biomarker for reproductive function in fish is the production of vitellogenin. Vitellogenin is synthesized by hepatocytes in response to rising levels of plasma estradiol (E2) and incorporated by maturing oocytes [327]. Vitellogenin synthesis is an extremely sensitive indicator of hormone levels as plasma vitellogenin concentrations change by up to a million fold in response to estrogens [328]. Most importantly vitellogenin is considered a specific and ecologically relevant indicator of fish reproductive response to chemical toxicants [329], including PCBs [323].

The mechanisms of PCB mediated reproductive disruption are largely unknown although various hypotheses link some types of pollutant induced disruption to the induction of CYP1A. Blocking CYP1A activity in fish blocks toxicant effects on reproductive hormones [330], indicating a link between CYP1A and reproductive function. Possible mechanisms include increased metabolism of E2 by induced levels of CYP1A [331], interaction of AhR or AhR dependent modulatory factors with estrogen dependent genes [332], and competitive binding of the estrogen receptor by CYP1A

ligand metabolites [331, 333]. However, none of these mechanisms have been demonstrated in fish, leaving the link between PCB exposure and reproductive effects unclear.

Different groups of organisms are known to display differing sensitivity to pollutant exposure. Certain populations of organisms appear to be pollution tolerant as a result of genetic selection after many generations of exposure [19, 334, 335] while other species appear to be naturally tolerant, or resistant, to certain pollutants [168, 188, 336]. Both varieties of resistance are characterized by lack of CYP1A induction following pollutant exposure [336] and appear to enable organisms to survive and reproduce in the presence of toxic chemicals [336]. Reduced CYP1A expression is likely responsible for chemical resistance in vertebrates [107] and CYP1A-suppressed fish experience reduced mortality rates, reduced rates of developmental deformities, and fewer teratogenic lesions when exposed to environmental pollutants [104, 337]. However, the consequences of resistance on a number of other targets of toxicity are largely unknown.

The present study will examine the effects of PCBs on reproduction in fish species that both lack resistance to and have 'natural resistance' to PCB mediated CYP1A induction. I hypothesize that green sunfish, an organism resistant to the CYP1A inducing effects of PCBs, will also demonstrate resistance to the antiestrogenic effects of PCBs.

7.3 Methods

Materials

7-Ethoxyresorufin (7-ER) and resorufin were obtained from Molecular Probes (Eugene, OR, USA). PCB 126 was purchased from AccuStandard (New Haven, CT). Purina Gamefish Chow was purchased from Southern States Feed. All other reagents were obtained from Sigma, Fisher Scientific (Fair Lawn, NJ), or Invitrogen. 48-well cell culture plates were purchased from Fisher Scientific.

Animals

Juvenile (12-13 month old) rainbow trout (*Oncorhynchus mykiss)* were obtained from Wolf Creek National Fish Hatchery in Jamestown, KY and held in the laboratory for seven weeks prior to caging. Fish were held in 948 l (250-gallon) flow-through tanks

at approximately 14^0C, 14/10 light/dark, and fed Purina Gamefish Chow ad libitum approximately every other day.

Green sunfish were collected via electro-shocking from the South Elkhorn (Woodford County) or Silver Creek (Madison County), relatively clean streams draining primarily non-intensive agricultural land in Central Kentucky. Fish were returned to the laboratory and held for at least 30 days prior to the experiment. Fish were housed in 948 l (250-gallon) flow-through tanks at approximately 20^0C tanks and fed Purina Gamefish Chow ad libitum approximately every other day.

Tissue Culture

Primary hepatocytes were isolated in the manner described by Willet et al. [338]. Fish were anesthetized in buffered water containing 150 ppm 3-aminobenzoic acid ethyl ether (MS-222). Anesthesia was maintained throughout the procedure by a recirculating system that passed MS-222 (150 ppm) continuously over the gills. The fish were placed ventral side up on a surgical table and an incision made from anus to transverse septum. Two lateral incisions were made along the caudal edge of the transverse septum to expose the liver. An angiocath was used to perfuse the liver by entering through a hepatic vein. Cells were isolated by a two-step collagenase perfusion method. Initially, buffer A (0.5 mM EDTA, 110 mM NaCl, 4 mM KCl, 25 mM NaHCO , pH 7.4) was pumped through the liver to flush out blood and prevent coagulation. After an adequate perfusion was obtained the liver was perfused with 150 ml buffer B (50 mg collagenase 2.5 mM CaCl , 110 mM NaCl, 4 mM KCl, 25 mM NaHCO , pH 7.4). When the liver was thoroughly blanched and softened it was excised and placed in a sterile glass petri dish containing buffer B. The liver was minced with sterile razor blades and passed through a sterile sieve to remove connective tissue. Cells were pelleted and rinsed twice with Leibovitz L-15 (Gibco BRL, Grand Island, NY) supplemented with 20 mM HEPES and 10 grams per milliliter of each gentamicin, streptomycin and tetracyline. Cells were resuspended, counted and evaluated for viability by trypan blue exclusion. Cells were diluted so that there were 7.5×10^6 cells in 10 ml L-15 and plated in 48 –well culture dishes (Costar, Corning Incorporated, Corning, NY), 0.5 ml per well. Cells were incubated in the dark in temperature controlled chambers at 14^0C (rainbow trout) and 20^0C (green sunfish).

Cell Dosing

Cells were treated with either vehicle control (DMSO), or PCB 126 at 10^{-10} or 10^{-11} molar 24 hours following isolation. PCB doses were selected based on previous work in our laboratory indicating they resulted in maximal CYP1A induction in cultured rainbow trout hepatocytes. CYP1A activity and VTG production measured 48 hours following treatment. The final DMSO concentration in the wells was 0.5%. Cells were also simultaneously treated with either vehicle control (ethanol) or 17-β estradiol at 10^{-6}molar. The final ethanol concentration in all wells was 0.3%.

Measurement of CYP1A activity

Media was aspirated from cells using a 9 inch Pasteur pipet attached to a vacuum pump. Cells were rinsed with 100 µL PBS, aspirated, and replaced with 185 µL PBS. 50 µL 7-ER, the substrate for CYP1A, was then added to all wells resulting in a final 7-ER concentration of 1.6 µM and the plate was immediately placed in a CytoFlour plate reader and fluorescence measured at 530 nm/590 nm for resorufin production for 15 minutes. Immediately following cessation of the CYP1A activity measurements 100 µL fluorescamine was added to each well. The plate was incubated in the dark for fifteen minutes before readings were taken at 400 nm/460 nm for fluorescence protein.

Vitellogenin measurement

Vtg concentrations in cell supernatants of rainbow trout and green sunfish were determined by direct ELISA for the using monoclonal antibodies developed by the Denslow and UF Hybridoma labs (UF) labs. Rainbow trout and green sunfish Vtg was measured using the Mabs: 1C8 (HL 1364), and a mixture of 3G2 (HL 1393) and 1C8, respectively

The direct ELISA assay was run as follows. Cell supernatant samples were diluted 1:2 and 1:10 in PBSZ (10 mM phosphate, 150 mM NaCl, 0.02% azide, pH 7.2) containing the protease inhibitor- Aprotinin (10 KIU/ml) and loaded onto a 96-well ELISA plate (Nunc ^{TM}, Nalge). A standard curve using ion exchange purified Vtg (0, 0.001, 0.004, 0.01, 0.02, 0.04, 0.06, 0.08, 0.1, 0.2, 0.4, 0.6, 0.8, and 1.0 µg/ml) , negative controls (normal male), and positive controls (developing female) to be used for intra and

inter-assay determination were also added. Each sample and standard was loaded in triplicate (50 µl/well) and incubated overnight (4 ºC) in a humidified tupperware container. The plate was washed four times with PBSTZ (PBSZ with 0.05% tween) using a plate washer (Skan washer, Molecular Devices). The plate was blocked with 1% BSA in TBSTZ (20 mM tris, 150 mM NaCl, 0.05% tween-20, 0.02% azide, pH 7.4) for 2 hours at room temperature. The plate was re-washed and the primary antibody (Mab- 1C8 or the pooled Mabs- 1C8 & 3G2 for the rainbow trout and green sunfish, respectively) was added to each well at 0.1 µg/ml in block buffer. By mixing the two Mabs, the sensitivity of the green sunfish ELISA was enhanced (Kroll, per observation). The plates were incubated overnight in a humidified chamber at 4ºC.

The following day, the plates were washed, and the secondary antibody -goat-anti-rabbit IgG- $F(ab)_2$- alkaline phosphatase (Pierce) diluted 1:1000 in blocking buffer was added and incubated for 2 hours at room temperature. The plate was washed and then 1.0 mg/ml of the substrate, para-nitro phenyl phosphate (Sigma), in carbonate buffer (30 mM carbonate, 2 mM $MgCl_2$, pH 9.6) was added to each well (100 µl/well). The plate was scanned at 405 nm using using a reader (SpectraMax 384, Molecular Devices) and the data interpreted by the SoftMax Pro program.

Cell viability

Lactate dehydrogenase (LDH) was measured from the cell culture medium and cell homogenate to estimate cell viability basically as described by Scholz and Segner [339]. The reaction buffer contained 50 mM Tris/HCl, pH 7.5, 1.1 m pyruvate, and 0.14 mM NADH. Activity was determined using 50 µL of the cell culture supernatant in a total reaction volume of 315 µL. The reaction was started with pyruvate and enzyme activities were recorded at 25^0C in a microplate spectrophotometer (Spectra Max 190). High LDH activities in media relative to cell homogenate indicate decreased membrane integrity indicating a decline in cell viability.

Statistical Treatment of Data

Statistical analyses were performed using SPSS. Data were analyzed using mixed models analysis to compare treatment response between species. All differences were considered significant at $p <$ or $= 0.05$.

7.4 Results

Lactose dehydrogenase assay

Cells are considered viable if the percentage LDH activity in the media is less than 20% of that found in the cells. For green sunfish cells the range of the percentage activity in the media relative to the cells was 0.01 to 3.0 % with a mean of 1.5%. Rainbow trout cells appeared only slightly less viable as the percentage activity in media versus cells ranged from 0.1 to 6.6 with a mean of 2.5%.

CYP1A activity

CYP1A activity in rainbow trout cells was significantly elevated over controls following treatment with PCB 126 at 10^{-11} M and 10^{-10} M (Figure 7.1). Co-treatment with estradiol (1 μM) and PCB 126 at both concentrations likewise resulted in induction relative to controls (Figure 7.1). Significant suppression of CYP1A activity was observed in estradiol/PCB 10^{-11} M treated trout cells (Figure 7.1). As anticipated CYP1A activity in green sunfish treated hepatocytes was relatively similar among treatments with no significant differences noted (Figure 7.2).

Vitellogenin levels

Vitellogenin levels in rainbow trout hepatocytes were strongly elevated in response to estradiol treatment as expected but unexpectedly also elevated in response to vehicle control treatment (Figure 7.3). Treatment with PCB 126 at both doses suppressed VTG concentrations below vehicle control levels (Figure 7.3). Green sunfish cell VTG levels were homogenous among treatments with no significant differences noted (Figure 7.4).

7.5 Discussion

The objectives of this study were to test the hypothesis that green sunfish, an organism resistant to the CYP1A inducing effects of PCBs, would also demonstrate resistance to the antiestrogenic effects of PCBs. Although the unexpected lack of VTG response in green sunfish prevents evaluation of this hypothesis several significant findings were obtained. The observance of *in vitro* VTG suppression by a PCB congener in rainbow trout is a significant observation as the two existing studies in carp and rainbow trout respectively [324, 341], examining this phenomenon provide conflicting data. This phenomenon may at least partially contribute to the many reported impacts of PCBs on fish reproduction *in vivo* [75-78]. This study is also the first reported *in vitro* study utilizing primary hepatocytes of any member of the family Centrarchidae, perhaps the most abundant family of freshwater fish in North America. Although the techniques employed produced viable cultures, the results clearly indicate further optimization is necessary. While the original hypothesis could not be addressed, this study does provide novel information valuable to research in this field.

In the rainbow trout hepatocytes of the present study significant suppression was observed in the PCB alone treatment relative to vehicle control cells, indicating significant suppression of VTG by PCB 126. The antiestrogenicity of CYP1A inducing compounds such as 2,3,7,8 – tetrachlorodibenzo-*p*-dioxin (TCDD) is well established in mammalian systems [342] and is a recently reported *in vitro* phenomenon in teleosts. Navas and Segner [330] report suppression of estradiol induced VTG production in rainbow trout hepatocytes co-exposed to the PAHs 3-methylcholanthrene and β-naphthoflavone. Likewise, Anderson et al. [340] reported significantly reduced vitellogenisis in rainbow trout hepatocytes exposed to the potent CYP1A inducing compound TCDD. Villalobos et al. [343] reported reduced VTG production in rainbow trout hepatocytes exposed to estradiol and CYP1A inducing metabolites of trichoroethane. The suppression of VTG levels by PCB 126 in the present study is consistent with previous reports of VTG suppression by CYP1A inducing compounds.

The lack of significant suppression of estradiol induced VTG observed in all PCB/estradiol co-treated rainbow trout hepatocytes in the present study is likely a consequence of dose. Smeets et al. [324] observed significant suppression of estradiol

induced VTG production in cultured carp hepatocytes co-exposed to several PCB congeners, including PCB 126 (10^{-8}M). However, Anderson et al. [340, 341] failed to see significant suppression of estradiol induced VTG production following treatment of rainbow trout hepatocytes with PCB 126 (10^{-8} M). PCB doses were similar among these two studies suggesting the explanation to the observed differences may relate to the estradiol concentrations utilized, 0.2 micromolar in the Smeets et al. [324] study and 1 micromolar in the Anderson study [340, 341]. This theory is supported by the fact that the antiestrogenic action of CYP1A inducing compounds has been shown to vary, depending on both the dose of estradiol and the CYP1A inducer. Lower concentrations of CYP1A inducing compounds fail to suppress VTG production while higher concentrations demonstrate suppression [340, 341]. The suppression of carrier induced VTG by PCB 126 in this study strongly indicates PCB 126 is antiestrogenic and likely would have suppressed estradiol induced VTG production if cells had been exposed to lower concentrations of estradiol or higher concentrations of PCB 126.

Suppression of PCB induced CYP1A activity by estradiol (10^{-11}M) was an anticipated result consistent with a number of previous studies. Spawning female fish are known to display suppressed levels of CYP1A relative to non-spawning females, juveniles, and male fish [344] and this phenomenon can be mimicked, although to a lesser degree, by the artificial administration of estradiol [344]. Several studies report suppression of CYP1A levels in teleost *in vitro* hepatic preparations following treatment with estradiol [340, 345]. The mechanism of this suppression is unclear but appears to be pretranslational [208] and involve the estrogen receptor [346].

The elevated levels of VTG in vehicle control treated rainbow trout hepatocytes was unanticipated and difficult to explain. To my knowledge no other studies examining VTG production in teleost primary hepatocytes have incorporated an untreated control, only vehicle controls. The carrier utilized in this study was similar to those used in previous reported studies, ethanol at a final concentration of 0.3% in all wells. Although ethanol indirectly leads to upregulation of the estrogen receptor in a number of organisms [347], agonistic binding of the estrogen receptor by ethanol has not been reported. A number of manufactured compounds such as components of plastic [348, 349] are known

to bind the estrogen receptor. It is possible that contamination of our vehicle control led to the observed increases in VTG production in the vehicle control.

In sharp contrast to green sunfish hepatocytes in the present study, exposure of isolated hepatocytes from a number of other teleost species to estradiol concentrations similar to those utilized in this study has been reported to result in significant increases in VTG production. Consistent with our observations, several studies with rainbow trout hepatocytes report VTG induction following exposure to estradiol concentrations equal to or lower than (0.22 μm) those utilized in this study (1 μM) [330, 340, 350]. Likewise, treatment with 1 micromolar concentrations of estradiol induced VTG synthesis in Japanese eel (*Anguilla japonica)* [351]. Significant induction of VTG in carp (*Cyprinus carpio*) hepatocytes was reported following treatment with estradiol concentrations of 0.1 nanomolar by Letcher et al. [352] and by 2 nanomolar by Smeets et al. [349]. Kim and Takemura [353] reported significant elevation of VTG levels in tilapia (*Oreochromis mossambicus*) hepatocytes exposed to estradiol concentrations as low as 0.1 micromolar.

Considering numerous studies measuring VTG in a wide variety of species, the complete lack of VTG response to estradiol in green sunfish hepatocytes in this study is puzzling. Although decreased cell viability would contribute to lack of vitellogenic response, it seems unlikely it played a role in this experiment. The lactose dehydrogenase assay indicated all cells tested were highly viable, vastly exceeding the miminum criteria for viable cells. In addition, although CYP1A activity response was minimal as anticipated, in at least one trial estradiol appeared to actively and significantly suppress CYP1A activity (Figure 7.1B) strongly suggesting cells were viable and capable of altering enzyme expression. The explanation for the lack of VTG response in green sunfish hepatocytes appears to be unrelated to decreased cell viability.

Another potential explanation for the lack of response observed in the green sunfish hepatocytes is the possibility that green sunfish hepatocytes were incubated at a temperature below the optimum temperature for vitellogenin production. Rainbow trout exhibit optimum growth at temperatures close to 16^0C [354], although certain strains may exhibit higher optimum temperatures [355]. Accordingly Pawlowski et al. [356] reported VTG production in cultured rainbow trout hepatocytes exposed to estradiol increased between 14 and 18^0C. Kim and Takemura [353] reported VTG production in estradiol

treated tilapia hepatocytes peaked at 28^0C, a temperature very close to the optimum growth temperature of tilapia (29-31^0C) [357]. Although no optimal growth temperature is available for green sunfish, they have been reported to exhibit an optimal temperature preference of 28.2^0C [358]. In addition closely related species of sunfish, longear sunfish (*Lepomis megalotis*) and pumpkinseed sunfish (*Lepomis gibbosus*) have been reported to spawn at 25^0C [359]. The reports previously discussed suggest that species specific temperature optimum for VTG production may exist, if true, the low incubation temperature (20^0C) of the hepatocytes in this study might help explain the lack of VTG production in green sunfish.

Additional environmental factors are also known to differentially affect cultured primary hepatocytes in various fish species. Kim and Takemura [353] reported VTG production in estradiol treated hepatocytes that varied with cell culture media and $NaHCO_3$ concentrations and theorize that cell culture systems utilizing primary hepatocytes need to be optimized for each species. To my knowledge no other reports on cell culture in green sunfish or any species in the family Centrarchidae currently exist. It is possible that some pH or nutritional requirement was not met in the current study, leading to the lack of VTG production in green sunfish hepatocytes. Future studies examining optimal conditions for VTG production in *Lepomis* species would be beneficial.

The results of this study, while insufficient to address the original hypothesis, may serve as a guide for future research. The observation of suppression of carrier induced VTG production strongly suggests PCB 126 is antiestrogenic. The *in vitro* studies utilizing a *Lepomis* species will serve as a pilot study for further research utilizing *Lepomis* hepatocytes and illustrate the need to take a number of variables into consideration. Future research on these topics should be enhanced as result of the finding of this study.

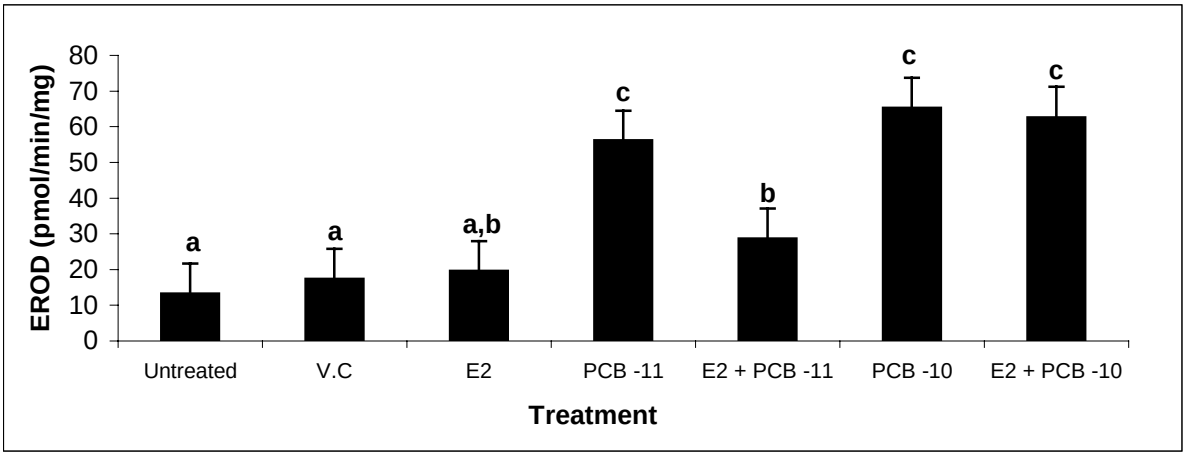


Figure 7.1. EROD activity (pmol resorufin/min/mg) in rainbow trout hepatocytes isolated from sexually immature trout. Three trials are represented in the graph in which hepatocytes were isolated from three separate fish. Hepatocytes were either untreated or treated with a vehicle control, 17-β-estradiol at 1 μM, PCB 126 at 10^{-11} M, PCB 126 at 10^{-10} M, or cotreated with PCB 126 and estradiol. Bars represent means ± SE for (18) wells. Means with the same letter are not significantly different at $p < 0.05$.

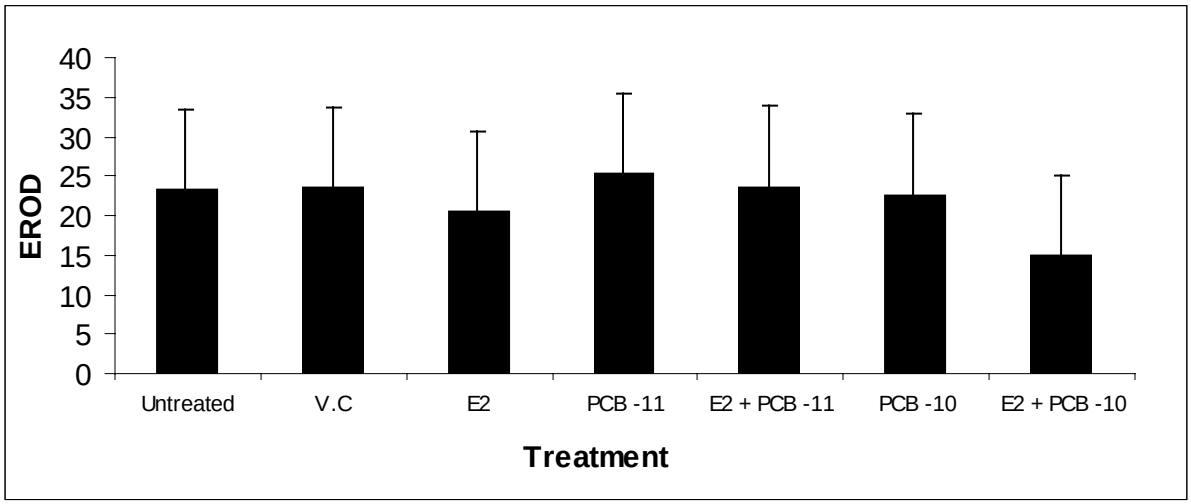


Figure 7.2. EROD activity (pmol resorufin/min/mg)in green sunfish hepatocytes isolated from male green sunfish. Two trials are represented in the graph in which hepatocytes were isolated from two separate fish. Hepatocytes were either untreated or treated with a vehicle control, 17-β-estradiol at 1 μM, PCB 126 at 10^{-11} M, PCB 126 at 10^{-10} M, or cotreated with PCB 126 and estradiol. Bars represent means ± SE for (12) wells. All means are similar at $p \leq 0.05$.

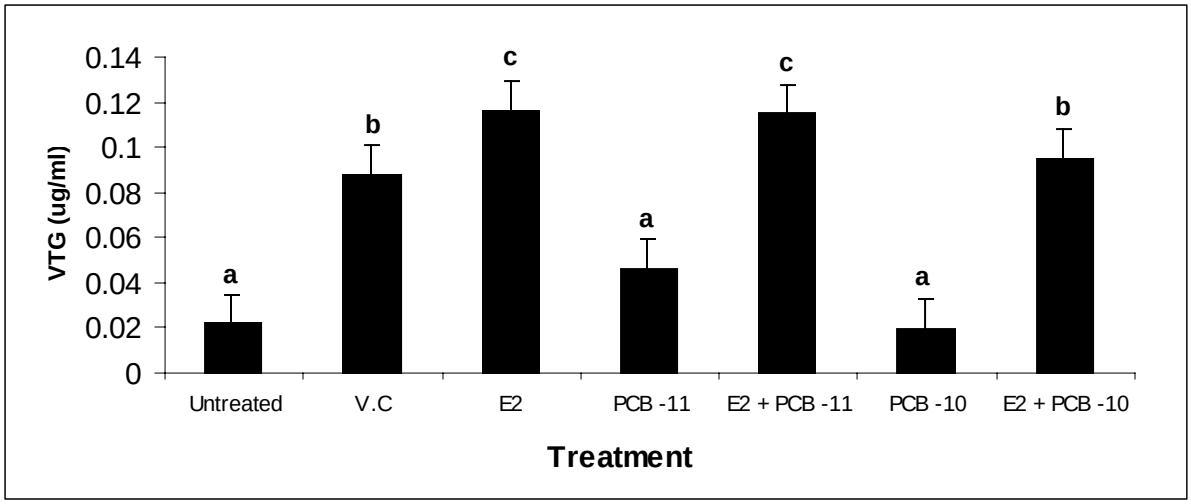


Figure 7.3. Vitellogenin levels in rainbow trout hepatocytes isolated from sexually immature trout. Three trials are represented in the graph in which hepatocytes were isolated from three separate fish. Hepatocytes were either untreated or treated with a vehicle control, 17-β-estradiol at 1 μM, PCB 126 at 10^{-11} M, PCB 126 at 10^{-10} M, or cotreated with PCB and estradiol. Bars represent means ± SE for (12) wells. Means with the same letter are not significantly different at $p \leq 0.05$.

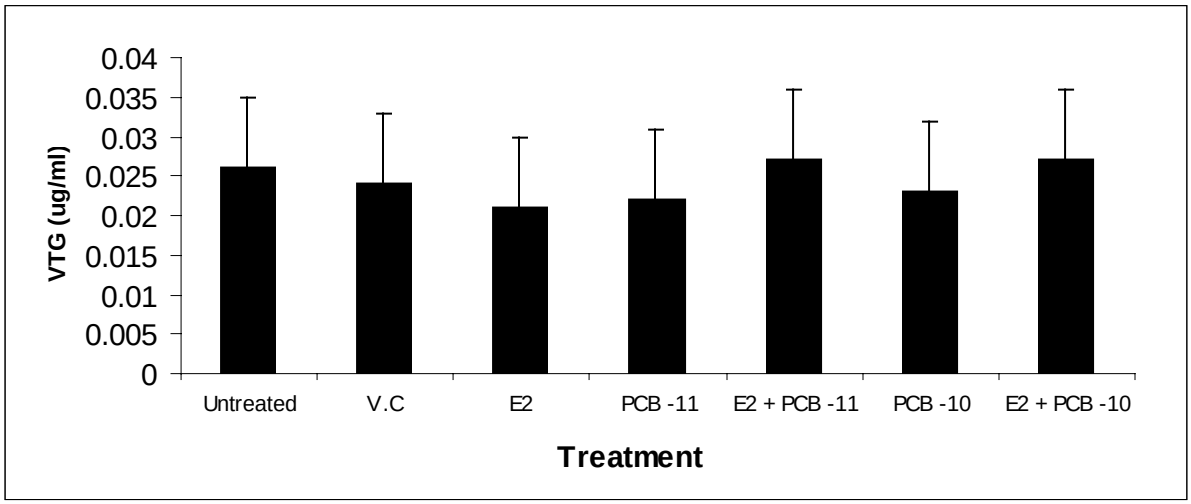


Figure 7.4. Vitellogenin levels released into the media by green sunfish hepatocytes isolated from two male green sunfish. Hepatocytes were either untreated or treated with a vehicle control, 17-β-estradiol at 1 μM, PCB 126 at 10^{-11} M, PCB 126 at 10^{-10} M, or cotreated with PCB 126 and estradiol. Bars represent means ± SE for (12) wells. All means are similar at $p < 0.05$.

Chapter 8: Conclusions

The studies of this work enhance the current understanding of the effects of chemical exposure on teleosts, particularly in organisms inhabiting chronically contaminated environments. The principle findings are the discovery of chemical resistance in a teleost species (and Family) in which this phenomenon was previously undocumented, the characterization of differing pollutant response to halogenated and non-halogenated inducers in several *Lepomis* species, and the characterization of the toxicity of an emerging contaminant, PBDEs, in different teleost life stages. These data provide novel and useful information to researchers and resources managers, significantly broadening the current knowledge base concerning teleost pollutant response.

The caging study (Chapter 2) of this dissertation not only demonstrates the utility of CYP1A expression in caged rainbow trout as a biomonitoring tool but also confirms the presence of significant and bioavailable levels of PCBs in the Town Branch/Mud River (TB/MR) System, providing data necessary for subsequent studies on this system. A number of caging studies have been conducted examining CYP1A protein [120], activity [360], or mRNA levels [126], however, the present study is one of the few to incorporate all three endpoints, allowing comparison of these different levels of CYP1A expression. The results of the caging study clearly demonstrate the utility of all three of these parameters as sensitive biomarkers of PCBs.

One of the principle findings of this work is the characterization of an Ictalurid species (yellow bullhead, *Amerius natalis*) that displays chemical resistance to the CYP1A inducing effects of PCBs. Previous work in this field demonstrated chemical resistance in several teleost species residing in highly contaminated environments including killifish from New Bay, NJ [106], yellow perch from a PCB contaminated lake in Sweden [110], and Atlantic Tomcod from the Hudson River, NY [107] . Laboratory studies supported my hypothesis that yellow bullhead in the TB/MR system have reduced sensitivity to CYP1A induction following PCB exposure relative to reference fish. This population may now provide a useful model system by which to examine the biological consequences of resistance.

The effects of this resistance may not be wholly beneficial as indicated by previous research on resistant populations. Acquired resistance presumably occurs as a result of selection for the more resistant individuals in a population, resulting in altered allele frequencies and possibly an overall reduction in genetic variation [111, 335]. Resistant populations are reported to display characteristics such as reduced fertilization success [361], decreased tolerance for low dissolved oxygen [335], compromised immune function and reduced growth rate [362]. The above "costs" may occur as a result of energy expenditure from a limited budget [362]. The organism must devote more energy to resistance (possibly including metabolism, elimination, or sequestration) so less is available for normal physiological tasks [362]. Although resistance may at first appear to be wholly beneficial for the organism, hidden costs are often associated with resistance.

The presence of chemically resistant species, whether natural or acquired, has the potential to impact the surrounding ecosystem. Resistant organisms survive in the presence of contaminants instead of succumbing in a manner similar to responsive organisms. As a results these organisms may bioaccumulate high levels of lipophilic contaminants such as PCBs. Monosson et al. [158] reported high levels of contaminants in PCB resistant killifish from Newark Bay. These contaminants may then be passed on to non-resistant predators that may experience toxicity [239] and/or pass the contaminants through trophic transfer to higher levels of the food web [335].

The discovery of a differential response to chlorinated and non-chlorinated inducers in *Lepomis* species is a significant finding with multiple implications. *Lepomis* species are perhaps the most common genera of North American fish with representatives found in a wide range of habitats across the continent [190]. Furthermore these fish typically have highly restricted home ranges and are often sexually dimorphic [190], characteristics highly desirable in species used for biomonitoring purposes. Currently several studies report the use of *Lepomis* species as biomonitoring tools [197, 363]. The results of this study provide valuable data to the resource manager. The results of the present study indicate at least three members of this genus (bluegill, longear sunfish, and green sunfish) are poor sentinel species for PCB exposure, exhibiting a reduced or

complete lack of phase I and II hepatic enzyme response apparently resulting from a natural low responsivity of CYP1A.

The altered developmental parameters in responsive, but not resistant, killifish embryos following PBDE exposure strongly indicate PBDE interference with developmental processes and suggests PCB resistant fish may be resistant to the toxic effects of PBDEs. Previous studies have found PBDEs may be relatively innocuous in fish [226, 244]. Although the juvenile catfish study supports this hypothesis, the embryo portion of this study suggests certain life stages may be impacted by these contaminants. The embryo results of this study are also in agreement with other recent research reporting altered rates of development in PBDE exposed killifish embryos [267]. The results of these studies indicate further research on the effects of PBDEs on developmental processes is necessary.

Our observation of vitellogenin (VTG) suppression following PCB exposure is a significant finding and one of the first observations of this phenomenon in a teleost *in vitro* model. The adverse reproductive effects of PCBs are well documented and known to include: [319]reduced fecundity [320], decreased hatching success [319, 364], decreased gonad size [203, 321], and lower offspring survival [264, 318, 319]. Although the mechanism underlying these effects currently remains unknown, a reduction in VTG production through the anti-estrogenic effects of PCBs is likely at least a contributing factor.

Future research at the primary field site of this work has the potential to yield valuable results. In the highly contaminated Town Branch system we have documented species displaying responsiveness (creek chubs, field study only), natural resistance (green sunfish), and acquired resistance (yellow bullheads). Non-quantitative field observations indicate high densities of both varieties of resistant fish in the contaminated area of this system where few responsive fish are found. Conversely, a short distance away in the reference section, large numbers of responsive fish are present along with moderate numbers of the aforementioned resistant species. Certain species, such as the green sunfish, have long been considered highly tolerant of degraded habitats [150, 170, 171], a characteristic utilized in a number of bioassesment protocols such as the fish utilizing Indice of Biotic Integrity [150]. Comparison of physiological endpoints in these

fish during critical life stages such as development and reproduction has the potential to yield valuable data elucidating the specific characteristics and possible mechanisms of pollutant tolerance.

Taking these and previous experiments into consideration it appears clear that a number of variables dictate pollutant response in a given teleost species. While contaminant exposure has long been known to affect pollutant response, this work demonstrates that certain species never exposed to a pollutant may show a relative lack of sensitivity to that contaminant. Biomarkers of contamination should be laboratory validated in new species before commencement of field studies. In addition, contaminant sensitivity to certain compounds appears to vary with life stage. Certain life stages may be seemingly immune to effects while others are sensitive. The data gained from this work will allow environmental managers and regulators to make more informed decisions concerning the impact of pollution on resident populations by improving their ability to make predictions concerning the effects of chronic contaminant exposure on population health.

Appendix 1: Protocols

MICROSOME, NUCLEAR & CYTOSOL PREPARATION

MAY 2001

Dissect out fish livers without gall bladder. If gb breaks, rinse livers IMMEDIATELY with ice cold homogenization buffer (50 mM Tris, pH 7.4).

Weigh liver quickly, record, and place in test tube containing appropriate amount of homogenization buffer (10 ml/g for usomes, use 4 ml/g if saving cytosol). When have at least 0.2 g wet liver in tube, homogenize.

Homogenize livers in ice cold Homo buffer using 5-8 passes of the homogenizer tube. Keep the tube in ICE+WATER during the homogenization.

Pour homogenate into high speed, 15 mL COREX centrifuge tubes and spin at 4°C:

4500 rpm (1,500 g) 10 min nuclear and cell membranes

(for DNA remove nuclear pellet, place in cryovial, and store in LN2).

11,000 rpm (10,000 g) 10 min mitochondria

Remove tube from centrifuge and pour supernatant into ultra centrifuge tube (kept on ice) and spin:

40,000 rpm (100,000 g) 1.5 hr microsomes = pellet
cytosol=supernatant

CYTOSOL
Transfer the supernatant into cryovials. Some people add glycerol to cytosol, others do not. If you do, add enough glycerol to make the cytosol 20% glycerol (e.g. 5 mL cytosol + 1.2 mL glycerol) and store at –192°C. in cryovials.

MICROSOMES
The microsomal pellet may be underlain by a clear glycogen pellet. *Try to take only the microsomes (amber colored), but if are integrated with glycogen, take whole thing.*

Place pellet into small homogenization tube (1 - 5 ml) and hand-homogenize ON ICE in resuspension buffer (50 mM Tris, 1 mM EDTA, 1 mM DTT and 20% glycerol) at a ratio of 1 ml buffer per gram of wet liver weight. Use a teflon pestle for 3 - 4 passes.

Pipet into cryotube (split between 1.0 cryotubes if have > 1.0 ml of microsomes), cap securely and immediately freeze in liquid nitrogen. (VWR low temp freezer vials @ 50/bag; 66008-251 1.2 mL; 66008-284 2.0 mL; 66008-400 5.0mL). Once all tubes are filled and frozen, transfer to LN2 Dewar for storage.
Mark location in Dewar Log book.

HOMOGENIZATION BUFFER (for making microsomes)-*store at 4°C*

50 mM Tris, pH 7.4 (1 liter)

6.61 g Tris HCL
0.97 g Tris Base

bring to 900 mL with dH20

adjust pH to 7.4
bring to 1 liter with dH20
store at 4°C

MICROSOME RESUSPENSION BUFFER – ***store at 4°C.***

50 mM Tris
1 mM EDTA (MW = 292.2 g)
1 mM DTT * (FW = 154.2 g)
20% (v/v) glycerol (add last)

For 500 mL:

Dissolve 0.146 g EDTA in 250 mL 50 mM Tris, pH 7.4

It is critical that the EDTA be dissolved first, because it takes a long time to dissolve and once other reagents are added, they slow down that dissolution.

Once EDTA is dissolved, add:
0.078 g DTT*
100 mL glycerol

Bring to 450 mL with 50 mM Tris
Adjust pH to 7,4

* should smell awful. If doesn't, has lost its potency and you need to buy new batch.

KINETIC EROD MICROPLATE ASSAY
(48-Well Costar plate)

protocol citation: Hahn, M. E., T. M. Lamb, M. E. Schultz, R. M. Smolowitz, and J. J. Stegeman.Cytochrome P4501A induction and inhibition by 3,3',4,4'-tetrachlorobiphenyl in an Ah receptor-containing fish hepatoma cell line (PLHC-1). Aquat. Toxicol. ***26:****185-208 (1993).*

Assay is performed at ~ RT so be sure that the 48 well plate containing samples is at RT before you add the NADPH!!

SET TEMP ON Plate Reader (~ 26°C optimal for fish).

Prepare 2.67 uM 7-ER buffer/substrate and 6.68 mM NADPH cofactor.
Dilute high activity samples 1:10 (or 1:20) with TEDG
Low activity samples can be assayed neat (10 uL undiluted microsomes)

150 µl of 7-ER buffer/substrate to wells of 48 well Costar plate (Fisher # 07-200-86).
10 µl of microsome sample (neat or diluted).
All Samples and a positive control (e.g. BNF-treated trout) are assayed in **triplicate.**
Initiate assay with 40 µl NADPH (final conc in assay 1.34 mM. NADPH)

Briefly shake plate and immediately measure fluorescence in the plate reader once per minute over 6-10 cycles (~ 6- 10 min) **or longer**; (530 nm excitation, 590 nm emission).

CYTOFLUOR PROGRAM

KINETIC (EROD)

Mix time	0
EX/EM	530/590
Reads per well	3
Cycles	6 - 10 (choose one number)
Time	00:00:00|
Scans per cycle	2
Gain	50, 70

REAGENTS

EROD BUFFER (pH 7.8)

2.66 g Trizma HCl
0.99 g Trizma Base
2.92 g NaCl
Bring to 500 mL with DI water and check that pH = 7.8
Store at 4°C

NADPH (6.68 mM)

55.7 mg NADPH (Sigma N1630)/10 mL EROD buffer
Store at -20°C, can be freeze/thawed

RESORUFIN (RR) STANDARD

Prepare a STOCK solution of RR in MeOH (store stock in amber bottle at -20°C):
- Dissolve crystals in 5 mL MeOH (heat in 65°C bath or under hot tap water)
- Measure absorbance of 1:100 dilutions in MeOH (10 uL RR + 990 uL MeOH) in triplicate @ 574 nM
- Extinction coefficient for RR @ 574 nm = 45.78 mM^{-1} cm^{-1}

Calculate concentration (c=Abs/E) *(e.g. Abs 574 = 0.297; c = 0.0400/0.04578 * 100 = 87.4 uM)*
Prepare 10 mL of 1 uM RR in EROD buffer (V1C1 = V2C2)
(e.g. (x)(87.4 uM) = (10 mL)(1 uM); x = 0.114 mL @87.4uM brought to 10 mL w/EROD buff)

Wrap in foil to protect from light- store on ice for day

7-ETHOXYRESORUFIN SUBSTRATE (7-ER)

Prepare a STOCK solution of 7ER in MeOH (store stock in amber bottle at -20°C):
- Dissolve crystals in 5 mL MeOH (heat in 65°C bath or under hot tap water)
(must be dk orange (saturated)!! If not, add more crystals)
- Measure absorbance of 1:100 dilutions in MeOH (10 uL 7ER + 990 uL MeOH) in triplicate @ 482 nM
- Extinction coefficient for 7ER @ 482 nm =22.5 mM^{-1} cm^{-1}

Calculate concentration (c=Abs/E) *(e.g. Abs 482 = 0.0320; c = 0.0320/0.0225*100 = 142.2 uM)*
Prepare 8 mL of 2.67 uM 7ER in EROD buffer (V1C1=V2C2), 8 mL is enough for one plate.
(e.g. (x)(142.2 uM) = (8 mL)(2.67 uM); x = 0.15 mL@142.2 uM brought to 8 mL w/EROD buff)

Wrap in foil to protect from light - store on ice; 7ER in buffer is stable only for1 day

TEDG (microsome resuspension buffer) **(500 mL**)

50 mM Tris (pH 7.4)	400 mL
1 mM EDTA (MW = 372.24 g)	0.186 g

-dissolve the EDTA in the tris FIRST before adding DTT or glycerol - will take awhile to dissolve

1 mM DTT * (FW = 154.2 g)	0.077 g
20% (v/v) glycerol	100 mL

Store at 4°C

IMPORTANT NOTES

- If nature of samples is known, put high activity samples (and positive control) last in the plate so less time elapses between initiation of reaction and scanning of plate.

- More than 10 µl of neat microsomes can be added for very low activity samples, but 7-ER buffer/substrate must be prepared at a higher concentration to yield a 2 µM final assay concentration.

- A induced fish liver sample of **known** activity is included in every plate as positive control.

- The slope of the standard curve (fluorescence units/pmol) at the appropriate sensitivity is used to calculate the activity of kinetic assays.

- Standard curves can be determined in the absence of BSA, NADPH, 7-ER cells or microsomes because these factors are relatively unchanging at the fluorescence maximum for RR and so can be ignored for std curve purposes. It is,however, essential to run the RR std curve in the same final volume as the samples (200uL), diluted in the EROD assay buffer. **pH is critical**, so use the same batch of EROD buffer for the samples as for the RR standards.

- To account for instrument variability, the RR standard curve can either be run when the samples are run, or can be run on a different day as long as you include a known 'hot' (high EROD activity) sample in each plate for standardization between days and between plates.

-7ER/MeOH STOCK solution must be SATURATED (very dark orange color). You do not want too much MeOH in the final 7ER buffer as it will kill the microsomes.

Slot Blot Protocol

June 2002

1. Thaw antibodies on ice (~ 2hrs).
2. Rinse slot plate with ethanol and then distilled water. Dry off.
3. Prepare sample dilutions in 1X TBS.
4. Set up blotting apparatus according to manual instructions – the slot plate has been known to crack when being tightened under vacuum, try to be gentle, but make sure a good vacuum is formed.
5. Wash slots with 100µl of 1X TBS prior to sample loading.
6. Add samples and standards to slots (no less than 200µl, 250µl is good)
   - Make sure all the membrane surface shown through the blot is covered and there are no bubbles.
   - Add sample in middle of slot to ensure even distribution.
7. Apply gentle vacuum (see manual instructions on position of manifold)
   - If sample goes through membrane too fast, it may not adhere well and come off during incubation/washes.
   - .If slots empty unevenly, keep adding 1X TBS to empty slots until all samples have gone through to avoid letting parts of the membrane go dry.
8. Wash slots with 200µl of 1X TBS; again, apply gentle vacuum.
9. Remove membrane and place it in milk block for 1h on shaker at RT
10. Wash membrane 4 times with dH_2O (rinse 5x each time) and 3 times with TBS-Tween (5min in shaker), alternating, starting with water.
11. **Using a new container**, incubate the membrane with the first antibody (100ml) for 1h on shaker at room temperature.
12. Wash membrane as in 10.
13. Using a new container, incubate the membrane with the second antibody for 1h on shaker at RT.
14. Wash membrane as in 10, plus additional water rinses to ensure all soap is gone.
15. Keep membrane in **a new container** with 1X TBS, at 4°C **protected from light** until scanned.

Solutions and materials

5X TBS

100mM Tris, 2.5M NaCl, pH 7.5
12.7g Trizma HCl
2.36g Trizma Base
(Based on recipe for a desired pH of 7.5)
146g NaCl

Check pH and adjust if necessary
Bring to 1 liter with dH2O
Store at 4°C

5% Milk Block
5g powdered milk dissolved in 100ml of 1X TBS

1 x TBS-0.1%Tween

1,000ml 1X TBS + 1ml Tween 20
Stir on stir-plate
Store at 4°C

Membrane

Nitrocellulose (0.45µ), 9x12cm

Notes

1. Thaw Abs on ice to prolong their life. They are proteins , and eventually break down at RT
2. Rinse the apparatus with ethanol or methanol, then DI water to make sure all the alcoholl is gone ~ then dry it. This will ensure any residual protein is removed.
3. At the last water wash after each incubation always rinse the membrane more than 5x just to be sure all detergent (Tween is a soap) is gone.

January 2004

Cytofluor Protein Assay

(Lorenzen, A. and Kennedy, S.W. (1993) *Anal. Biochem.* **214**: 346-348.)

Reagents:

1. 0.05 M sodium phosphate buffer, pH 8.0

2. 0.9% NaCl

3. BSA standards, 2.0 mg/ml in 0.9% NaCl. Use good quality BSA and check A278. $\square^{1\%} = 6.67$ (Hayes, Principles and Methods of Toxicology, p. 610). *Make up a 1 mg/ml std, which should have a 0.667 OD at 278 nm. The usual contaminants are fatty acids which affect weight, hence the need for the Absorbance calibration.*

4. 1.08 mM fluorescamine (Sigma F-9015; FW=278.3)
   add 3.0 mg to 10 ml acetone (need 4.8 ml per plate)

Procedure:

A. Standard Curve

1. Add BSA and NaCl to 48-well plate in QUADRUPLICATE as follows:

well #	ug protein/well	0.9% NaCl (ul)*	BSA (ul) (2 mg BSA/mL 0.9% NaCl)
A1-4	0	100	0
A5-8	20	90	10
B1-4	40	80	20
B5-8	60	70	30
C1-4	80	60	40
C5-8	100	50	50
D1-4	120	40	60
D5-8	140	30	70

* Need 2080 uL 0.9% NaCl for BSA curve; ~ 5mL per plate of samples.

2. Add 300 ul sodium phosphate buffer to each well

3. Add 100 ul fluorescamine solution to each well, with plate shaking on shaker.

4. Shake for 1 min after last addition, then let sit for 5 min PROTECTED FROM LIGHT.

5. Measure fluorescence on cytofluor using the following settings:
   excitation = E 409 (20)
   emission= A 460 (40)
   gain = 50, 60

B. Samples:

1. Prepare 1:10 dilutions of each sample in TEGD (e.g. 10 uL usomes + 90 uL TEDG). Keep on ice.

2. Pipet diluted samples into wells (2 concentrations: 10 uL & 20 uL of 1:10 dilution)

We use 10 uL and 20 uL of 1:10 dilution. *Why? B/c microsomal protein typically comprises 5 - 10% of liver weight and we want to load protein levels that will fall within the standard curve. Thus, if liver weighs 0.2 g, and microsomes were resuspended in TEDG 1:1 (TEDG vol: liver wt) then 0.2 g liver x 0.05 to 0.10 = 0.01 to 0.02 g protein/mL = 10 - 20 mg/mL = 10 - 20 ug/uL microsomal protein. 10 and 20 uL loadings of a 1:10 dilution would theoretically yield between 10 & 40 ug/well (10 uL x 1 - 2 ug/uL = 10 -20 ug; 20 uL x 1 - 2 ug/uL = 20 - 40 ug).*

3. Add NaCl to bring each sample to final volume of 100 uL

4. Add 300 ul sodium phosphate buffer to each well

5. Add 100 ul fluorescamine solution to each well, with plate shaking on shaker.

6. Shake for 1 min after last addition, then let sit for 5 min IN THE DARK.

7. Measure fluorescence on cytofluor using the following settings:

excitation = E 409 (20)
emission= A 460 (40)
sensitivity = 2,3

EROD & Protein Assays w/ CELLS
(48 well plates)

modified from: Hahn ME, Woodward BL, Stegeman JJ and Kennedy SW, Rapid assessment of induced cytochrome P4501A protein and catalytic activity in fish hepatoma cells grown in multiwell plates: response to TCDD, TCDF, and two planar PCBs. Environ. Toxicol. Chem. ***15:*** *582-591, 1996.*

Need: **Resorufin stock** (50 u M) in MeOH (pink) -20°C
Fluorescamine (30 mg/50 mL) in acetonitrile (ACN) (yellow) -20°C
7-Ethoxyresorufin (7-ER) (113 uM) in MeOH (orange) -20°C
PBS pH 8.0 ***at RT***
BSA Sigma A8806 (2 mg/mL) in dH2O

IMPORTANT: Fluorescamine, RR and 7ER are light sensitive!

1. Make working solutions (*only good for day they are made*)
RR = 175 uL PBS + 75 uL of 50 mM RR (3 plates)
7ER = 7 mL PBS + 498 uL of 113 uM 7ER (3 plates)

CELL WELLS

2. Aspirate media off cells w/ sterile pasteur pipet (CAREFULLY, trying to remove as few cells as possible)

3. To rinse cells, add 100 uL PBS (**to wall** not directly to cells)

4. Aspirate off (CAREFULLY).

5. Add 185 uL PBS to cells

STANDARD WELLS

Add 50 uL PBS to each std well
Add in triplicate (48 well):
PBS: 135uL, 124 uL, 113 uL, 91 uL, 70 uL, 47 uL
BSA: 0 uL, 10 uL, 20 uL, 40 uL, 60 uL, 80 uL (change tip b/w concs)
RR: 0 uL, 0.5 uL, 1 uL, 2 uL, 4 uL, 5 uL (change tip b/w concs)

48 WELL PLATE pmoles RR/**ug BSA**

	1	2	3	4	5	6	7	8
A	0	7.5/**20**	15/**40**					
B	0	7.5/**20**	15/**40**					
C	0	7.5/**20**	15/**40**					
D	30/**80**	60/**120**	75/**160**					
E	30/**80**	60/**120**	75/**160**					
F	30/**80**	60/**120**	75/**160**					

EROD ASSAY

To all CELLS & STD wells:

Add 50 uL 7 ER working solution (final conc in well 1.6 uM 7ER)
Read in cytofluor immediately **RECORD TEMPERATURE**

Mix time	0
EX/EM	**530/590**
Reads per well	3
Cycles	10
Time	00:00:00
Scans per cycle	2
Gain	50, 70

Save EROD file

PROTEIN ASSAY

To all CELLS & STD wells

Add 100 uL fluorescamine.
LET SIT FOR 15 MIN IN DARK.

REMOVE LID before reading in cytofluor

Open NEW FILE on cytofluor

Mix time	0
EX/EM	**409/460**
Reads per well	3
Cycles	1
Time	00:00:00
Scans per cycle	3
Gain	50, 70

Save PROTEIN file

RR AND 7ER **STOCKS**

7ER STOCK in MeOH (5 mL)
Final concentration of 7-ethoxyresorufin (7-ER) in the assay is ~ 1.6 µM.

1. Add a few crystals of 7-ER (Molecular Probes) to 2 ml methanol.
2. Make triplicate 1:100 dilutions in MeOH (10 uL in 990 uL MeOH); read at 482 nM
3. Determine concentration using extinction coefficient (22.5 mM-1 cm-1)
4. Calculate (Abs482 x 100)/22.5 = mM 7ER
E.g. 0.020/0.018/0.016 avg = 0.018 x 100/22.5 = 0.113mM = 113 uM

Store STOCK in brown bottle, lid wrapped in parafilm, at –20°C.

RESORUFIN STOCK in MeOH

Dissolve 0.0587 g RR in 5 mL MeOH = 50 mM solution
Make 1:1000 dilution w/ MeOH = 50 uM solution

Store STOCKs in brown bottle, lid wrapped in parafilm, at –20°C.

EROD Calculations

Slope of EROD in each well (AFU/min)
Protein in each well (in mg)
Slope/protein for each well (AFU/min/mg)
Average of 3 replicate wells = AFU/min/mg

Convert to pmoles RR:
AFU/min/mg = pmoles RR/min/mg
AFU/pmole RR

NOTES:
Can keep dipping pipet tip into PBS aliquot – just toss PBS at end of day
Can keep dipping pipet tip into fluorescamine in the bottle, no problem
For BSA and RR stds, however, change tips between concentrations

If you have one, use a Repeat Pipettor, or a multichannel pipettor, to add:
50 uL PBS to all std wells
100 uL fluorescamine to all wells
50 uL 7ER to all wells

Check RR and 7ER stock concentrations periodically using spec

FISH HEPTOCYTE PERFUSION PROTOCOL
(detailed protocol follows)

IN BRIEF

*Sterile Buffer A, **Buffer B (at RT),** L15+, PBS*
*Sterile **B+** (25 mg collagenase/75 mL buffer B) @ RT*
Sterilized hemostats, scapel, scissors, petri dishes, tissue sieve, beakers, razor blades
Perfusion system cleaned w/ 70 - 95% EtOH.
*MS-222 Initial in bucket (**0.6 g** MS222/**1.2** g $NaHC0_3$ in **4 liters**)*
*MS-222 Maintenance in perfusion basin (**0.8 g** MS222/**1.6 g** $NaHC0_3$ in **8 liters**)*
Angiocath (B-D Ref # 381134; but I have also used 23 g needles instead of the catheter)
bucket of ice
Sterile 10 mL disposable pipets
Autopipettor

Run **Buffer A** through the perfusion system to rinse out EtOH.
Anesthesize fish in **MS222 'Initial'** & **WEIGH**
Place fish, ventral side up, in wire-cradle, in a basin w/ recirculating **MS-222 'Maintenance'** flowing over gills.
Fill angiocath with **BUFFER A**.
Make incisions to expose liver, check for heart beat, insert buffer-filled angiocath
Watch for blanching of tissue (1-2 minutes).
Perfuse for 5-10 minutes with **Buffer A** (Ca-free).
Switch to **Buffer B+ (collagenase) at RT (impt!)** and perfuse another ~ 15 min.
Once soft, clip aorta, remove liver (w/o gb) to small beaker w/ **BUFFER B+**

IN LAMINAR FLOW HOOD

Transfer liver to glass petri dish containing **Buffer B-,** remove mesenchyme
Transfer to new glass petri dish containing **Buffer B-**, dice with razor blades
Place minced liver into **metal tissue sieve** sitting atop a 250 mL beaker, nestled in ice
Gently 'stir/press' tissue through sieve with glass pestle, adding **Buffer B-** ***sparingly***.
When liver mush turns a milky color you have only fat and mesenchyme left.
Pour cell mixture (thick, porridge beige color) into 50 mL tubes (2 or 4 as needed).
SPIN ~ 100 x g, 4 min, 4°C (~ 600 rpm)
Cells should be at bottom, may have a layer of rbcs on top
If supernatant is not clear, remove and keep on ice in separate tube in case contains cells
Resuspend pellet in **PBS** (10 mL) - SPIN
Resuspend pellet in **PBS** (10 mL) - SPIN
Resuspend pellet in **L15+** (10 mL) - SPIN
Resuspend **ONE tube** with 10 mL **L15+**, resuspend & transfer to all other tubes **-SPIN**
Discard supernatant, add **EXACTLY 10 mL L15+** and resuspend ***thoroughly*** in **L15+**
COUNT CELLS - put 10 uL of the following mixture onto each side of a cytometer
100 uL cells+ 300 uL L15 + 100 uL 0.4% trypan blue solution
5 x #cells x 10^4 = cells/mL (use conversion factor for your particular cytometer)

THAW MATRIGEL ON ICE

ISOLATION OF CHANNEL CATFISH LIVER CELLS USING COLLAGENASE PERFUSION

- adapted from Willett/DiGuilio protocol of 2/13/095 -

INTRODUCTION

CC hepatocytes are isolated following perfusion through the portal vein of the liver.

REFERENCES

The original method of liver perfusion was described by MJ Meredith for rat (Meredith, 1998. Cell Biol Toxicol 4:405-425). For catfish, DiGuilio used the retrograde perfuison procedure of in which hepatocytes are isolated following perfusion through the atrium and sinus venosus which lie directly caudal to the liver through the transverse septum. This technique has successfully been used in William Prosser's lab at the U of Illinois in Urbana with channel catfish (Seddon, W PhD thesis, 1993). Some modifications are from the method used by MJ Miller for the isolation of trout hepatocytes (Blair, JB et al. 1990. In Vitro Cell Dev Biol 26:237-249).

RECIPES for buffers are in RECIPE file.

DAY BEFORE

Make up and sterile-filter Buffer A, Buffer B- (w/o collagenase), L15 medium and store at 4°C
Sterilize hemostats, scapel, scissors, petri dish, tissue sieve, other as listed below
Clean perfusion system tubing & degasser by pumping 70 - 95% EtOH through it for a few minutes.

PERFUSION OF LIVERS

Have ready:
MS-222 Initial in bucket
MS-222 Maintenance in basin
bucket of ice

Following should be sterile:
Buffer A (Ca+ free), Buffer B- (w/o collagenase), **Buffer B+ (w/ collagenase) at RT**[1]
L15 & L15+ antibiotics
PBS
hemostats, scalpel, surgical scissors, razor blades, 4 x 250 mL beakers

1. Run **Buffer A** through the perfusion system to thoroughly rinse out the EtOH from the system.

2. The fish is anesthesized in deionized water containing water dosed with **MS222 'Initial'** at a concentrated of 150 ppm and buffered with sodium bicarbonate. The fish should be well-anesthesized in a few minutes.

3. The fish is weighed and placed ventral side up in a wire-cradle, nestled in a basin containing a recirculating aquarium pump (Powerhead 1000 works well) and **MS-222 'Maintenance'** solution (100 ppm) buffered with sodium bicarbonate. Tubing from the pump is placed into the fish's mouth securely, enabling the MS222 (100 ppm) solution to pass continuously over the gills. The heart should still be beating.

[1] Collagenase works better at RT than at 4·C

4. The following incisions are made: a longitudinal incision is made from the anus to the transverse septum. Two lateral incisions along the caudal edge of the transverse septum to expose the liver. The skin flaps are lifted clear or removed.

5. Attach the angiocath to the out-flow tubing from the de-gasser, turn on the perfusion pump, and fill catheter with **Buffer A**. Unhook it from the tubing, filled with Buffer A.

6. Insert the buffer-filled angiocath needle (still unhooked from the tubing) into a portal vein.

CAREFULLY remove the needle from the angiocath, maintaining the angiocath position in the vein. Have an assistant start the perfusion pump - let a few drops exit from the tubing before attaching it to the angiocath.

Hold angiocath in place by hand - watch for blanching of tissue. This should occur within 1-2 minutes.

Note: One vein may infuse only a portion, or all, of the liver ~ if only get partial perfusion, can try selecting another vein, or simply discard later those portions of the liver which did not get perfused adequately.

Soon after perfusion starts, sever one or more of the small portal veins to allow perfusate to flow out of the liver (I never do this, actually, because unlike catfish, trout have only one good portal vein).

NOTES:

~ you can spend 10 min hunting for a good vein before you need to worry about the liver becoming either necrotic or so full of blood clots that it can no longer be well perfused.

*~ watch that the gall bladder is **not** filling with perfusion medium*

7. Perfuse for 5-10 minutes with Buffer A (Ca-free).
Switch to **Buffer B+ (collagenase)** and perfuse another ~ 15 min.

8. Once liver is relatively well-blanched (won't be completely blanched) and softened (translucent), keep the catheter in place (to prevent heart from pumping blood back into the liver), and have assistant clip the aorta. Clip mesenchymal & other attachments.

CAREFULLY lift liver out of body, leaving the gall bladder behind[2], and place into beaker containing remaining Buffer B+ at RT so that outer areas of liver are exposed to collagenase.

After a few minutes, transfer liver to beaker with Buffer B- (do not want to over-digest tissue!) and put on ICE.

IN LAMINAR FLOW HOOD

9. Bring liver in **Buffer B-** on ice immediately to laminar flow hood. Transfer to glass petri dish containing **Buffer B-**

Cut away mesenchyme, and poorly perfused regions of liver and discard.

Transfer to second petri dish with **Buffer B-** and dice with razor blades held with hemostats.

10. Place minced liver into **metal tissue sieve** sitting atop a 250 mL beaker, nestled in ice

Stir and gently 'coax/press' tissue through sieve with glass pestle, adding **Buffer B-** ***sparingly*** as needed - do not want to add more than 200 mL at most! In any case, be sure it doesn't reach the bottom of the sieve.

After 15-20 minutes the liver mush turns a milky color indicating you have only fat and mesenchyme left and that fat is clogging sieve.

11. Pour cell mixture (thick, porridge beige color) into 50 mL tubes (2 or 4 as needed)

SPIN ~ 120 x g, 4 min, 4°C (~ 800-900 rpm)
Cells should be at bottom, may have a layer of rbcs on top - not a problem. Supernatant may not be clear. If is not clear, remove with electric pipettor and keep on ice in separate tube in case there are cells in there.
Resuspend pellet in **PBS** (10 mL)
SPIN
Resuspend pellet in **PBS** (10 mL)
SPIN
Resuspend pellet in **L15+** (10 mL)
SPIN
Resuspend ONE tube with 10 mL **L15+**, add to second (3^{rd}, 4^{th}) tube & resuspend til all tubes combined for a total volume of 10 mL in a single tube
SPIN

12. Discard supernatant, add EXACTLY 10 mL **L15+** and resuspend ***thoroughly*** in **L15+** using electric pipettor and 10 mL pipet to minimize clumps and disperse cells thoroughly. Could take 15 minutes or more.

COUNT CELLS

100 uL cells
300 uL L15
100 uL 0.4% trypan blue solution

Pipet 10 uL onto each side of the hemocytometer

[2] *If gall bladder breaks, quickly move liver to a new petri dish containing Buffer B+.*

Add coverslip

13. Determine amount of CELLS needed for experiment:

FOR FISH LIVER CELLS (from Willett):

48 WELL PLATE (500 uL cells/well) ~ also used for 96 well plates (note: Sue Bello used 4 x 10^5 cells/well for 96 well plates)

2.9 x 10^5 cells/well
5.8 x 10^5 cells/mL

6 WELL PLATE (2 mL cells/well)

10 x 10^6 cells/well
5 x 10^6 cells/mL

1. Volume of cells/well x # cells/mL = number of cells needed per well
2. Number of treatments x number of replicates/treatment = # wells needed
3. Number of cells needed per well x # wells needed = TOTAL # cells needed for expt.

Example:
1. 2 ml/well (for 6 well plate) x (5 x 10^6 cells/mL) = **100 x 10^6 cells/well**

2. Treatments = vehicle, estradiol (0.5 uM), untreated = 3
Timepoints = 6,12,24,48 = 4
Replicates per timepoint = 3

3 x 4 x 3 = 36 treatments = **36 wells** = 6 x 6 well plates

3. (**100 x 10^6 cells/well**) x **36 wells** = 360 x 10^6 cells TOTAL NEEDED
Cells need to be in a FINAL VOLUME of 2mL x # wells = 2 x 36 = 72 mL L15+

Average yield (in our hands) ~ 100 x 10^6 cells/liver
(10 x 10^6 cells/mL in 10 mL suspension).

Thus, for this experiment, would need to perfuse 2-3 fish.

CALCULATING YIELDS

1. COUNT CELLS

A cell count of **150** means:
5 x **150** x 10^4 = 750 x 10^4 cells/mL = 7.5 x 10^6 cells/mL
(dilution factor x **cell count** x hemocytometer calibration = cells/mL)

2. DILUTE CELLS TO ACHIEVE DESIRED CONCENTRATION

For 6 Well plate ~ need 5 x 10^6 cells/mL

HAVE: $\underline{7.5 \times 10^6 \text{ cells/mL}}$ = **1.5**x too concentrated.
NEED: 5×10^6 cells/mL

DILUTION:
20 mL cell suspension x **1.5** = 30 mL ; So add 30-20 = **10 mL*** of L15+ to the 20mL cell suspension.

* IMPORTANT: Calculate account the amount of **Matrigel** you need to add (15 uL/mL cells). Then add L15+ minus the Matrigel amount. Example, if final cell volume is to be 30 mL, then 15 uL matrigel/mL cells x 30 mL cells = 450 uL Matrigel. So add 9.5 mL L15+ & 450 uL Matrigel to the 20 mL cell suspension.

NOTE: Need a total of 72 mL for this experiment, so will need to perfuse another fish, maybe two more, and combine cells, recount, dilute as needed, then ADD MATRIGEL (15 uL Matrigel/mL cells), & plate the cells

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