ABSTRACT

Title of Document: EFFECTS OF PCB EXPOSURE ON HEPATIC GENE EXPRESSION AND ENZYME ACTIVITY IN AVIAN SPECIES

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Polychlorinated biphenyls (PCBs) are a class of anthropogenic chemical compounds used by industry from the 1940s until the 1970s. Two General Electric plants at Fort Edward and Hudson Falls, NY contaminated the Hudson River by disposing roughly 604,500 kg of PCBs into the waterway during that time. This research focused on using whole-genome screening to find novel biochemical responses in laboratory and wild birds that are exposed to PCB mixtures relevant to the Hudson River. We used two PCB congener profiles, found in spotted sandpiper eggs and in tree swallow eggs from the Hudson River area of concern. We also tested PCB 126 and PCB 77 singly, since both have very high avian TEQs compared to other PCB congeners. Microarray technology was used to assess the spotted sandpiper mixture in Japanese quail. Pathways of interest were identified and qPCR was performed on a suite of genes to assess the response levels of Japanese quail that were exposed to both mixtures as well as the single congeners. Ethoxyresorufin-O-deethylase (EROD) was assessed in

Japanese quail with all treatments, and in tree swallow and bluebird populations at the Hudson River site, and at three reference sites. Major findings from the microarray study revealed that the pathways for xenobiotic metabolism, oxidative damage, endocrine disruption, and energy balance were all impacted with PCB exposure. For the four compounds tested with both sexes, EROD activity increased in laboratory birds for seven of those eight sex/compound combinations. Cytochrome P450 1A4 and cytochrome P450 1A5 were the most consistently responsive genes for all sex/compound combinations. All other genes showed varied responses that changed with concentration, compound, and sex, however there were few differences seen at the level of significance ($p<0.05$). EROD exhibited mild response to PCB exposure in wild birds, with significant ($p<0.05$) differential expression in environmentally exposed birds at the Hudson River across years, 2006-2008. In summary, this research demonstrates that xenobiotic metabolism remains a highly responsive pathway with PCB exposure and that this pathway responds to PCB mixtures in a manner that does not mirror the toxic equivalency of the component PCBs.

EFFECTS OF PCB EXPOSURE ON HEPATIC GENE EXPRESSION AND ENZYME ACTIVITY IN AVIAN SPECIES

By

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Dissertation submitted to the Faculty of the Graduate School of the
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Professor Mary Ann Ottinger, Chair
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Dedication

To my parents…
You have been the guiding stars by which I sail my vessel, all my life;

To David…
My partner, my best pal;

And to Jackson, Lily, and Baby #3…
You are proof positive that God loves me and wants me to be happy.

Personal Acknowledgements

Praise to the Lord in Heaven, Creator and Sustainer of life, Author and Perfector of my faith! All good things come from You, and You have brought this work to completion. You saved me from the depths, and You brought me to life. You have shown me immeasurable blessings with a calling and a family. You have given me abundant life. I will praise Your Name forever.

To David. You have been in the trenches with me. You have talked me out of quitting every single time I wanted to. You had faith in me when I had absolutely no more faith in myself. You let me disappear on the weekends to get work done. You fed the kids when I wasn't there to do so. You took days off from work so I could go to the lab. You have taken a financial hit to let me pursue this lifetime dream. You believed that I was capable of and deserving of "having it all". Please know that I would not have anything if I did not have you. You have shown me that life is so much more than a job, or even a passion for a calling, but that a good home life makes that calling worth having. Nothing can replace what I have with you, and no future career success will ever mean more to me than our life together. I know you will always keep me on track to remember that when I forget. You'll do it not by verbally telling me, but by your daily affection, provision, leadership, and example. You have found a place for my calling in the larger vision you have for our family, and that is unmatchable.

To my wonderful children. You have been my therapy. At the time of writing this, Jack, you are 4; Lily, you are 19 months; and Baby #3, we don't even know what to call you yet. We don't know who you are, but we love you so deeply,

and we can't wait to meet you. Hopefully one day you will read this and be able to get an idea of what life was like in the early days of our family. It was harried. It was hurried. It was filled with pumped milk, dirty diapers, ridiculous amounts of laundry, late and long nights, board books, trips to the zoo and train museum, and dried up Cheerios in the Mazda. And it was filled with love. We love and celebrate each one of you for exactly who you were created to be. We were planning on one baby during my time in graduate school, and we were blessed with two and a little bit of #3. I was pretty freaked out when we found out we were pregnant with Jackson, and I was scared out of my mind when we found out we were pregnant with Lily. I had no idea how we were going to make it all work financially or how I was going to manage to be a good mom without going insane. But I cannot point to anyone other than God as the sustainer of our lives. He gave us the power to endure when we were so tired and worn out neither of us could stay awake through bedtime. He wanted us to have you, at the exact moments in time when we did. I can only hope that we did an okay job with you guys. I want you to understand that, despite how scared I was, each one of you has been a boon to my soul during my time in graduate school. When I got crappy results, or when I couldn't get a lab procedure to work, or when I was too darned tired even to try to make it into the lab that day, the necessity of putting it all down to come home and do the mind-numbingly simple tasks of taking care of you, feeding you, changing your diapers, playing with you, reading to you, and putting you to bed was the best therapy I could have asked for. It forced me to gain perspective, not to worry about the future, and not to worry about the events of the day. As long as there was you, there was life. While there were times when I

wanted to trade it all in for two hours of sleep, I know that if I could do it differently, I never would. You were exactly what I needed during this incredibly busy time of life. I also want you to understand that we wanted to raise you with the appreciation that you CAN have a rich, full, abundant life with careers that you're passionate about, hobbies, and friends, all while you start a family. This is the "having it all" that generations past have called it. Don't be mistaken, it is incredibly difficult to achieve and maintain, but the reason that is achievable and maintainable is that you WANT it. You want it so badly you are willing to do a lot to get it. It is hard, but anything worth having is going to be hard, and having an abundant life is the thing above all else worth having.

To my parents. You were the first who showed me what love looks like. You wanted for Ashley and me what you wanted for yourselves. Living up to your standards has been a good and desirable thing. You instilled in us the passion for a calling and the desire for a life filled with good things (family, hobbies, friends). You gave us the best of everything that was to be had, and you did not withhold your affection and love. You helped us when we fell, and you let go of our hands when it was time for us to do things on our own. Not everything you did was perfect, and through your humanity we've seen examples of being true to oneself. And that's not easy to display to one's children (as I'm learning). You have also instilled the love of finding out about the world, especially the natural world. You raised us as the nerds we are, and I am so grateful you did. Because of that, passing that on to my own children comes completely naturally, and for that legacy I cannot express enough gratitude and appreciation. You both have shown me the value of being my own

person. That comes with the nerdiness, but also from having given me the authority over my own life. I think primarily of my decision to go to St. John's for college. Dad, I know you weren't initially happy with that decision, but I also know you came to a place where you trusted me with that decision not only because you wanted me to take ownership of my life's decisions, but also because you taught me to be a well-rounded person, not just one who wanted to know only about science. And part of the consequence of that method of raising me was that I had to find out everything that the ivory tower held for me and not simply relying on the efficient training of a marketable scientific degree. You wanted me to be educated, not merely trained. Mom, you spent the time making sure I got that education. You taught me biology, music, the Bible, and even a little bit of Latin for a while. You have also been the main thrust of dealing with the biological questions asked in this dissertation, both statistically and philosophically, a contribution not easily found in other places. And as I've gone through graduate school, you both have iterated time and again the importance of asking the harder questions, the questions that transcend the biological questions. The most pertinent one of those is probably, why are we doing this, this thing called science? And why is it important? And in this way, the things important to you are important to me.

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the unknown of supporting me, and have always been there for us as a family as we try to balance it all.

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We therefore must not recoil with childish aversion from the examination of the humbler animals. Every realm of nature is marvelous...so we should venture on the study of every kind of animal without distaste.

- Aristotle, *Parts of Animals*

By doubting we come to inquiry, by inquiry we come to truth.

- Peter Abelard

If I speak in the tongues of men and of angels, but have not love, I am only a resounding gong or a clanging symbol. If I have the gift of prophesy and can fathom all mysteries and all knowledge, and if I have a faith that can move mountains, but have not love, I am nothing.

- 1 Corinthians 13:1-2

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List of Abbreviations

AhR	aryl hydrocarbon receptor
AHR1	aryl hydrocarbon receptor 1
ALDOB	fructose biphosphate aldolase B
CAT	catalase
CR	Cobleskill Reservoir
CYB5	cytochrome b5
CYP1A5	cytochrome P450 1A5
DDT	dichlorodiphenyltrichloroethane
DIO2	deiodinase type 2
Ct	threshold count
DNT	2,4-dinitrotoluene
DRC	dose-response curve
ED	embryonic day
ER	estrogen receptor
EROD	ethoxyresorufin-*O*-deethylase
G2/M	growth phase 2-to-mitosis
GADPH	glyceraldehyde 3-phosphate dehydrogenase
GE	General Electric
GLEMEDS	Great Lakes Embryo Mortality, Edema, Deformity Syndrome
GPx	glutathione peroxidase
GR	glutathione reductase
GSH	glutathione
GST	glutathione-S-transferase
GSTA	glutathione S-transferase alpha
HR	Hudson River
IPA	ingenuity pathway analaysis
PCA	principal components analysis
PCB	polychlorinated biphenyls

PCR	polymerase chain reaction
PROD	pentoxyresorufin-*O*-deethylase
PRR	Patuxent Research Refuge
qPCR	quantitative polymerase chain reaction
RDX	cyclotrimethylenetrinitramine
ROS	reactive oxygen Species
SAS	Statistical Analysis Software
SL	Sacandaga Lake
SOMs	self-organizing maps
SPSA	spotted sandpiper
TCDD	dioxin, tetrachlorodibenzo-*p*-dioxin
TEQ	toxicity equivalency quotient
TRES	tree swallow
TSCA	U.S. Toxic Substances Control Act
TXN	thioredoxin
UHR	Upper Hudson River
USEPA	United States Environmental Protection Agency

Chapter 1: Background

Part I: PCBs as a Compound of Interest

Chemistry and History of PCBs

PCBs are a family of chlorinated hydrocarbons that were commercially manufactured in the U.S. starting in the 1920s for use in hydraulic fluids, die-casting equipment, the manufacture of transformers, and as insulating fluid for the assembly of capacitors (Frame et al., 1996a; 1996b; Safe, 1990; reviewed in Jensen, 1972 and Rice et al., 2003). There are 209 possible congeners of PCBs, which are determined by the number and placement of chlorine atoms around the two-phenyl ring backbone. Steric hindrance is caused by close placement of chlorine atoms to the connecting carbons. Low steric hindrance allows the rings to lie in the same plane as each other and therefore the non-ortho-substituted, i.e. the meta- and para-substituted, PCBs have a more coplanar structure (Brunström and Halldin, 1998; Rice et al., 2003; Van den Berg et al., 1994). This conformation makes these congeners very structurally similar to tetrachlorodibenzo-p-dioxin (TCDD, i.e. dioxin). Accordingly, they behave similarly to TCDD in terms of activating the aryl hydrocarbon receptor (AhR) in a dioxin-like manner, and are subsequently referred to as being dioxin-like.

PCBs rarely, if ever, exist as single congeners in the fluids and matrices that contain them (Niimi, 1996; Rice et al., 2003). Most are mixtures of several congeners, and many such mixtures were manufactured and commercially available. They have been marketed under several different commercial names including Aroclor (U.S.), Pheneclor (France), Kaneclor (Japan), and Sovol (Russia; Frame et

al., 1996a; Rice et al., 2003). It has been estimated that total worldwide production of PCBs through 1976 was $6.1x10^{11}$ kg, roughly 93% of which was manufactured by the American company Monsanto (Durfee et al., 1976), and roughly $6.4x10^{8}$ kg. of halogenated biphenyls manufactured worldwide has been reported (Safe, 1984). The U.S. Toxic Substances Control Act (TSCA) was enacted in 1976 and authorized the U.S. Environmental Protection Agency (USEPA) to regulate chemicals that were determined to cause unreasonable risk to public health or the environment. In July 1979, TSCA banned the manufacture, processing and distribution of PCBs in the U.S. and regulated the phase-out of their existing uses (Bremer, 1983; USEPA, 2010).

Mechanisms of Action of PCBs

In general, many dioxin-like compounds bind the aryl hydrocarbon receptor (AhR), including 12 out of 209 PCB congeners that are known to activate the AhR. The ligand-bound AhR causes a genomic upregulation of any number of cytochrome P450 (CYP450) enzymes, but specifically the CYP450 1A enzymes that have detoxification activity through several biochemical reactions, including deethylation and hydroxylation, among others (Hutzinger et al., 1972; Safe, 1984). In mammals, the CYP1A1 and CYP1A2 genes are involved with these detoxification reactions and the CYP1A4 and CYP1A5 genes are their orthologs in birds (Gilday et al., 1996; Kubota et al., 2006; Mahajan et al., 1999; reviewed in Head and Kennedy, 2007).

Ethoxyresorufin-O-deethylase (EROD), a hepatic Phase I biotransformation enzyme involved in detoxification (Melancon et al., 2003; 2006; Wright and Welbourn, 2002), is one of several enzymes that is upregulated in response to exposure to dioxin-like compounds (Sanderson et al., 1998) and its induction is

correlated with exposure to coplanar PCBs (Rattner et al., 1994; 1996). EROD activity is catalyzed by the enzyme encoded by the CYP1A4 gene, which is upregulated via the AhR (Head and Kennedy, 2007). Therefore, it is considered a reliable and sensitive measure of exposure to dioxin-like compounds (Head and Kennedy, 2007; Rattner et al., 1989). The CYP450 enzymes are part of a much larger enzyme family that have many physiological roles throughout the body, including steroidogenesis. For example, CYP19 codes for aromatase, the enzyme involved with converting testosterone into estradiol (Ojeda, 2004). At non-steroidogenic sites, including the liver, aromatase is involved with steroid metabolism and homeostasis, and has been shown to be upregulated in response to compounds such as DDE (You et al., 2001). In addition, it has been shown that there is cross-talk between the AhR and the estrogen receptor (ER), in that they can dimerize with each other to cause either transcriptional changes in estrogen-responsive genes, or to cause the ubiquitination of the ER for break-down in proteosomes (Ohtake et al., 2003). These data demonstrate significant cross talk between the detoxification and endocrine systems.

In contrast, non-coplanar PCBs are less likely to activate the AhR but instead may act through non-AhR-related systems (Fischer et al., 1998). These systems include oxidative damage and the antioxidant enzymes that respond to it. Oxidative stress refers to the overall effects of reactive oxygen species (ROS) build-up in an organism (Mitchelmore et al., 1998; Gutierrez, 2000; reviewed in Andreyev et al., 2005). This process is exacerbated by exposure to environmental pollutants. Pollutants accelerate ROS accumulation through hydroxylation reactions of the

contaminant by the CYP450 enzymes. This combined with impairment of the oxidative phosphorylation cascade by organic contaminants and metals will result in synergistically elevated levels of ROS, eventually leading to intracellular oxidation and reduction of the xenobiotic compound to a free radical (Shertzer et al.,2006). This cycle repeats because the parent compound can be subsequently reformed as is the case with quinone-based anticancer drugs, with a superoxide anion being a by-product of the reaction. This redox cycling of the compound leads to hydrogen peroxide and hydroxyl ion production (Gutierrez, 2000). Accumulated ROS react with many biological macromolecules, leading to DNA damage, cancer, pulmonary disease, and neurodegenerative disorders (Ames et al., 1993; Cadenas and Davies, 2000; Ghio et al., 2012; Mitchelmore et al., 1998). Glutathione aides in the recycling of ROS through many roles, including being an intermediate step in reduction reactions via glutathione peroxidase (GPx) and glutathione reductase (GR) reactions. Studies have found an increase in these enzymes in response to PCB exposure (Aly and Domènech, 2009; Brown et al., 2007; Hoffman et al., 1998). It stands to reason that this enzyme system is controlled through transcriptional regulation, since it has been demonstrated that there is already one genomic receptor system – the AhR – that regulates enzymes transcriptionally upon exposure and subsequent activation by PCBs.

PCBs in the Environment

Although PCBs were released and persist as mixtures in the environment, the congeners are metabolized as they move through the food chain, thereby altering the composition of the remaining mixtures (Brown et al., 1984). As a result, the specific

congener profile that toxicologists encounter in the field is characteristic of a particular site based on the source mixture and transforming biota. The mixtures found as body burdens in animals differ between species at the same contaminated site (Custer et al., 2004; Echols et al., 2004). Since individual congeners have different effects (for example the activation of the AhR by the more coplanar congeners), specific mixtures have different net effects, thereby confounding the problem of studying the effects at a particular site, or of predicting the effects of a particular mixture.

PCBs have entered the environment largely through aquatic systems. From there the less chlorinated congeners can disperse into the air due to their relatively high volatility. Incineration has been a low source of environmental exposure, since only 4.4% of PCBs purchased by US industry until 1976 were incinerated (Durfee et al., 1976). Atmospheric dispersion causes rapid movement of PCBs to otherwise pristine ecosystems such as the Arctic, where they then have deleterious effects after moving back into the water and/or soil, and from there moving into biota (reviewed in Wright and Welbourn, 2002). Once in the water, they settle into the soil and are taken up by microbes and other biota in the soil (i.e. worms and snails). Those organisms are then eaten by higher-order organisms, often larger soil-dwelling creatures or fish, which are then in turn eaten by larger organisms, including higher-trophic level fish, terrestrial animals, and humans. In this way, PCBs will bioaccumulate in individuals and will biomagnify as they move up the food chain. As a result, higher trophic level organisms become more at risk to the damaging effects of PCBs. For example, Safe (1989) reported that in Lake Ontario,

concentrations increased from 0.05 ng/g PCB in water to 150 ng/g in sediment, 1800 ng/g in plankton, 11,580 ng/g in catfish, and finally 3,530,000 ng/g in herring gull. Similarly, Chiu et al. (2000) discusses PCB bioaccumulation in the food chain from algae to polar bears in three steps.

Life cycle analyses have documented the transport and distribution of PCBs through the environment. Generally, they are released into an aquatic system where they usually settle into the sediment because they do not remain well-suspended in aqueous matrices. However, a small fraction may remain in the water column depending on the level of hydrophobicity. Once in the sediment, some fraction remains there while the rest is taken up by benthic life. From the sediment, PCB residues will pass from organism to organism, trophic level to trophic level. Therefore, aquatic environments and their biota have a high absolute amount of residues, however high-trophic level organisms, including mammals, birds, and humans have especially concentrated body burdens (Safe, 1990). This is exacerbated by higher concentrations of residues stored in blubber because these compounds are lipophilic, therefore high-trophic level animals, and especially those that live in extremely cold climates, i.e. the Arctic and Antarctic, are of concern because concentrations in polar species indicate a higher body burden. These animals will include polar bears, seals, whales, and penguins, among others. Bustnes and colleagues (2006) reported average total PCB loads of 124 ng/g wet weight with a range of 9.8-781.2 in the subarctic great black-backed gull (*Larus marinus*), compared to an average of 448.7 ng/g wet weight with a range of 84.3-1576.1 in the arctic glaucous gull (*Larus hyperboreus*), In addition, it should not be overlooked that

some portion of PCBs will also be aerosolized, based on their volatility, and can spread even faster than by moving via water and biomass. When a chemical can move through so many types of matrices with varying degrees of ease, it can quickly spread through the global ecosystem, moving far away from its points of entry. PCB concentrations, in the Arctic Ocean ranged from <2 to 6 pg/L in 1992, 42-72 pg/L in the Antarctic Ocean in 1982, and 40-590 pg/L in the Pacific Ocean in 1984 (Hargrave et al., 1992; Tanabe et al., 1983; Tanabe et al., 1984). Therefore even allegedly pristine environments have been shown to carry high PCB loads.

In contrast, environments with known sources of contamination have higher ranges, as much as an order of magnitude above pristine areas, although these often decrease over time. PCB concentrations in the Great Lakes have been measured at 1 ng/L in Lake Michigan in 1987, 1-4 ng/L in Lake Superior in the late 1970s, and 1 ng/L in Lake Ontario (Swackhamer and Armstrong, 1987; Capel and Eisenreich, 1985; Oliver and Niimi, 1988). Wan and colleagues (2010) reported fish having body burdens ranging from 21 to 190 ng/g wet weight in the Saginaw and Tittabawassee Rivers in Michigan. Norstrom and Hebert (2006) report a decline in total PCBs in gull eggs from 1971-1982 (from 196 down to 39 ug/kg at Scotch Bonnet Island in Lake Ontario; from 133 down to 34 μg/kg at Big Sister Island in Lake Michigan), possibly reflecting the ban on PCBs in the late 1970s as residues are buried in sediment.

Biological Effects of PCBs

Birds

Avian populations started declining in the 1960s as a response to chemicals such as dichlorodiphenyltrichloroethane (DDT), and since then the effects of man-made compounds on birds has been of interest to toxicologists (Scanes and McNabb, 2003). The effects of avian exposure to PCBs have been studied both in the field and in the laboratory. PCB-related effects in birds have been reported to include decreased reproduction and developmental abnormalities (Fernie and Bortolotti, 2003; Fernie et al., 2003b), altered behavior (Fernie and Bortolotti, 2003); reduced hatchability in both maternal feeding and egg injection studies (Brunström, 1989), embryo mortality (Halldin et al., 2005), endocrine disruption affecting thyroid (McNabb, 2005; Webb and McNabb, 2008) gonadal effects including reduced testis weight and imbalanced sex hormones (Biessmann, 1982) and hypothalamic hormone level changes (reviewed in Ottinger et al., 2009a-b). Other studies have reported hepatic cytochrome P450 (CYP450) enzyme induction (via AhR; Brunström, 1989; Brunström and Halldin, 1998; Elliott et al., 1990; Melancon, 2003) and differential expression of related genes (Head and Kennedy, 2007); immune effects including follicle and cell count reduction in the bursa and thymus (Nikolaidis et al., 1989) and immunosuppression (Lavoie and Grasman 2007); and changes in song production (Hoogesteijn et al., 2008). Many of these effects can emerge in the second generation after parental exposure (Fernie et al., 2003a).

Mammals

PCBs have been implicated in adverse biological effects in several terrestrial and marine mammal species including seals, polar bears, mink, otters, and cetaceans (Basu et al., 2007; Beland et al., 1988; Chiu et al., 2000; Harding et al., 1999; Mos et

al., 2007). This demonstrates further that these compounds are ubiquitous across the earth and through all biota.

General effects of PCBs have been gained from extensive laboratory studies using mice and rats, yielding similar results to those found in birds and other mammals, including decreased and altered detoxification, immune, reproductive, neurological, and endocrine system function. Mariussen and Fonnum (2006) review a number of neurochemical effects in laboratory animals, including changes to neurotransmitters, behavior, the neuroendocrine system, long-term potentiation, signal transduction pathways, and calcium homeostasis. Brown and colleagues (2007) reported increased hepatic tumors, superoxide production, and CYP450 induction, which changed in a correlated manner with PCB dose. Aly and Domènech (2009) found that rat hepatocytes were exposed to Aroclor 1254 produced increased reactive oxygen species (ROS) and lipid peroxidation; in addition, glutathione reductase (GR), glutathione peroxidase (GPx), and glutathione (GSH) production decreased. There was a correlated increase in ethoxyresorufin-*O*-deethylase (EROD) and pentoxyresorufin-O-deethylase (PROD) activities, which provide biomarkers of exposure.

The Upper Hudson River: A Case Study

Historic manufacturing and disposal facilities have been identified as the chief point-sources of PCBs to the environment. The upper Hudson River in New York State is one of the largest sites of PCB contamination. Between 1940 and 1977, two General Electric (GE) capacitor manufacturing plants located in the towns of Fort Edward and Hudson Falls, NY, released 95,000 to 604,500 kg PCBs into the river

(NYSDEC; Carlson et al., 2009; Feng et al., 1998; Sanders, 1989). As a result, the entire 200 mile downstream stretch of the river, between Hudson Falls and the Battery in New York City, was placed on the USEPA's National Priorities List in 1984 and is now a Superfund site (Feng et al., 1998; Hettling et al., 1978; Sanders, 1989).

Although PCB levels in the Hudson River have dropped from 540 ng/L in 1977 to 130 ng/L by 1981 (Sloan et al., 1983), contamination persists to this day. Echols and colleagues (2004) reported PCB concentrations in insects, tree swallow (*Tachycineta bicolor*, TRES) eggs , and hatchlings at two contaminated sites (Remnant Site 4 and Special Area 13) on the upper Hudson River. Mean concentrations in two families of insects (Odonata and Diptera) at one of the sites were 1.2 and 18 μg/g, respectively. Mean concentrations at another site for those insects were 0.56 and 6.7 μg/g, respectively. Mean tree swallow egg concentrations at those two sites were 24 and 13 μg/g respectively (Echols et al., 2004). Mean day-5 nestling concentrations were 14 and 19 μg/g. Mean day-10 nestling concentrations were 48 and 32 μg/g. Mean day-15 nestling concentrations were 96 and 32 μg/g. Mean adult concentrations at one of the sites were 152 μg/g. This is compared to an upstream reference site where day 10 nestlings and adults had mean concentrations of 1.5 and 53 μg/g, respectively. In 1999, PCB concentrations in TRES eggs were 9.3-29.5 μg/g wet weight (ww), while adults and hatchlings had concentrations at 114 and 3.7-62.2 μg/g ww respectively (Secord et al., 1999). Spotted sandpiper (*Actitis macularius,* SPSA) eggs had a concentration average of 15.2 μg/g ww, while 56.2 μg/g ww was the highest concentration measured in an individual egg (Hudson River

Natural Resources Trustees, 2005). Impaired reproduction has been observed in bald eagles, white-tailed eagles, and peregrine falcons with respective egg burdens of 20, 25, and 40 μg/g ww (Custer et al., 2010).

Part II: Transcriptomics as a PCB Toxicity Endpoint

Use of Genomic and Molecular Tools in Toxicity Endpoints

Functional genomics has recently entered the scientific arena as an important tool of study for the purpose of delving deeper into mechanisms of action for physiological processes and the processes of disease. Toxicologists have also recently started using these tools to study the mechanisms by which manmade environmental contaminants exert their toxicity on organisms, referring to this new field as toxicogenomics. Iguchi and colleagues (2007) made two points about the usefulness of these genomic tools, specifically 1) transcript, protein, and metabolite profiles aids in profiling classes of toxicants, and 2) elucidating modes of action of toxicants.

Use of a reliable profile of molecular and physiological biomarkers will be useful in field studies involving exposure and risk assessment. For example, assessment of a population's health, robustness, and reproductivity will be more efficient at the subcellular level where potentially a small sample can be collected instead of sacrificing large numbers of animals. Whole-genome screening can be used to identify biomarkers and measures of contaminant effects. It is also critical to

develop a deep understanding of the multitude of biological pathways that are affected by a given toxicant, as pointed out by Iguchi and colleagues (2007).

Microarray Technology

Microarray technology is a reliable tool of ecotoxicological assessment of gene transcription. Gust and colleagues (2009) conducted a study that considered effects of two munitions constituents, cyclotrimethylenetrinitramine (RDX) and 2,4-dinitrotoluene (DNT), on neurological gene expression in Northern bobwhite quail (*Colinus virginianus;* Gust et al., 2009). Four experimental trials were carried out, with a 14-day DNT exposure of five doses, a 14-day RDX exposure with five high doses, a 14-day RDX exposure with five low doses, and a 60-day RDX exposure with low doses. It was found that DNT had little effect, although some RDX treated birds experienced seizures while others did not. Interestingly, the seizing birds had transcriptome changes that clustered together, contrary to the non-seizing birds. This study highlights the fact that a toxicogenomic study can accomplish both goals outlined by Iguchi et al. (2007) mentioned above, specifically that a profile of exposure can be seen between different chemicals, and a mechanism of action can be found from these studies.

A study using DNA microarrays assessed the toxicogenomic response in C. elegans to PCB52 (Menzel et al., 2007). These investigators make a point of using PCB52 because it is non-planar and therefore is most likely not to interact with the AhR in a TCDD-like fashion. The findings of this study demonstrated that certain classes of genes, specifically cytochrome P450 genes, short-chain dehydrogenases,

and genes involved with lipid homeostasis, are potential targets of PCB toxicity. This points to potential profiling of PCB52 gene induction.

Another study involving tomcod, a species of freshwater fish found in the upper Hudson River (New York, USA) near the sites of two General Electric factories, used a microarray designed from a cDNA library of tomcod heart tissue (Carlson et al., 2009). The goal of this study was to find new genes in tomcod that point to species differences in response to PCB exposure. Tomcod is a unique species in that this fish is a wintertime spawner in the river and are the dominant prey in deeper waters during the summertime months. This would indicate a different physiological response as well as different level or timing of exposure to PCB contamination. Researchers dosed tomcod from the Hudson River, as well as Shinnecock Bay (New York, USA) and Miramichi Bay (Canada), with two levels of PCB mixture and ran a cDNA microarray with heart tissue. This raised an interesting hypothesis that the population from the Hudson may exhibit fewer changes in gene expression than the fish from the relatively pristine sites upon exposure to PCBs due to continuous environmental exposures to these compoundss. The microarray was non-normalized and showed a high preponderance of ribosomal proteins as well as alpha and beta globins. As a result, few genes were differentially expressed, though 143 clones were found to be differentially expressed using a one-way ANOVA. A principal component analysis revealed that the different populations showed different levels of expression compared to each other, however the different doses of the PCB mixture did not. The main finding of this paper indicates that a normalized cDNA

library would help greatly with finding more dramatic findings in differences of each population to the different levels of PCB mixtures.

Many studies rely on rat and mouse models, as in one study by Silkworth and colleagues (2008) in which doses of TCDD and Aroclor 1254 with similar TEQs were given to rats in order to elucidate gender-specific differences in the carcinogenicity of Aroclor 1254, specifically where mechanisms of action for carcinogenicity are concerned. Aroclor 1254 exposure is related to a higher rate of liver tumors in females than in males. Ultimately, this study revealed several interesting profiles of gene expression along gender lines, as well as showing the TEQs, as they are defined by the World Health Organization (reviewed in Silkworth et al., 2008), did not explain gender differences. Accordingly, one aspect that was examined in this research was if the TEQ would predict effects (see p. 31 for discussion of expected compound differences in gene expression).

A microarray study investigating the role of AhR in gene regulation by AhR-activating compounds (TCDD, pentachlorodibenzofuran [PeCDF], and PCB 126) and one non-AhR-activating compound (PCB 153; Vezina et al., 2004). Several AhR-responsive genes were differentially impacted by the AhR-active compounds but not by PCB 153, which induced a very different expression profile. The investigators highlighted the crucial role of AhR-active compounds, but did not address the genes that were differentially expressed by PCB 153. This indicates that not enough attention is paid to the non-AhR effects of non-dioxin-like compounds, creating a path for study of non-dioxin like PCB effects.

Another rodent study investigated the difference in gene expression profiles of PCBs and methylmercury (MeHg) in a mixture together compared to each compound singly (Shimada et al., 2009) in newborn (postnatal-day 1) rat brains, using a micorarray. It was found that the single exposures (i.e. MeHg or PCBs alone) induced unique gene expression patterns that also differed from those induced by mixtures of the two chemicals. This is further indication of the conundrum associated with exposure to mixtures, and how difficult it is to tease out the effects thereof.

Padhi and colleagues (2008) also performed a microarray study looking at the effect of mixtures on neurodevelopment in postnatal-day 14 rat cerebella, using a mixture found in the Canadian arctic of MeHg, PCBs, and organochlorine contaminants. The three components of the mixture were also administered and screened for effects separately. Genes with differential expression included cell-cell communication, cellular differentiation, signal transduction, myelination, and synaptic transmission.

The strengths and weaknesses of these studies taken together illustrate a clear need for research into both the dioxin-like and non-dioxin-like effects on biological systems in investigating complex mixtures. Since little is known about the underlying effects of gross or morphological toxicity, developing a "footprint" of exposure could offer a detection method of physiologically upstream responses for exposure to toxicants often before a true biological detriment has affected the organism, population, or ecosystem, which can ultimately lead to the development of biomarkers not just of exposure but also of effect on individuals and possibly up through the population and ecosystem levels.

Use of Egg Injections in Avian Toxicity Studies

Dosing eggs with compounds of interest to mimic maternal deposition via injection into either the yolk or air cell has been established as a reliable, effective, and efficient method for toxicity assessment (Brunström, 1988; 1989; Brunström and Halldin, 1998; Hoffman 1996;1998; McKernan et al., 2009; 2010). The Japanese quail is a well-established animal model for toxicological investigation and reported to be less sensitive than the chicken (Head et al., 2008; Hervé et al., 2010; Ottinger et al., unpublished data).

Part III: Objectives and Significance of the Current Study

The aim of this study was to assess gene expression in biochemical pathways as affected by PCB exposure. AhR-mediated genes are known to be upregulated because dioxin-like compounds such as PCBs are ligands for AhR, however the mechanism by which PCBs affect other pathways is unknown, though evidence of whole-organism as well sub-cellular effects of PCBs on these pathways is available. Therefore, this study focused on PCB effects at the level of transcription, thereby lending evidence of their mechanism of action on non-canonical pathways. Two controlled experiments were conducted with the Japanese quail as an avian model to investigate the effects of PCB 126, PCB 77, and two PCB mixtures on hepatic gene expression. The dosing range of the two PCB mixtures encompassed the range of exposure as measured in eggs at UHR (Appendix 1). One PCB mixture was comprised of 58 congeners found in SPSA eggs at UHR (Custer et at., 2010; Appendix 2, Table 1). The other mixture was comprised of 66 congeners found in

TRES eggs at UHR (Echols et al., 2004; Appendix 2, Table 2). Japanese quail eggs were exposed *in ovo* via air cell injections. Livers for all studies were collected within 24 hours of hatching. In the first study, one PCB mixture was tested and microarray technology as well as qPCR was used to measure gene expression. In the second study, all four PCB compounds were tested and qPCR was used to measure gene expression. EROD enzyme activity was also measured in the second study. A third study was conducted to compare EROD in tree swallows and bluebirds at UHR and birds at three reference sites that were experimentally exposed to PCBs. These studies will help develop molecular tools for Japanese quail, establish gene expression profiles in Japanese quail exposed to these compounds, serve as a starting point for application of these techniques in the field, and eventually lead to the discovery of new and emerging biomarkers of toxicity in birds via early detection of differential gene expression.

Chapter 2: Identification of Differential Expression of Hepatic Genes with Embryonic Exposure to an Environmentally Relevant PCB Mixture Using Microarray Technology

Abstract

Biomarkers of exposure and effect provide valuable indices of potential damage to an organism or population exposed to environmental contaminants. Wild populations are often exposed to complex mixtures of compounds, the response to which is further complicated by life-stage and species differences in sensitivity. The upper Hudson River (UHR) was contaminated with PCB Aroclor mixtures from the 1940s until the late 1970s. Several well-established biomarkers, such as the induction of hepatic cytochrome P450 monooxygenases, have been used to measure exposure to PCBs and similar contaminants. In this study, Japanese quail eggs were injected with a PCB mixture based on a congener profile found in spotted sandpiper eggs at UHR. Hepatic gene expression profiles were analyzed using a customized chicken cDNA microarray. Results showed potentially useful biomarkers of both exposure and effect from PCB mixture. Biorag and Ingenuity Pathway Analysis® analyses revealed differentially expressed genes including those involved in glycolysis, xenobiotic metabolism, replication, protein degradation, and tumor regulation, including cytochrome P450 1A5 (*CYP1A5*), cytochrome b5 (*CYB5*), NADH-cytochrome b5 reductase, glutathione-S-transferase (*GST*), fructose bisphosphate aldolase (*ALDOB*), glycogen phosphorylase, carbonic anhydrase, and DNA topoisomerase II. *CYP1A5, CYB5, GSTA,* and *ALDOB* exhibited differential expression with increasing dosage,

as confirmed by qPCR. Findings from this study provide an initial transcriptional profile associated with an environmentally relevant PCB mixture.

Introduction

From the 1940s until 1977, nearly 604,500 kg of polychlorinated biphenyls (PCBs) were released into the upper Hudson River (UHR) from two factories above Fort Edwards, NY (Wirgin et al., 2011), exposing a total of almost 200 miles of the river downstream from the plants to these chemicals (Hudson River Natural Resources Trustees, 2013). PCBs are a class of manmade organic molecules comprised of two phenyl rings that have as many as ten chlorine substitutions on both rings. Specific PCBs were manufactured in factories such as those at Fort Edward on the UHR for industrial use and marketed as Aroclor mixtures (Rice et al., 2003; Frame et al., 1996a; 1996b; Brown et al., 1987) and were disposed into waterways without regulation or mitigation until 1979 when they were banned from manufacture in the United States (USEPA, 2010; Bremer, 1983). However due to the very stable and persistent nature of these compounds, they continue their legacy of contamination in biota to the present day (Johnson et al., 2000).

Physiological effects of dioxin and dioxin-like compounds (DLCs) include mortality, cancer, liver damage, decreased reproduction, low birth weight, neurotoxicity, immunotoxicity, and developmental abnormalities and delays (Fernie and Bortolotti, 2003; Fernie et al., 2003b; Giesy et al., 1994; Gilbertson et al., 1991; Grasman et al., 1996; 1998; Hoffman et al., 1998; Lavoie and Grasman 2007). Biochemical effects include neuroendocrine, sex steroid, thyroidal, and

glucocorticoid changes, and activation of the aryl hydrocarbon receptor (AhR) and its downstream enzyme products including several cytochrome P450 (CYP450) enzymes that are responsible for xenobiotic metabolism (Brunstrom and Halldin, 1998; Elliott et al., 1990; Manning et al., 2013; Melancon, 2003; Rattner et al., 1994; 1996; 1997). AhR-endocrine crosstalk such as dimerization with the estrogen receptor to upregulate estrogen-responsive genes as well as ubiquitination and proteolysis of the estrogen receptor (Ohtake et al., 2006) is also observed along with an increase in oxidative stress. Some PCB congeners have more deleterious effects than others based on their chlorination pattern. The less ortho-substituted congeners allow the two phenyl rings to lie planar to each other and therefore behave in a dioxin-like manner by binding the AhR (Hestermann et al., 2000). The ability of a compound to bind in this way gives it a high toxicity equivalency quotient (TEQ), which has been held as the standard for toxicity comparison across compounds, where tetrachlorodibenzo-p-dioxin (TCDD) is given a TEQ of 1. As reported by the World Health Organization, PCB 126 has a TEQ of 0.1 while PCB 77 has an avian TEQ of 0.05 (Van den Berg et al., 1998; 2006; reviewed in El-Shahawi et al., 2010). Knerr and Schrenk (2006) report a mammalian TEQ of 0.0001 for PCB 77, lending evidence to the possibility that birds are more susceptible to this congener than mammals. Both of these congeners are non-ortho-substituted, making them very planar and therefore hypothetically able to activate the AhR. However, different PCB congeners have varying levels of toxicity, and are typically found as mixtures in the environment, as is the case at UHR (Frame et al., 1996a; 1996b; reviewed in Dean et al., unpublished).

Mixtures of manmade compounds that are released into the environment and cause toxic effects in wildlife pose a challenge for toxicological investigations, since the different compounds will be chemically altered through metabolism as they move up the food chain (Brown et al., 1984). Therefore, it is necessary to determine the effects of each compound or of each kind of compound. Due to varying degrees of toxicity of PCB congeners, each newly discovered environmental mixture must be treated as a unique compound. In this study, we addressed the particularly complex 58-congener mixture found in spotted sandpiper (SPSA) eggs at UHR (Custer et al., 2010 and investigated the effect of this mixture on hepatic gene expression in the Japanese quail (*Coturnix coturnix japonica*) laboratory model. The expression of a number of genes are linked to well-established biomarkers of toxicological exposure, such as in the case of the cytochrome P450 1A4 (*CYP1A4*) gene, which correlates with the activity of its protein product, ethoxyresorufin-O-deethylase (EROD, Head and Kennedy, 2007; Herve et al., 2010). Cytochrome P450 1A5 (*CYP1A5*) is related to *CYP1A4*. However, there are also transcriptional effects that result from oxidative damage and endocrine changes. Therefore, this study utilized a high-throughput transcriptional approach to identify novel genomic biomarkers of exposure to the SPSA mixture. Genes that were differentially expressed with increasing doses of the mixture were clustered into self-organizing maps (SOMs), followed by Biorag pathway analysis and Ingenuity pathway analysis (IPA). Quantitative real-time PCR (qPCR) of genes of interest from the initial microarray analysis was performed for confirmation of microarray results.

The Del-Mar 14K Chicken Integrated System microarray encompasses a number of organs and biological systems including the neuroendocrine system (5929 genes), fat (4800), liver (2635), muscle (2398), the reproductive tract (2008), as well as quality control genes (64; Cogburn et al., 2004). The array has been validated for six species including chicken (*Gallus gallus*), Japanese quail, turkey (*Meleagris gallopavo)*, mallard duck (*Anas platyrhynchos)*, tree swallow (*Tachycineta bicolor*), and American kestrel (*Falco sparverius*), showing that 90% of the genes on the array were detected in quail, indicating that the chicken cDNA microarray canbe used to effectively profile mRNA levels in quail samples (Cogburn and Porter, unpublished data).

Methods and Materials

Compound Doses, Egg Injections, and Incubation

The 58-congener PCB mixture doses were formulated by Dr. Don Tillitt's lab at USGS Columbia Environmental Research Center (Columbia, MO). The stock solution of 246 µg PCBs/µL was prepared as an emulsion in a 0.75% corn oil: propylene glycol solvent. This solution was then diluted serially into 123, 62, 31, 15, and 8 µg PCBs/µL. An egg weight of 10g was assumed, and 0.4 µL solution/g egg was injected. Therefore, the amounts of PCB mixture delivered to the egg via air cell injections were 98, 49, 24, 12, 6, 3, and 0 µg/g egg. Doses were selected to encompass environmental concentrations of the mixture, which were found to be 9.128 ug/g egg (Custer et al., 2010). Untreated and vehicle control groups were also included for comparison. Sample sizes are reported in Table 1.

Quail eggs were collected from the University of Maryland colony and stored at 4ºC until incubation. Eggs were incubated at 37ºC and 50-60% humidity. Air cell injections took place on embryonic day (ED) 3, which is equivalent to ED 4 in chicken (McKernan et al., 2010). Eggs were randomly assigned to treatment groups, including one untreated group (no vehicle, no sham) and one vehicle control group (0.75% corn oil:propylene glycol). Eggs were staged during the course of incubation; infertile and dead eggs were removed from the study.

Tissue Collection and Storage

Animals used for this study were hatched and sacrificed under the University of Maryland Institutional Animal Care and Use protocol R-04-57. Hatchlings were sacrificed within 24 hours of hatch by decapitation, and livers were harvested, immediately snap frozen in liquid nitrogen, and stored at -80ºC until RNA extraction.

RNA Extraction

Total RNA was extracted from the liver using the RNeasy Midi Kit (Qiagen, Valencia, CA) according to the manufacturer's protocol. The protocol was modified to use a 50% ethanol solution instead of 70% for RNA binding to the column, according to a modification made by Head and Kennedy (2007). RNA was quantified by measuring absorbance at 260 nm with reference at 280 nm; sample quality was assessed with 1X 3-[N-morpholino]propanesulfonic acid (MOPS)/3% formaldehyde/1% agarose gel electrophoresis.

Microarray Hybridization and Data Analysis

Female samples were hybridized to the Del-Mar 14K Chicken Integrated Systems microarray with a reference RNA hybridization design using a Cy3/Cy5 system as previously described (Porter and Ellestad, 2005). Each individual sample was labeled with Cy3, while a pool of all samples was labeled with Cy5. The reference sample consisted of a pool of all individual samples (n=16) and was applied to each array along with an individual sample. Microarray hybridization was performed at University of Maryland's Biotechnology Institute Microarray Core Facility (Rockville, MD). Micorarray data were processed and normalized using freely available software from the TM4 suite of microarray data analysis applications (Saeed et al., 2003) offered by The Institute for Genomic Research (TIGR, Rockville, MD). The two images from each slide were aligned using SpotFinder version 2.2.4. Numeric data were exported for normalization to Microarray Data Analysis system (MIDAS, version 2.18). Data from the Cy3 channel, which represent individual experimental samples, were Lowess normalized without background correction, followed by standard deviation regularization by slide, with Cy5 as the reference.

The spot mean was calculated by dividing spot intensity by spot area. This value was then corrected for background by subtracting the mean of 8 salmon DNA spots on the array from the spot average. If the corrected average was <0, the spot was removed from the analysis for that sample. The spot average was then normalized by subtracting log of Cy5-labelled reference sample from log of Cy3-labelled individual samples. This produced a log ratio for each spot, which was submitted to a one-way analysis of variance (ANOVA) analysis using SAS software.

The means from each PCB dose for differentially expressed spots ($p<0.05$, $n>7$) were organized into 12 clusters [cluster 0 (c0) to cluster 15 (c15)] SOMS analysis using GeneCluster version 2.0 (Reich et al., 2004) developed by the Broad Institute (Cambridge, MA) and freely available on their website (http://www.broad.mit.edu/cancer/software/genecluster2/gc2). There was little redundancy between clusters, therefore a 4x3 grid was chosen for ease of reporting.

Annotated genes were submitted to a pathway analysis using BioRag (Bioresource for array genes) at www.biorag.org. Ingenuity Pathway Analysis (IPA, Ingenuity® Systems version 9.0) was used to perform a Core, Network, and Toxicology analysis. The software's algorithms identified biological function or pathways that were most significant to the dataset. Genes from the dataset that were recognized as human, mouse, or rat orthologs and met a p-value cut-off of 0.05 were used in the analysis.

Quantitative PCR

Reverse Transcription

First strand reverse transcription was performed using 1 µg of RNA and SuperScript III (Invitrogen), oligo(dT)12-18 primers (Invitrogen), and random hexamers (Life Technologies), using the protocol for the SuperScript III with the addition of a 5 minutes, 25°C incubation for the random hexamers.

Primer Design and PCR Optimization

Primers for qPCR were designed against the chicken genome using Primer3 web-based software, and were designed as far 3` as possible and spanning an intron. Optimal annealing temperature and primer concentration were assessed. A serial

dilution using a pool of all experimental samples was run with each primer set in order to calculate efficiency, which was acceptable at 0.95-1.05 for all genes. If any of the above measures failed (i.e. no product or too high or too low efficiency), the primers were redesigned. Gel bands of the PCR products were extracted and sequenced. Sequences were aligned to the chicken genome using BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi; see Appendix 2,Table 1 for primer sequences).

PCR Assay Conditions

Only male samples were were analyzed by qPCR due to inadequate amounts of RNA from female samples. Reactions were run in a 96-well nuclease-free qPCR plate with final concentration of 1X Bio-rad 2X SYBR Supermix, 800 nM of each primer set, 1 µL cDNA template, and water, for a total volume of 15 µL. PCR parameters consisted of 95°C for 3 minutes; 40 cycles of 95°C for 15 seconds, primer-specific annealing temperature for 30 seconds, and 72°C for 30 seconds; a melt curve starting at 55°C and stopping at 95°C, moving 0.5°C every 10 seconds. A No-RT and a No-Template sample were run in each PCR plate as negative controls.

Calculation of Expression Values and Statistical Analysis

Samples were run in triplicate, and a CV value of 1-2% was used to determine the precision of the Cts measured for the replicates of each sample. Genex (MultiD) was used to calculate log-2 transformed relative quantities. Cts of the replicates for each sample were first corrected for efficiency as calculated by the standard curve, then averaged together for each sample. Averaged Cts were then normalized to beta actin (*ACTB*) and glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*). Finally,

quantities relative to the untreated samples were calculated and log-transformed. It was verified that all samples fell within the validated range of dilutions used to determine efficiency. If they did not, they were removed from the analysis for that gene. Quantities were analyzed in SAS® a non-parametric one-way ANOVA followed by Wilcoxon post-hoc test in order to test differences between doses.

Results

Differential Gene Expression Based on Microarray Analysis

In female samples, microarray hybridization yielded 285 spots that showed differential expression levels across treatment groups ($p<0.05$). 159 of those spots yielded values for at least 8 of the 16 samples analyzed (Table 2), which were carried to further analysis.

Cluster Analysis

A cluster analysis created 12 self-organizing maps (SOMs) from 159 spots (Figure 1). Cluster 0 contained 5 spots, 4 of which aligned to fructose bisphosphate aldolase B (*ALDOB*), the fifth of which did not BLAST to anything. Of the 12 SOMs, Cluster 6 showed a canonically positive dose-response curve, while Clusters 8 and 9 showed canonically negative dose-response curves. Other SOMs were difficult to interpret in their expression patterns.

Pathway Analysis

A pathway analysis was performed using BioRag Pathway Miner to relate genes biochemically, and a table categorizing genes in larger biological functions was

generated (Table 3). The order of biological categories from the largest to the smallest number of genes were Cell Communication, Adhesion, Transport, and Signaling (15 genes); Glycolysis, Gluconeogenesis, and Energy Balance (10 genes); Replication, Mitosis, Protein Synthesis, Gene Regulation, and Cell Cycle (9 genes); Stress and Immunity (7 genes); Protein Degradation and Proteasome Complex (6 genes); Xenobiotic Metabolism (4 genes); and DNA Damage/Repair and Tumor Regulation (2 genes).

The number of genes that were up-regulated compared to controls increased with PCB dose, while the number of genes that were down-regulated compared to controls decreased with dose (Figure 2). This revealed a positive dose-response in up-regulated genes and a negative dose-response in down-regulated genes using a logistic regression ($p=0.002$).

Ingenuity Pathway Analysis

A core analysis and biomarker finder analysis were performed on the 159 spots using IPA (Ingenuity® Systems). The top five networks that were identified in this analysis included the Endocrine System Development and Function, Small Molecule Biochemistry, Lipid Metabolism network (Table 4). This network contained 29 of the 159 genes submitted to the analysis (Figure 3). The top toxicology lists included Genes Down-Regulated In Response To Chronic Renal Failure, Aryl Hydrocarbon Receptor Signaling, Cytochrome P450 Panel, and PXR/RXR Activation, all of which have biological significance for PCB exposure (Table 5).

PCR Confirmation

In male samples, four genes were evaluated by quantitative PCR (qPCR; Figure 4A-D, Table 6): cytochrome P450 1A5 (*CYP1A5*), fructose bisphosphate aldolase B (*ALDOB*), cytochrome b5 (*CYB5*), and glutathione S-transferase alpha (*GSTA*). These genes were chosen based on cluster analysis placement, pathway category of interest, maximum fold-difference seen on the array, and interest in them based on prior knowledge. Due to heteroscedastic data, a Wolcoxon non-parametric one-way analysis was run in SAS® using the NPAR1WAY procedure. All genes had significant differences across doses ($p<0.05$) as follows. Response of CYP1A5 was significant between the high and all other doses except the low dose (Figure 4A); ALDOB response was significant between the untreated and high doses (Figure 4B); CYB5 response was significant between the untreated and high doses (Figure 4C); GSTA response was significant between the medium and high doses (Figure 4D).

Maximum difference between doses was 28-fold between high and vehicle doses for CYP1A5; 2.5-fold difference between the medium and vehicle doses for ALDOB; 7.1-fold difference between vehicle and untreated doses for CYB5; 0.1-fold difference between high and vehicle doses for GSTA (Figure 5; Table 6). These expression levels were compared to the maximal expression levels seen on the array (Table 6); it was found that while the expression levels were proportional in qPCR data as compared to the microarray data for the same gene, the response level was greater in the qPCR data, further demonstrating that qPCR is a much more sensitive technology for detecting changes in gene expression. It was also found that of the genes tested, CYP1A5, GSTA, and CYB5 were three of the four genes that were

placed in the xenobiotic metabolism pathway category (Table 2), and all three were placed in Cluster 11. The fourth xenobiotic metabolism gene, NADH-b5 reductase, was placed in Cluster 6.

Discussion

Here we have used a customized multi-system cDNA microarray to assess global hepatic gene expression in Japanese quail hatchlings exposed to environmentally relevant concentrations of an environmentally relevant PCB mixture. Pathways with toxicological implications such as xenobiotic metabolism, stress, immunity, AhR activation, endocrine system development and function, and the cytochrome P450 panel were affected by PCB exposure. This is consistent with previous studies that have shown that biochemical effects of PCBs include activation of the AhR and subsequent induction of downstream Phase I detoxifying enzymes (EROD), increase in antioxidant enzymes (glutathione reductase), and impacts on the endocrine system such as a decrease in thyroid levels and neuroendocrine effects (Brunström and Halldin, 1998; Cesh et al., 2010; Elliot et al., 1990; Letcher et al., 2010; Manning et al., 2013; Melancon, 2003; Ottinger et al., 2013; 2009a; 2009b; Rattner et al., 1994; 1996; 1997; Schlezinger et al., 2000).

This study also focused on novel pathways of toxicity with exposure to PCBs. Many of the pathways affected are important for proper organ function regardless of the presence of a stressor. The non-canonical pathways found in this study include the growth phase 2-to-mitosis (G2/M) DNA damage checkpoint, cell cycle, communication, signaling, growth, and proliferation; DNA replication; mitosis;

protein synthesis and degradation; gene regulation; and glycolysis, gluconeogenesis, and lipid metabolism. Previous work has shown that pathways of cell communication, metabolism of lipids, fatty acids, and steroids, cell cycle, and cell proliferation and differentiation are affected in response to PCB 126 (Faust et al., 2013). The results here showing that pathways involved with cell growth and proliferation as well as the cell cycle are consistent with previous findings that PCBs have also been shown to cause delayed growth and development as well as DNA damage.

Our results indicated that PCB exposure was limiting the animal's ability to express those genes, potentially resulting in systemic failure. Overall gene expression increased with PCB dose, indicating that the animal's ability to mount a response remains intact with increasing doses of the mixture, while the mixture was minimally detrimental to the system. However, it is important to note that there was high mortality in the two highest doses with only two survivors of each sex in each of those doses. This suggests a survivor effect and the presence of genetic differences among animals that permit individuals to survive high dose exposures.

With the exception of the four genes involved with xenobiotic metabolism, genes did not cluster with their biological function as revealed by the Biorag pathway analysis (Supplemental Figure 2). Xenobiotic metabolism is an important hepatic function and one that would be highlighted in a study of PCB exposure. Finding only four genes involved in xenobiotic metabolism that were detected by the array might be a function of other genes not being on the array, or that microarray technology is not sensitive enough to detect changes in other genes in this pathway.

The mixture in this study was composed of 58 PCB congeners, including several non-ortho-substituted that have a planar conformation. The biological activities of most of these congeners may not directly affect hepatic gene expression, especially via traditional AhR activation. As such, the composition of this mixture makes the biological responses of hepatic gene expression unpredictable. Because the responses of most genes analyzed by the cluster analysis were not monotonic, it is reasonable to conclude that the composition and dose of the compound will greatly affect the trend of each response, as each congener may have differing effects on individual responses. It should be noted that there was an overall increase in gene expression with dose, indicating that exposure to an increasing dose of this PCB mixture will cause an increase in biochemical activity. These data taken together indicate that the liver experiences a dramatic activity increase in normal functions when the organism is exposed to this particular PCB mixture.

Expression levels of genes tested with qPCR did not mimic microarray expression values or patterns. Since females were used for the array and males were used for qPCR, sex difference accounts for a large part of this discrepancy. Normally, the same samples would be used in both qPCR and the microarray assays, however due to a technical complication, there was not enough remaining sample to run qPCR. Therefore, the decision was made to test male samples for qPCR. While there were no significant differences between untreated and vehicle treated samples in the qPCR assay, there is a possible vehicle effect in all of the genes except CYP1A4 due to the fact that the response seen in the vehicle-dosed group was greater

than in the untreated group (Figure 4). This indicates that the vehicle used should be carefully evaluated in the context of treatment comparisons.

Fold changes were calculated between each PCB dose and the vehicle, and it is clear that CYP1A5 is the most responsive gene (Figure 6). CYB5 and ALDOB were responsive at lower doses. GSTA exhibited the least amount of change. However, the data exhibited high heteroscedasticity, which allowed for few significant differences to be found. Therefore, this study was underpowered for differences in response to PCBs. However, it should be noted that qPCR for all genes exhibited higher sensitivity to expression changes than did the microarray (Table 5). Despite the lack of sufficient sample sizes, a clear trend of differential gene expression was still seen with qPCR analysis.

Due to the complexity as well as the spatial and temporal specificity of this mixture, there is a dearth of data to which to compare these results. While several studies have shown effects caused by specific industrially produced Aroclor mixtures, the biotic metabolism of these compounds produces metabolites of unknown biological activity, the effects of which are not taken into account in these studies. Furthermore, we recognize that this mixture is temporally specific because it is constantly being metabolized further and further. Therefore, each wild animal, each generation, and the biota within each area within the region of concern will experience a slightly different compound.

Taken together, these results corroborate previous findings that PCBs activate traditional pathways such as AhR activation and cytochrome P450 induction as well as endocrine pathways; they also point to new pathways of interest, such as energy

metabolism as well as cell function and cycle. Further study of the expression profiles of these genes is needed to assess risk and to identify biomarkers for investigating mechanisms of action.

Figures

Table 1: Numbers of eggs dosed for each dose as well as numbers of samples used in the microarray and qPCR experiments. Female samples were used in the microarray study, and male samples were used in the qPCR study. These numbers represent survival except in the case of 3 µg/g egg, in which they were removed from the study due to lack of enough microarray slides.

Dose	Total Eggs Dosed	Microarray Study	qPCR Study
Untreated	13	0	6
Vehicle	22	7	4
3	10	0	0
6	16	5	2
12	23	2	3
24	?	0	0
49	20	2	7
98	?	0	0

Table 2: Global gene expression changes that occurred upon exposure to a PCB congener mixture in quail hatchlings on the Integrated Systems 14K Del_Mar Chicken Array. Microarrays were hybridized with a Cy3-labelled sample and a Cy5-labelled reference sample. Each microarray image was aligned to a grid using SpotFinder® software and the spot mean was obtained by dividing spot intensity by spot area. Background was eliminated by subtracting the mean of 8 salmon DNA spots from the spot mean. This value was normalized by subtracting the lob of the reference sample from the log of the individual samples. The log ratio was submitted to a one-way analysis of variance with SAS®.

Category	Number of Spots
Total Spots on the array	19,200
Spots with chicken cDNA	17,834
Significant Spots ($p<0.05$)	285
Significant Spots ($p<0.05$, $n > 7$)	159
Unique Genes Represented	156
Self-Organizing Maps	12

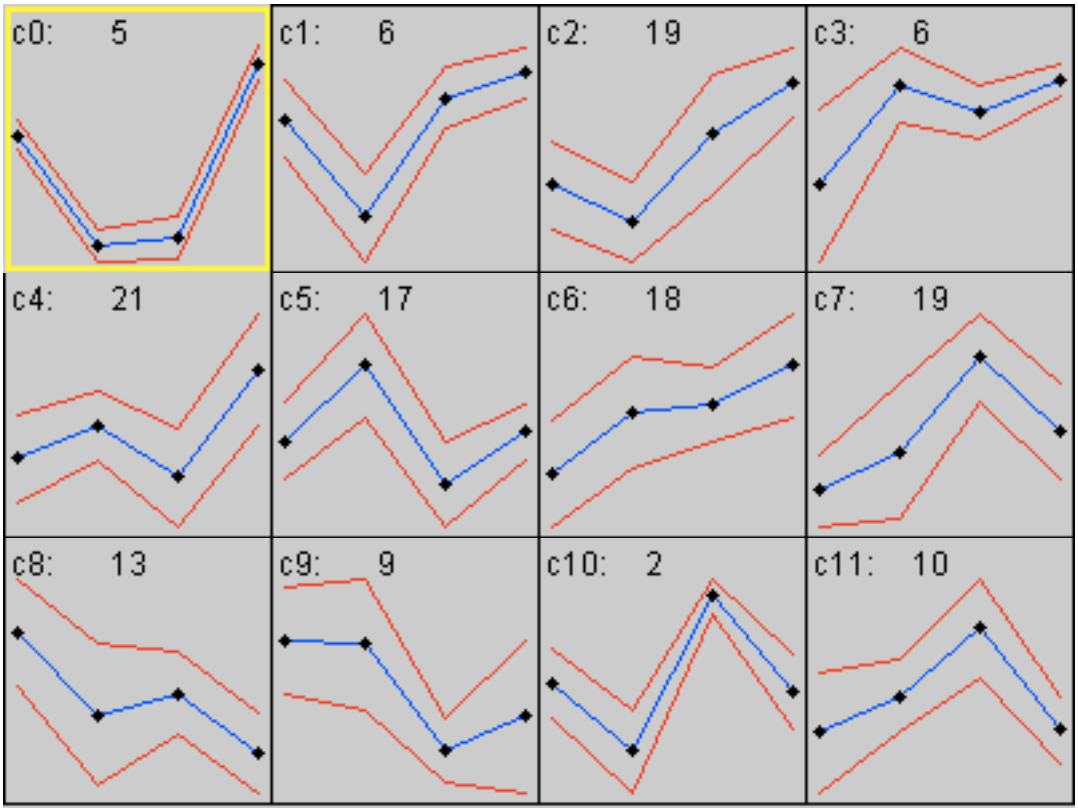


Figure 1: 12 SOMs of 159 differentially expressed spots ($p<0.05$, $n>7$) were generated performing a cluster analysis with GeneCluster version 2.0. Gene expression levels are relative. In each SOM, the points from left to right represent control, low, medium, and high doses, respectively. The number in the top center of each SOM is the number of genes in that cluster. Blue lines represent average change between two treatments, and red lines represent standard errors.

Table 3: Pathway analysis of 40 annotated spots using Biorag Pathway Miner. Genes were further categorized into seven broad categories of pathways.

Pathway Category	# Genes
Protein Degradation and Proteosome Complex	6
Stress and Immunity	7
DNA Damage/Repair and Tumor Regulation	2
Replication, Mitosis, Protein Synthesis, Gene Regulation, and Cell Cycle	9
Cell Communication, Adhesion, Transport, and Signaling	15
Xenobiotic Metabolism	4
Glycolysis, Gluconeogenesis, and Energy Balance	10

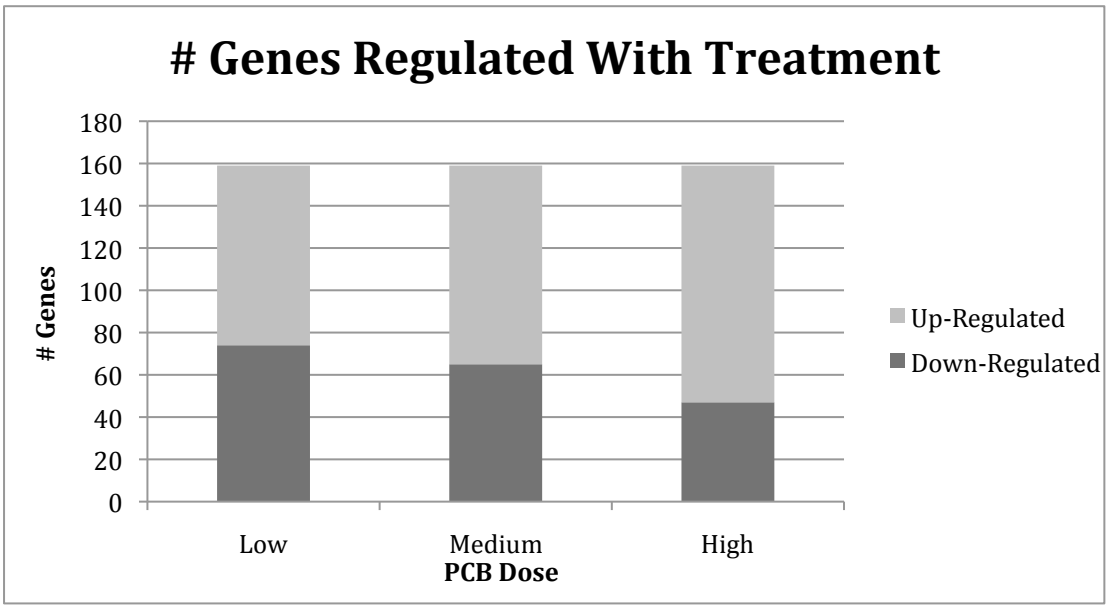


Figure 2: The number of genes that were up- and down-regulated in each treatment group compared to the controls were significantly different across treatment groups using a logistic regression (p=0.002).

Table 4: A core analysis in Ingenuity Pathway Analysis (IPA) identified top networks affected by PCB exposure.

Associated Network Functions	Score
Endocrine System Development and Function, Small Molecule Biochemistry, Lipid Metabolism	53
Cellular Growth and Proliferation, Nervous System Development and Function, Tissue Morphology	44
Cellular Assembly and Organization, Cellular Development, Cellular Growth and Proliferation	29
Hereditary Disorder, Neurological Disease, Cell Cycle	28
Cell-mediated Immune Response, Cellular Movement, Hematological System Development and Function	27

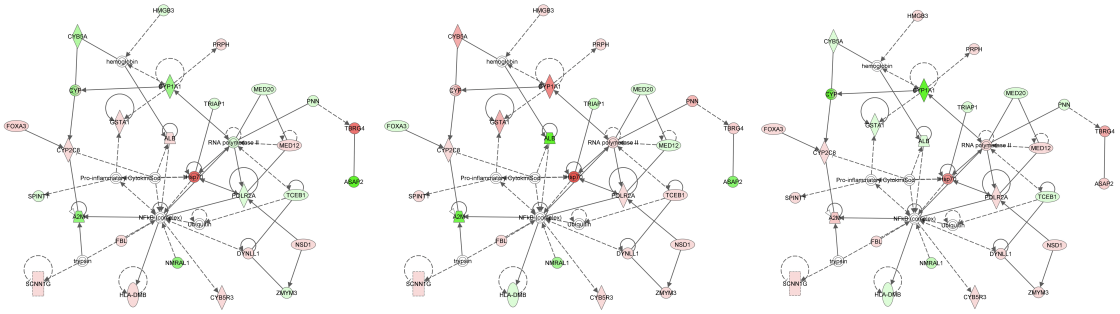


Figure 3: The Endocrine System Development and Function, Small Molecule Biochemistry, Lipid Metabolism network included 29 genes from the 159 annotated spots that were significantly expressed on the array, using Ingenuity Pathway Analysis (IPA). This network includes several genes of interest, including cytochrome P450 1A5 (CYP1A5), cytochrome b5 (CYB5), and glutathione S-transferase (GSTA).

Table 5: A toxicology analysis identified top tox lists in IPA.

Name	p-value	Ratio
Genes Downregulated in Response to Chronic Renal Failure (Rat)	2.69E-03	2/10 (0.2)
Cell Cycle: G2/M DNA Damage Checkpoint Regulation	6.11E-03	3/47 (0.064)
Aryl Hydrocarbon Receptor Signaling	8.47E-03	5/158 (0.032)
Cytochrome P450 Panel - Substrate is a Xenobiotic (Human)	9.75E-03	2/19 (0.105)
PXR/RXR Activation	1.61E-02	3/67 (0.045)

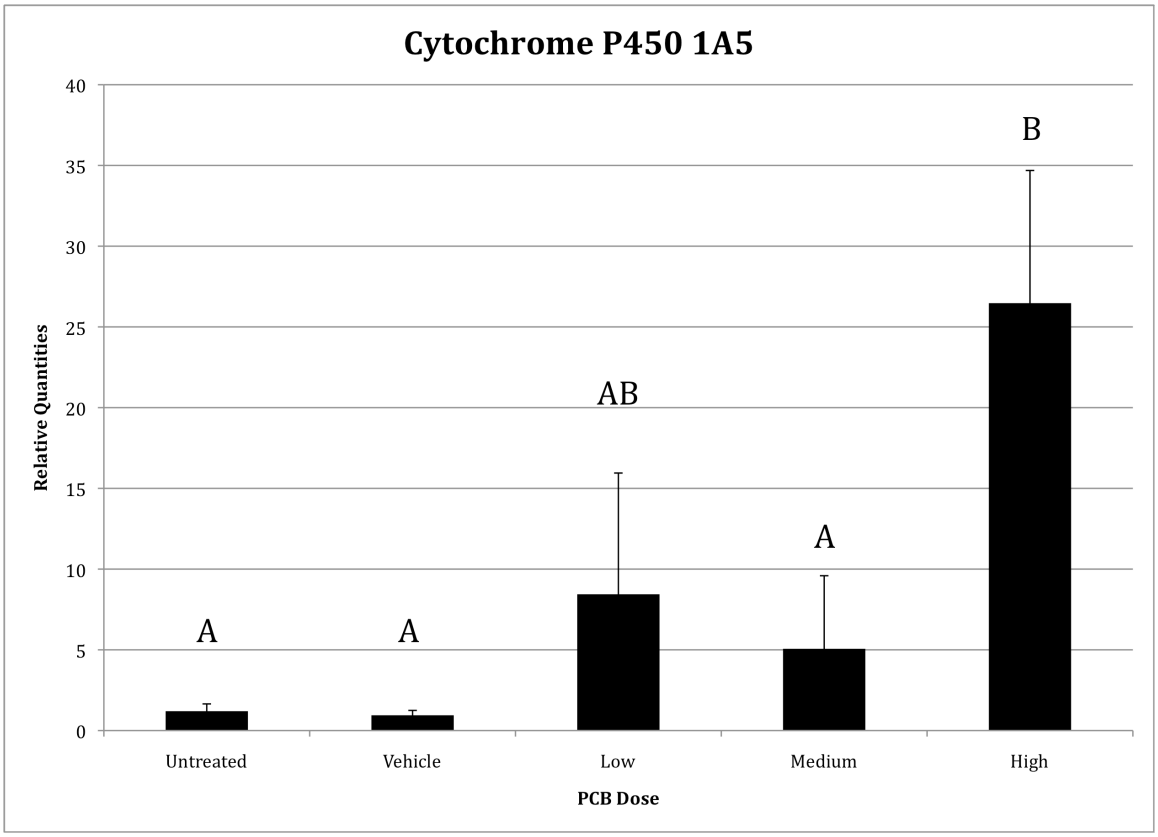


Figure 4A: Quantities relative to the untreated group (set at 1 or close to 1) of cytochrome P450 1A5 (CYP1A5) measured in hatchling males via qPCR and calculated using GENEX software. Differential expression was detected at the high dose compared to untreated, vehicle, and medium-dosed birds ($p<0.05$) using a Wilcoxon nonparametric one-way analysis.

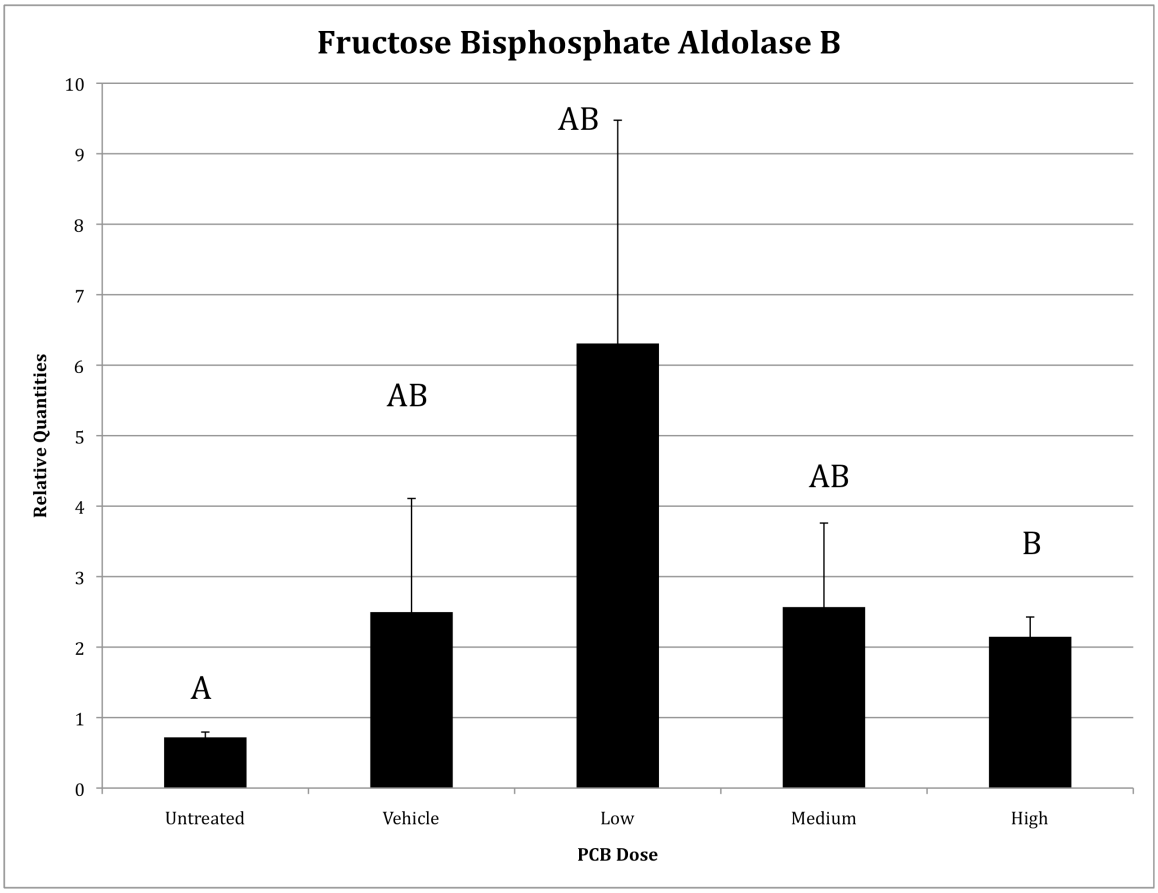


Figure 4B: Quantities relative to the untreated group (set at 1 or close to 1) of fructose bisphosphate aldolase B (ALDOB) measured in hatchling males via qPCR and calculated using GENEX software. The low-dose increase was not significant ($p \geq 0.05$). Significance was seen between untreated and high-dosed birds ($p < 0.05$) using a Wilcoxon nonparametric one-way analysis.

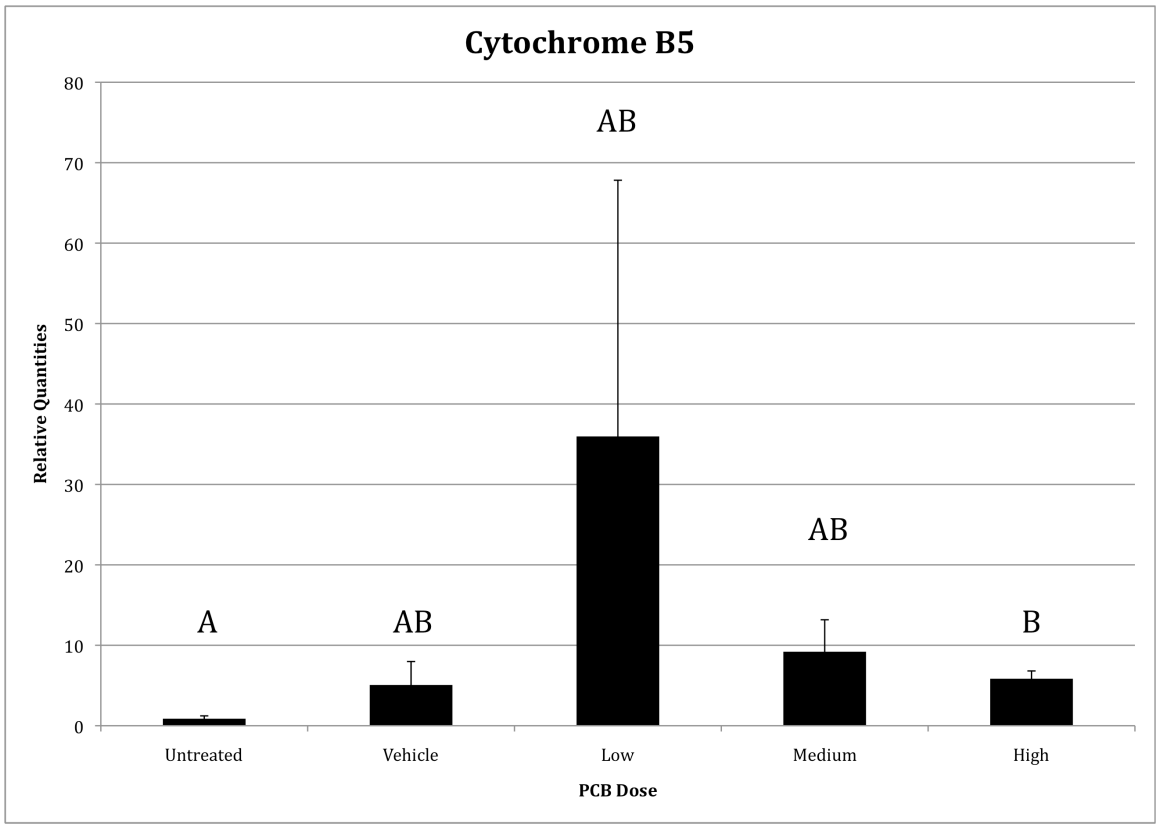


Figure 4C: Quantities relative to the untreated group (set at 1 or close to 1) of cytochrome B5 (CYB5) measured in hatchling males via qPCR and calculated using GENEX software. Differential expression was detected between untreated and medium-dosed birds as well as between untreated and high-dosed birds ($p<0.05$) using a Wilcoxon nonparametric one-way analysis.

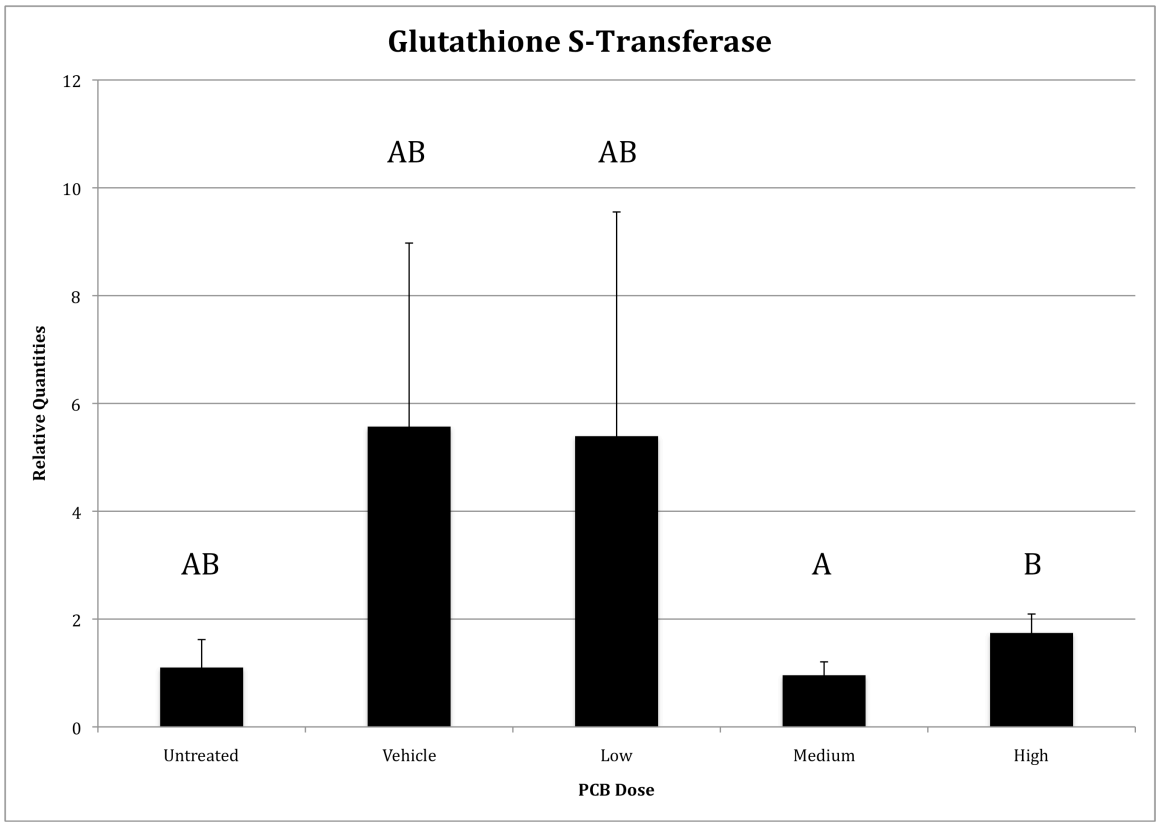


Figure 4D: Quantities relative to the untreated group (set at 1 or close to 1) of glutathione S-transferase (GSTA) measured in hatchling males via qPCR and calculated using GENEX software. Although a dramatic increase in vehicle and low-dose treated birds was observed compared to the untreated group, this difference was not significant ($p \geq 0.05$). Significance was seen between the medium and high doses ($p < 0.05$).

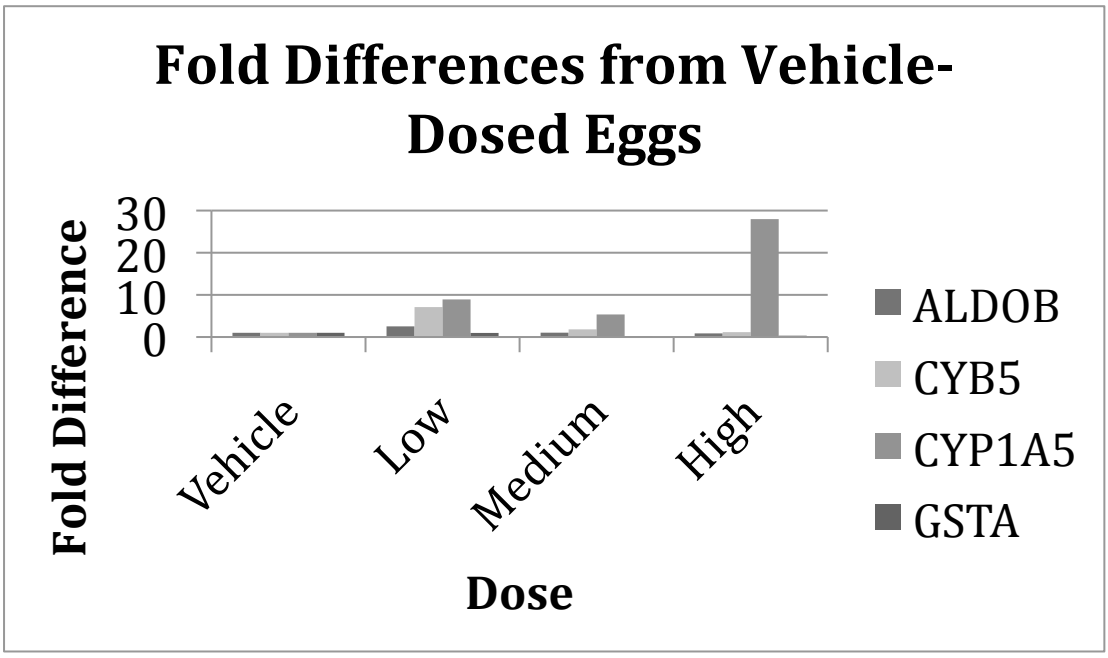


Figure 5: Fold differences of PCB-dosed eggs from vehicle-dosed eggs showed CYP1A5 to be the most responsive gene measured by qPCR.

Table 6: Four genes were used to confirm microarray results. Comparison of the two technologies (array vs. qPCR) reveals proportional levels of change. All genes used in qPCR were differentially expressed with PCB exposure.

Gene Name	Pathway Category of Interest	Cluster	Max Fold Diff seen on Array	Max Fold Diff seen on qPCR
Cytochrome P450 1A5	Xenobiotic Metabolism	11	2.4	28
Fructose bisphosphate aldolase B	Glycolysis	0	1.1	2.5
Cytochrome b5	Xenobiotic Metabolism	11	0.92	7.1
Glutathione S-Transferase alpha	Xenobiotic Metabolism	11	0.62	0.1

Chapter 3: Hepatic Gene Expression and Downstream Biomarkers of Biochemical Pathways Affected by Exposure of Two Environmentally Relevant PCB Mixtures

Abstract

Previous work considering global gene expression revealed several pathways affected by PCB exposure to a PCB mixture found in spotted sandpiper (*Actitis macularis*; SPSA) eggs at the Hudson River (HR) superfund site. Of the pathways affected, xenobiotic metabolism, oxidative damage, energy balance, and endocrine disruption were the focus of this current study. The purpose of this study was to evaluate sub-lethal effects of PCB exposure seen at the level of gene expression as well as in ethoxyresorufin-O-deethylase (EROD) activity in order to characterize pathways affected by PCB exposure. Japanese quail (*Coturnix japonica*) eggs from the University of Maryland colony were air-cell injected with either one of two environmental mixtures identified in SPSA and tree swallow (*Tachycineta bicolor*; TRES) eggs at HR, PCB 126 or PCB 77. Control groups included untreated, sham-injected, or vehicle-injected (charcoal-stripped corn oil) eggs. The GLM procedure in SAS software was used to evaluate the nature of the dose response curves for each sex/compound combination by testing the cubic, quadratic, and linear components of each curve. Principal component analysis was performed in order to cluster the genes together based on their expression patterns. For 13 endpoints across 8 sex/compound combinations giving a total of 104 dose-response curves, the cubic response pattern was significant 13 times, the quadratic response pattern was significant 16 times, and

the linear response was significant 10 times. Of 52 dose-response curves for each sex, males responded 21 times and females responded 18 times. Of 26 dose-response curves for each compound, there were 6 responses to PCB 126, 10 responses to PCB 77, 13 responses to the TRES mixture, and 10 responses to the SPSA mixture. Of the 13 endpoints, EROD and *cytochrome P450 1A4* (*CYP1A4*) and *cytochrome P450 1A5* (*CYP1A5*) were most responsive. Of the 8 sex/compound combinations, EROD was responsive 8 times, and *CYP1A4* as well as *CYP1A5* were responsive 7 times.

Introduction

Previous work using a microarray to screen hepatic gene expression in day-old Japanese quail (*Coturnix japonica*) hatchlings revealed several pathways that were globally affected by an environmentally relevant PCB mixture found in spotted sandpiper (*Actitis macularis*; SPSA) eggs at the Upper Hudson River (UHR; Bohannon et al., unpublished data). The affected pathways included xenobiotic metabolism; cell signaling, cycle, and communication; endocrine function; immunity and stress; and glycolysis, gluconeogensis, and energy balance. Of these, xenobiotic metabolism is a well-characterized response to xenobiotic exposure, with EROD being a widely used biomarker of that pathway (Brunström and Anderson, 1988; Brunström and Halldin, 1998; Custer et al., 2000; Head and Kennedy, 2007; Melancon et al., 2006; Rattner et al., 1993; 1994). Many polyaromatic environmental contaminants such as 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) and coplanar PCBs activate the aryl hydrocarbon receptor (AhR) which binds to a xenobiotic response element in the promotor regions of many cytochome P450

(CYP450) genes. The enzyme products of these genes will chemically alter the contaminants that served as the AhR ligand, often by hydroxylation, to render the contaminant more hydrophilic and therefore more likely to clear the body rather than be sequestered in lipid tissue (Head et al., 2008; Head and Kennedy, 2007; Hervé et al., 2010; Rattner et al., 1997; 1996; 1994; 1989; Safe, 2001; 1990, 1980). The purpose of this study is to characterize the response of AhR-mediated gene expression to two environmentally relevant PCB mixtures, as well as to investigate the responsiveness of processes that are not mediated through the AhR and are involved with other physiological processes that PCBs alter to the detriment of the organism. The processes chosen for this study included oxidative damage, energy balance, and hepatic endocrine function.

Experimental compounds included PCB 126 and PCB 77 as single-congener treatments, as well as two mixtures relevant to UHR. One mixture was based on a PCB profile from tree swallow (*Tachycineta bicolor*; TRES) eggs found at UHR (Echols et al., 2004), while the other was based on a PCB congener profile from spotted sandpiper (SPSA) eggs found at UHR (Custer et al., 2010). PCB 126 and 77 are 2 of 20 non-ortho-substituted PCB congeners, resulting in a planar molecular structure. They also have the highest individual congener TEQs for avian species, at 0.1 and 0.05 respectively, as reported by the World Health Organization (WHO; Van den Berg et al., 2005). PCB 126 is a known AhR agonist, and PCB 77 is thought to have similar activity based on its TEQ. We hypothesized that PCB 126 and PCB 77 would account for a large proportion of the toxicity of the two mixtures.

Mortality data from this study, previously reported by Dean et al. (manuscript in review), showed that PCB 126 had an LC50 of 66.6 ng/g egg, PCB 77 had an LC50 of 800 ng/egg, the SPSA mixture had an LC50 of 10.995 ng/g egg, and the TRES mixture had an LC50 of 23.728 ng/g egg (Appendix 4).

For this study, 12 genes and EROD activity were chosen for investigating sub-lethal toxicity of these compounds in the Japanese quail laboratory model. This is important because it is hypothesized that while these mixtures are not lethal to all birds at the UHR site, they will take an additional physiological toll that will affect larger processes such as reproduction, homeostasis, and overall health. The xenobiotic metabolism pathway genes assessed are cytochrome P450 1A4 (*CYP1A4*), cytochrome P450 1A5 (*CYP1A5*), cytochrome b5 (*CYB5*), and aryl hydrocarbon receptor 1 (*AHR1*); the oxidative damage pathway genes were catalase (*CAT*), glutathione peroxidase (*GPX*), glutathione reductase (*GR*), glutathione S-transferase (*GSTA*), and thioredoxin (*TXN*); fructose bisphosphate aldolase B (*ALDOB*) was chosen for energy balance, deiodinase type 2 was chosen for endocrine disruption, and HMG-CoA synthase (*HCS*) was chosen for both energy balance and endocrine disruption. Ethoxyresorufin-O-deethylase (EROD) is the enzyme coded for by *CYP1A4*. It is a commonly used endpoint in toxicity studies (Melancon et al., 2006).

CYP1A4 and *CYP1A5* are directly upregulated by the AhR, however there is no evidence that any of the other genes tested in this study are directly upregulated by xenobiotic compounds in the ligand-dependent manner of the CYP450s. The mechanism of action of PCBs on the expression level of these genes is thought to be through an indirect mechanism as part of the organism's attempt to respond to a

stressor (Van den Berg et al., 1998; 2006). This is especially true of the genes involved in the repair of oxidative damage, as xenobiotic exposure increases the body's production of reactive oxygen species. GSTA is a Phase II detoxification enzyme and therefore induction has implications in the xenobiotic metabolism pathway (Jancova et al., 2010). However it is not mediated through the AhR (Scandalios, 2005; Bak et al., 2011). Therefore we hypothesize that the body increases production of antioxidant enzymes in an attempt to combat oxidative damage. However, biological responses will often decrease dramatically with higher and higher doses as the stressor effectively overwhelms homeostatic mechanisms (Calabrese and Baldwin, 2001). Therefore we predicted an increase in response at low doses and a dramatic decrease dipping below basal (i.e. control) levels at higher doses.

We hypothesized that genes involved with energy balance, namely *ALDOB* and *HCS*, would experience a similar expression pattern to that of the antioxidant enzymes, based on the idea that a small challenge would cause an uptick in basal metabolic rate in an attempt to fight off the adverse effects of the stressor. Both of these genes were differentially expressed on a microarray platform (Bohannon et al., unpublished data).

PCBs are known endocrine disruptors. The hepatic role of *DIO2* is to metabolize thyroid hormones. PCBs are known to lower circulating T4 and T3, therefore it is hypothesized that *DIO2* would be lower as well. HCS is involved with cholesterol production, ketone formation, and insulin processing. Therefore it also has implications for endocrine function in the body.

Methods and Materials

Animals, Egg Injections, Dosing, Incubation, and Tissue Collection

Japanese quail eggs were collected daily from the University of Maryland in-house colony of 240 breeding pairs. Animal housing and care were conducted under Institutional Animal Care and Use Committee approved protocols R-07-59 (Japanese quail breeding protocol) and R-08-02 (Toxicity of *in ovo* exposure to organochlorines in Japanese quail). Eggs were refrigerated until ready for injection on embryonic day (ED) 0. Eggs were randomly assigned to an experimental or control group, for a total of 30 eggs per treatment group (Dean et al., in review).

Four treatments included: PCB 126 (3,3',4,4',5-pentachlorobiphenyl), PCB 77 (3,3',4,4'-tetrachlorobiphenyl), and two PCB congener mixtures based on measured PCBs in spotted sandpiper eggs (Custer et al., 2004) and tree swallow eggs (Echols et al., 2004), respectively at the Upper Hudson River Remnant 3 NRDA superfund site. Dosing solutions were purchased from AccuStandard (New Haven, CT) and prepared in charcoal-stripped corn oil by Dr. D. Tillitt (Columbia Environmental Research Center) to deliver 0.1 μL/g egg. Eggs were weighed 24 hours prior to injection and also on the day of injection to ensure proper moisture loss and to calculate injection volume. Table 1 shows treatment doses. Table 2 shows sample sizes for gene expression.

Table 1: Treatment doses for four PCB compounds.

Dose ID	Treatment ID			
	LA	**LB**	**LC**	**LD**
	PCB 126 (ng/g)	**PCB 77 (ng/g)**	**PCB TRES* mix (µg/g)**	**PCB SPSA* mix (µg/g)**
UNT	untreated			
SHAM	sham			
VEH	vehicle (charcoal stripped corn oil)			
6	2500	800	120	120
5	1000	400	60	60
4	400	200	30	30
3	160	100	15	15
2	64	50	7.5	7.5
1	25.6	25	3.75	3.75

** TRES: tree swallow mixture; SPSA: spotted sandpiper mixture*.

Eggs were incubated at 38°C and 55% humidity for 14 days in a Georgia Quail Farms incubator. Viability was monitored every three days via egg candling. Humidity was increased to 65% on ED14. At ED16, eggs were transferred to hatching chambers.

Hatchlings were checked for deformities and sacrificed within 24 hours of hatching. Livers were quickly weighed, divided into three sections, snap frozen in liquid nitrogen, and stored at -80°C.

Quanitification of Gene Expression by qPCR

RNA Extraction and Quantification

Total RNA was extracted from frozen livers using the Qiagen RNeasy Plus Mini Kit (Qiagen Inc.), according to the manual for that kit. 50% ethanol was used instead of 70% ethanol for higher RNA yield. RNA was run on a denaturing agarose gel using 1X TAE buffer with samples prepared with NorthernMax®-Gly Sample Loading Dye (Ambion®) to determine purity. RNA was further quantified using a Nanodrop reader. An additional DNAse digestion was performed on the samples that had DNA contamination using Turbo DNA-*free* (Ambion®) and re-read on the nanodrop for accurate quantities.

cDNA Synthesis

First strand reverse transcription was performed using 1 ug of RNA and SuperScript III (Invitrogen), oligo(dT)12-18 primers (Invitrogen), and random hexamers (Life Technologies), using the protocol for the SuperScript III with the addition of a 5 minutes 25°C incubation for the random hexamers.

Primer Design and qPCR Validation

Coturnix sequences were designed using Primer 3. If no coturnix sequence was available, the chicken (*Gallus gallus*) and zebra finch (*Taeniopygia guttata*) sequences were uploaded into Geneious Ver.6.16 (Biomatters) software for sequence alignment. Primers were designed if sequences had long enough stretches of homology. Degenerate primers were designed if sequences did not have enough homology. Primers were subjected to BLAST analysis to confirm target gene, and annealing temperatures were confirmed using a web-based multiple primer analyzing software (Thermo Scientific). All primers were purchased from Sigma Life

Sciences. PCR products were sequenced for confirmation of amplicon identity. The primer set for deiodinase type 2 (DIO2) was kindly provided by Dr. Natalie Karouna-Renier.

Endpoint PCR was run on primer sets with a temperature gradient to confirm annealing temperature. A primer concentration gradient was run to confirm optimal primer concentration in the reaction. A standard curve was run to calculate efficiency. Efficiency was acceptable from 90-105% (see Appendix 3, Table 2).

qPCR and Data Processing

Quantitative PCR was performed on experimental samples using iQ SYBR Green Supermix (Bio-Rad), which had a final reaction concentration of 1X. 1 uL of sample was loaded into all reactions. The primer concentration was different for all genes (Appendix 3, Table 2). All reactions were run in 15-uL reactions on a 96 well plate. PCR program was run at 95°C for 3 minutes; 40 cycles of 95°C for 15 s, Tm of choice for 30 s, and 72°C for 30 seconds; a melt curve starting at 55°C and stopping at 95°C, moving 0.5°C every 10 seconds. A No-RT and a No-Template sample were used on each PCR plate as negative controls. Three interplate calibrator samples were run on all plates. If the standard deviation for the IPCs across the plates for one gene was above 0.6, the outlying plate or plates were re-run. Beta actin and GAPDH were used as normalizing genes. Raw threshold counts (Cts) were processed with Genex Ver. 5.4.4 (MultiD) by first correcting for efficiency, then correcting interplate calibrator values. Next, sample replicates were averaged, then normalizing to beta actin and GAPDH. Relative quantities were then calculated, and finally a log-2 transformation was performed with sample sizes shown in Table 2.

Table 2: Samples sizes for each dose within each compound for qPCR analysis.

Dose ID	Treatment ID			
	LA	LB	LC	LD
	PCB 126 (ng/g)	PCB 77 (ng/g)	PCB TRES* mix (µg/g)	PCB SPSA* mix (µg/g)
UNT	16, 8§			
SHAM	14, 12			
VEH	16, 2			
6	0, 0	12, 10	0, 0	0, 0
5	0, 0	10, 6	0, 0	0, 0
4	0, 0	10, 12	4, 6	0, 0
3	0, 0	6, 10	10, 12	4, 6
2	6, 6	8, 8	12, 8	14, 10
1	10, 16	10, 8	14, 17	20, 12

** TRES: tree swallow mixture; SPSA: spotted sandpiper mixture*.
§ # males, # females

EROD Assay

Microsomal Preparation

The frozen livers were homogenized in cold 0.2M potassium phosphate-monobasic/0.2M sodium phosphate-dibasic, pH 7.4 homogenization buffer. The homogenate was then centrifuged at 11,000 g at 4°C for 20 minutes. The supernatant was collected and centrifuged at 100,000 g at 4°C for 60 minutes. The supernatant was discarded and the pellet containing the microsomes was resuspended in 0.05M

Potassium phosphate/sodium phosphate/10^{-2}M EDTA resuspension buffer of pH 7.6, and frozen at -80°C for later EROD activity measurement.

Assay Validation

The assay was validated as follows. First, the appropriate dilution of the microsomal samples was ascertained for detection with a linear reaction rate.

Second, the fluorescence of the resuspension buffer was compared to the 0.066 M Tris-HCl assay buffer, pH 7.4. There was no difference in FLU readings between the two buffers. The CV for these two buffers used to run a standard curve was 5.1%.

The third aspect of the validation was to confirm that the homogenization buffer used yielded similar results to those previously published by Melancon (1997). A Japanese quail liver that had not been induced was used to test the two buffers, and it was found that once the final resuspended sample had been diluted 1:50, there was no difference between the activity readings. In samples that were diluted less than 1:50, the activity curves were optimal in the samples run with this study's buffer. Therefore the buffer used was chosen based on the weight of the livers, with tree swallow hatchling livers roughly 1/50th the weight of Japanese quail adult livers.

Intra- and inter-assay validation were run to test the precision and accuracy of the standard curve. The %CV for the intra-assay validation was within 15% for the replicates for each of the standards and within 20% for the blank. For the inter-assay validation, the %CV for each point on the standard curve was within 15% for five standard curves run on five plates and within 20% for the blank. The standard curve values were as follows (in uM): 0.1, 0.05. 0.01, 0.009, 0.007, 0.005, 0.003, 0.001, and 0.000. The limit of quantitation for the curve was 0.005 uM, since <15% CV was not

achieved for 0.003, 0.0001, and 0.000 uM points. All samples that fell below this limit were eliminated from the study.

Assay Procedure

Ethoxyresorufin-O-dealkylase (EROD) was assayed in triplicate on a fluorescence plate scanner. The assay utilized 200 µL of 1.25 µM ethoxyresorufin (Sigma-Aldrich Chemical) substrate made up in 0.066 M pH 7.4 Tris-HCl buffer, 10 µL of 0.125 mM NADPH (Sigma-Aldrich Chemical), and 50 µL of microsomal protein. The plate was read for a total of 12 readings over 18 minutes after incubation at 37°C. Reference Japanese quail microsomes that had not been induced were included with each plate. Change in fluorescence units over time were converted to the rate of product formation with the use of a 9-point standard curve (0.001-0.1µM). Protein was determined using the BCA Protein Assay kit (Pierce Chemical Company, Rockford, IL, USA). Ethoxyresorufin-O-dealkylase activity was calculated as picomoles of product formed/mg microsomal protein/min. Sample sizes for EROD analysis are included in Table 3.

Table 3: Sample sizes for each dose within each compound for EROD analysis.

Dose ID	Treatment ID			
	LA	LB	LC	LD
	PCB 126 (ng/g)	PCB 77 (ng/g)	PCB TRES* mix (µg/g)	PCB SPSA* mix (µg/g)
UNT	10,8			
SHAM	10,14			
VEH	14,3			
6	0,0	7,8	0,0	0,0
5	0,0	8,4	0,0	0,0
4	4,0	7,8	2,3	0,0
3	1,8	9,7	8,10	3,3
2	4,3	9,4	13,9	11,8
1	5,10	9,6	10,12	14,5

***** *TRES: tree swallow mixture; SPSA: spotted sandpiper mixture*.
§ # males, # females

Statistical Analysis

EROD and gene expression data were tested for normal distribution and log-2 transformed. The three control groups were tested for non-significance ($p \geq 0.05$). All control data were pooled and distributed across all treatment groups, taking sex into account (i.e. male controls were placed with treated males). The GLM procedure in SAS software was used to test if the dose-response curves of each compound/sex combination were cubic, quadratic, or linear in nature ($p < 0.05$). A p-value of 0.05 or greater signified no change with dose. Gender differences within compounds were

tested using the GLM procedure. The dose*gender interaction was first tested. If that was non-significant ($p \geq 0.05$), then the interaction was taken out of the model and gender and dose were tested.

A principal components analysis (PCA) was performed on the gene expression of all 12 genes together, for each treatment-gender group, partialed for concentration, to identify duplication or singularity of gene action over the 12 genes across the 4 compounds and 2 genders. Thus the locations as well as the shape of the curves were accounted for. The resulting groupings of the twelve genes was evaluated according to their loading into each principal component (PC) within each treatment-gender group and verified on the partial correlation values for each treatment-gender group, and then compared for similar clustering across the eight treatment-gender groups, for a final determination of the genes' mutual association into the principal components. Principal components analysis (PCA) was performed on the gene expression of all 12 genes together, for each treatment-gender group, past of the information of the most correlated genes was loaded into the first PC, at moderate eigenvalue levels: most of the first PC was loaded at about a 0.3 level for several genes, when in fact some of those genes loaded more strongly into the 5^{th} or 6^{th} PC (generally, a value of 1.0 was chosen as cut-off for the eigenvalue, indicating the sufficient number of PCs).

Results

Gene Expression

GLM Procedure

Expression levels for 12 genes and enzyme activity for EROD were measured via qPCR and analyzed with the GLM procedure. The cubic, quadratic, and linear components were tested for each compound/sex combination (Figure 1A-L). The nature of the response for each of these combinations was different based on the gene. For 12 endpoints across 8 sex/compound combinations giving a total of 96 dose-response curves, the cubic was significant 10 times, the quadratic was significant 14 times, and the linear was significant 8 times. Of 48 dose-response curves for each sex, males responded 18 times and females responded 14 times. Of 24 dose-response curves for each compound, there were 5 responses to PCB 126, 8 responses to PCB 77, 11 responses to the TRES mixture, and 8 responses to the SPSA mixture (Table 2). Of the 12 genes, *CYP1A4* and *CYP1A5* were responsive in 7 of the 8 sex/compound combinations. *CAT* was responsive in 4 of the 8; *DIO2* was responsive in 3 of the 8; *CYB5*, *ALDOB*, *GPX*, *GR*, and *GSTA* were responsive in 2 of 8; *AHR1* was responsive in only 1 of the 8; *HCS* and *TXN* did not respond at the level of significance (Table 3).

Principal Component Analysis

PCA was performed on each sex/compound combination, partialed for dose. Genes were grouped into principal components for each sex/compound combination (Figure 2). PCA groupings were inconsistent across sex/compound combinations, however *DIO2* was grouped by itself 6 out of 8 times, and *CYP1A4* and *CYP1A5*

were grouped together 7 out of 8 times. Other groupings were more common than others, such as *ALDOB* and *HCS* being grouped 5 out of 8 times (Figure 3).

EROD Analysis

EROD data were analyzed with the GENMOD procedure (Figure 3). The cubic component was significant ($p<0.05$) in the PCB 126-dose males, all of the birds dosed with the TRES mixture, as well as the SPSA mixture-dosed males. The quadratic component was significant ($p<0.05$) in PCB 126-dosed males and SPSA mixture-dosed females. Finally, the linear component was significant ($p<0.05$) in the PCB 126-dosed females as well and PCB 77-dosed males.

Discussion

Xenobiotic Metabolism

The purpose of this study was to characterize hepatic gene expression of responsive systems upon exposure to four PCB compounds. Most endpoints were largely unresponsive, with the most notable exceptions being observed in EROD, *CYP1A4*, and *CYP1A5* (Figures 1A, 1B, and 3). It was expected that these endpoints would respond because they are directly upregulated through AhR activation by PCBs and other xenobiotic compounds. Therefore, the higher the dose, the higher the level of expression of these and other CYP450 genes and their end-products such as EROD. An increasing dose response curve was seen primarily in the two mixtures of these three endpoints. This is potentially a survivor effect, as there was moderate survivability at the higher doses of the two mixtures, whereas a smaller increase is

seen with PCB 126 in these endpoints because there was not enough survivability beyond the first few doses to conduct an analysis at the higher doses. The lack of pronounced response with PCB 77 was surprising, since PCB 77 is thought to activate the AhR and was therefore hypothesized to be one of the greatest components of AhR activation in the two mixtures (WHO; Van den Berg et al., 2005). The fact that these genes respond as heavily as they do to the two mixtures indicates that a component of the mixtures is activating the AhR, however it is clear that PCB 77 is not a principal agent of response in either of the mixtures. A revised hypothesis is that PCB 126 and possibly other congeners in the mixture are in small enough concentrations to activate the AhR without high levels of mortality.

The GLM procedure provided a powerful analysis to explore the shape of the dose-response curves generated. The nature of the dose-response curves is important in considering the effect of these compounds. The cubic, quadratic, and linear responses indicate different physiological events. For instance, the response of EROD in both sexes to the TRES mixture is one of dramatic increase at low doses, followed by a plateauing response at medium doses and a second dramatic increase at high doses (Figure 3). This indicates that the animal responds to a low dose of PCBs, however the animal maintains the level of response without increasing it at the medium dose, however further challenge leads to an even more exaggerated response, as seen by the significant cubic component. However it is important to note that there has been pronounced mortality at the higher doses, indicating that what is still alive is alive primarily because it can mount this elevated response. Therefore, a survivor effect occurred at higher doses of the TRES mixture. It is further important to note

that the TRES mixture was the less lethal mixture (Appendix 4). The SPSA mixture, on the other hand, exhibits the same dramatic response at low doses with a plateauing at medium doses; however in most cases of the *CYP1A4*, *CYP1A5*, and EROD endpoints, the response to SPSA is a downward trend at higher doses, producing an inverted-U shape, as indicated by the significant quadratic component for these three endpoints in both sexes of this compound (the one exception being EROD in SPSA males). This combined with the higher lethality of the SPSA mixture is evidence that few birds were able to mount a response in these endpoints that helped keep them alive at higher doses. Therefore there appears to be a more lethal element in the SPSA mixture than in the TRES mixture. These non-monotonic dose-response curves play an important role in characterizing the effects of these xenobiotic compounds on living systems (Calabrese and Baldwin, 2001).

While PCB 77 did not elicit a strong response for any of these three endpoints in either sex, the significant responses seen in response to PCB 77 were mostly linear and quadratic (Table 2). This is evidence that PCB 77 displays low affinity for AhR that either increases mildly with dose monotonically, or exhibits a slight decrease at higher doses. Further investigation into the relative affinity of PCB 77 for the AhR is warranted.

CYB5 and *AHR1* were unresponsive, hypothetically since these genes are not directly mediated through a xenobiotic ligand-dependent receptor system. There were several p-values of the dose-response curves for *CYB5* that were below 0.10, indicating that these treatments were underpowered to find significance at the level of 0.05.

Oxidative Damage

Several genes explored in this study work to reduce the ROS load that accumulates with PCB exposure. This pathway utilizes glutathione, a molecule that is used to react with ROS in order to mitigate their deleterious effects (Scandalios, 2005). Studies have shown that oxidative damage increases with xenobiotic exposure, therefore we hypothesized that expression of antioxidative enzymes would increase in response to elevated ROS production (Aly and Domènech, 2009; Glauert et al., 2008). However, few significant differences were seen with *CAT*, *GPX*, *GR*, *GSTA*, and *TXN*, however a few dramatic trends are seen, for instance a drop in GSTA expression in SPSA females (Figure 1K), where there is an increase at low doses, with a dramatic decrease at medium doses. There are no obvious response differences between the two mixtures as there was with the CYP450 endpoints, indicating that the harmful element to be acting in the SPSA does not have the same effect on this pathway. However it remains clear that there are distinct if insignificant changes in expression of this pathway's genes. Many p-values of these endpoints indicate that the changes to be seen are lowly expressed enough that they are underpowered. Thioredoxin is a gene product that acts similarly to glutathione in its antioxidative capacity (Tanaka et al., 1997). There is limited understanding of a role for this potentially responsive element to xenobiotic exposure . We hypothesized that due to its close function with other enzymes that utilize glutathione, this would be a responsive gene to PCB exposure. This gene was remarkably unresponsive. This is evidence that glutathione is a more responsive mechanism to xenobiotic

challenge than thioredoxin. Future studies should focus on glutathione as a means of repairing oxidative stress rather than thioredoxin.

Endocrine Disruption and Energy Balance

DIO2, *ALDOB*, and *HCS* showed low responses to the PCB challenge, much in the same way as the genes involved with oxidative damage. This was unexpected, therefore it is suspected that mortality occurred too early for the full effect of these sub-lethal endpoints to be seen, or that a one-time *in ovo* dose does not allow enough time for these effects to be seen, with too high of a dose proving to be lethal before the animal can respond. It is reasonable to hypothesize that a chronic low-level dose instead of a one-time dose of either high or low levels will allow the effects of the contaminants on these endpoints to emerge slowly over the course of an animal's life. Therefore, future studies should be chronic exposure studies that follow these animals into adulthood, where development, sexual maturation, fecundity, overall health, and senescence can be monitored and measured with these and similar endpoints.

Figures

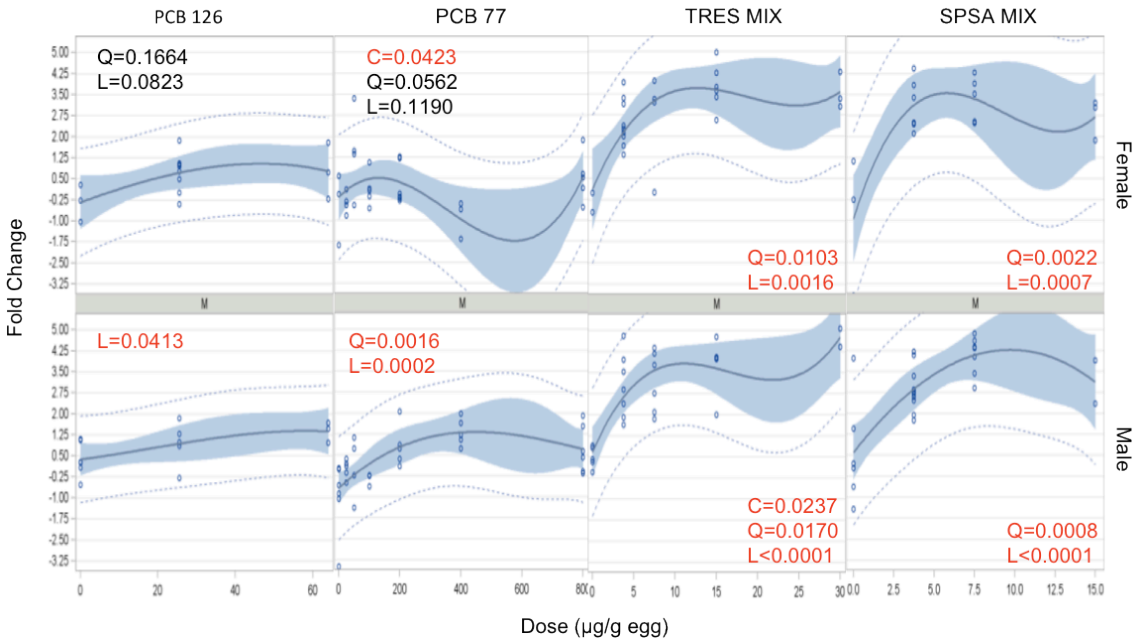


Figure 1A: CYP1A4 expression with PCB dose by compound and sex as analyzed using the GLM procedure in SAS®. Log2 values are calculated relative to the untreated group separated by sex, as processed in GENEX® software. The cubic, quadratic, and linear components were tested for each regression curve, which was by each treatment-sex combination. Top 4 panels represent female birds; bottom 4 panels represent male birds. "C" represents the p-value of the cubic component of the regression curve; "Q" represents the p-value of the quadratic component of the regression curve; "L" represents the p-value of the linear component of the regression curve. Significance was set at <0.05. Red values are the significant p-values. Open circles represent individual samples; solid blue line represents the mean of the regression curve for that treatment-sex combination; shaded blue region represents 95% confidence limit; dashed line represents 95% prediction limit. No significance was found in the PCB 126-dosed females. The linear component was the highest order of significance in the PCB 126-dosed males. The quadratic component was the highest order of significance in the PCB 77-dosed males, the TRES mixture-dosed females, and both sexes of the SPSA mixture-dosed males. The cubic component was the highest order of significance in the TRES mix-dosed males.

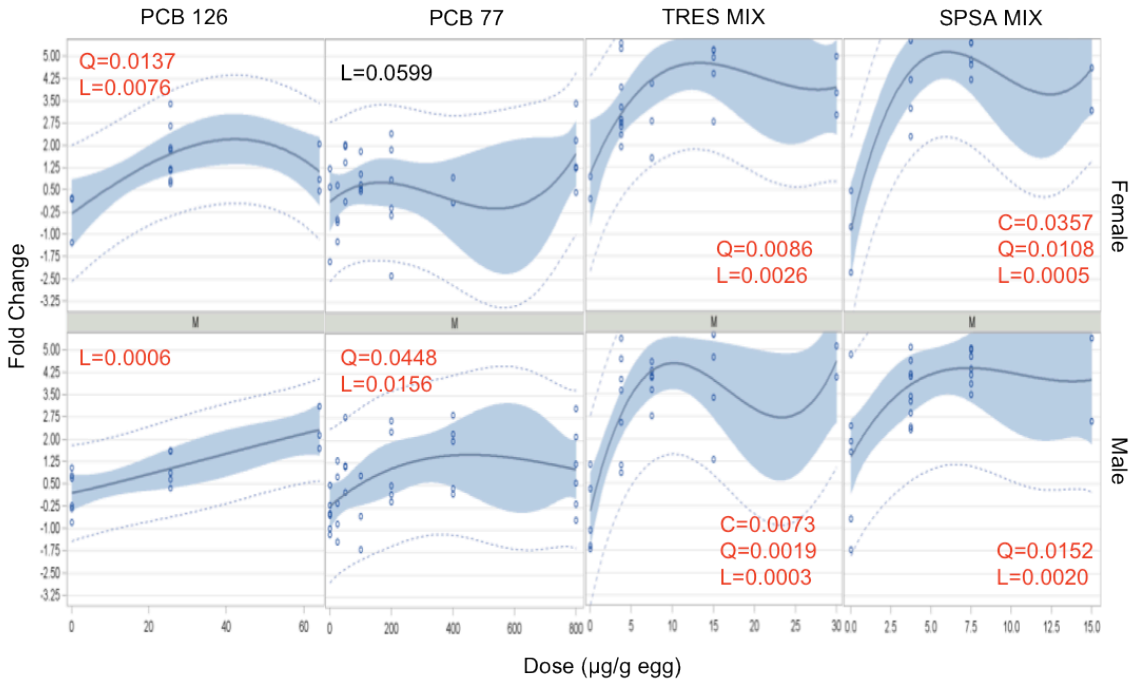


Figure 1B: CYP1A5 expression with PCB dose by compound and sex as analyzed using the GLM procedure in SAS®. See Figure 1A for pertinent details. No significance was found in the PCB 77-dosed females. The linear component was the highest order of significance in the PCB 126-dosed males. The quadratic component was the highest order of significance in the PCB 126-dosed females, PCB 77-dosed males, TRES mixture-dosed females, and SPSA mixture-dosed males. The cubic component was the highest order of significance in the TRES mixture-dosed males and the SPSA mixture-dosed females.

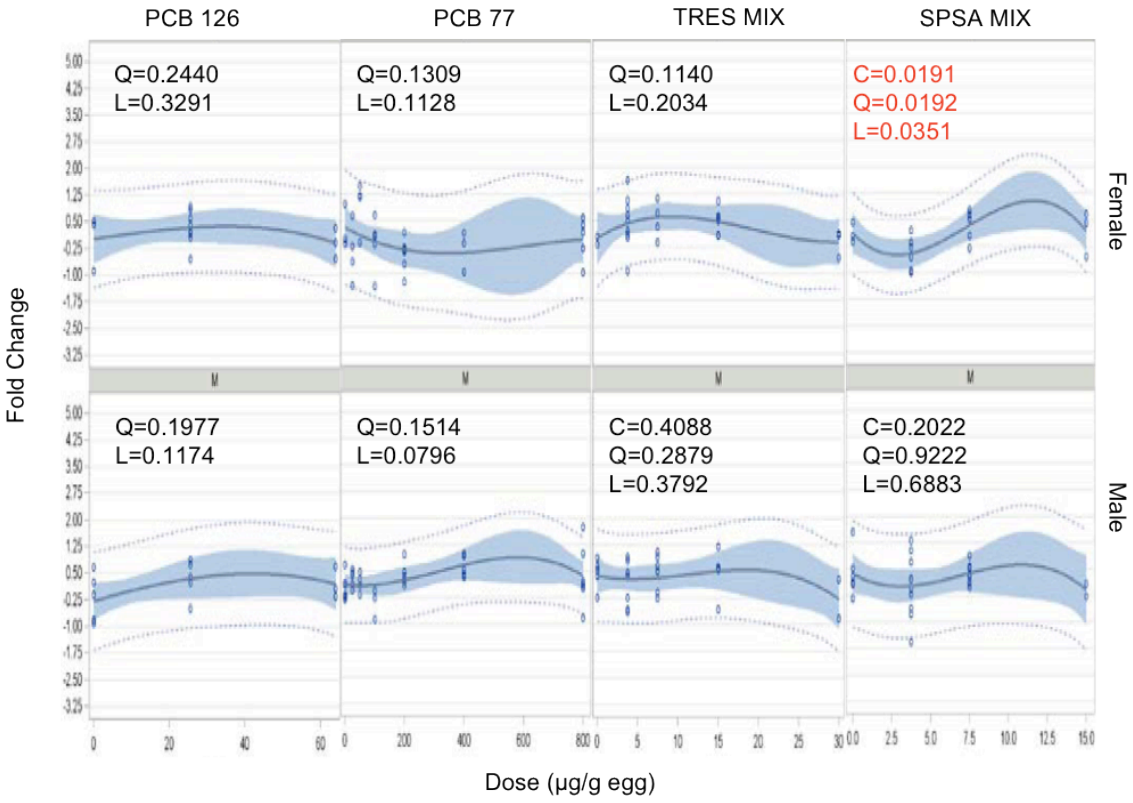


Figure 1C: AHR1 expression with PCB dose by compound and sex as analyzed using the GLM procedure in SAS®. See Figure 1A for pertinent details. The cubic component was the highest order of significance in the SPSA mixture-dosed females. No other significant differences were found.

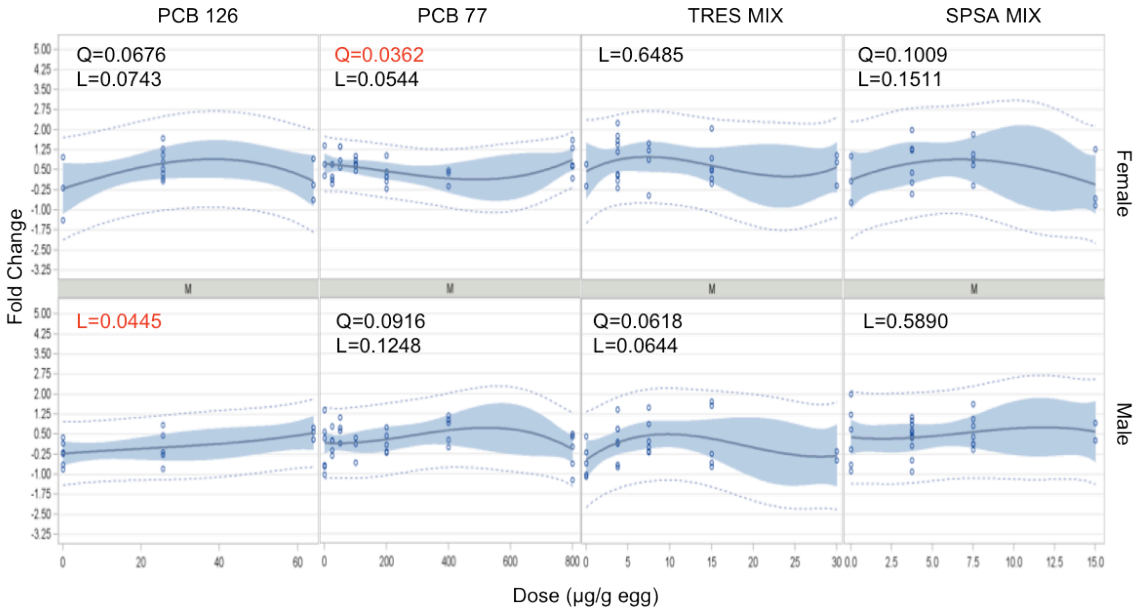


Figure 1D: CYB5 expression with PCB dose by compound and sex as analyzed using the GLM procedure in SAS®. See Figure 1A for pertinent details. The linear component was the highest order of significance in the PCB 126-dosed males. The quadratic component was the highest order of significance found in the PCB 77-dosed females. P-values below 0.10 were found in components of the PCB 126-dosed females as well as in the PCB 77-and TRES mixture-dosed males.

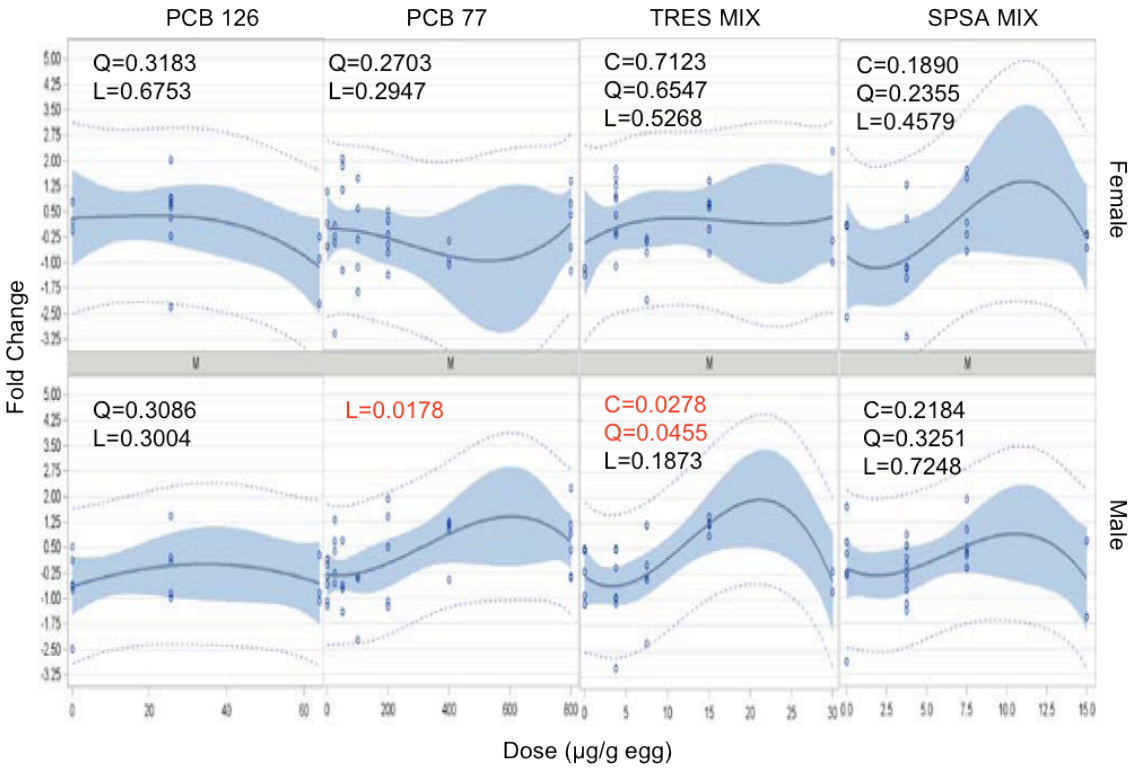


Figure 1E: ALDOB expression with PCB dose by compound and sex as analyzed using the GLM procedure in SAS®. See Figure 1A for pertinent details. The linear component was the highest order of significance in PCB 77-dosed males. The cubic component was the highest order of significance in TRES mix-dosed males. No other significant differences were found.

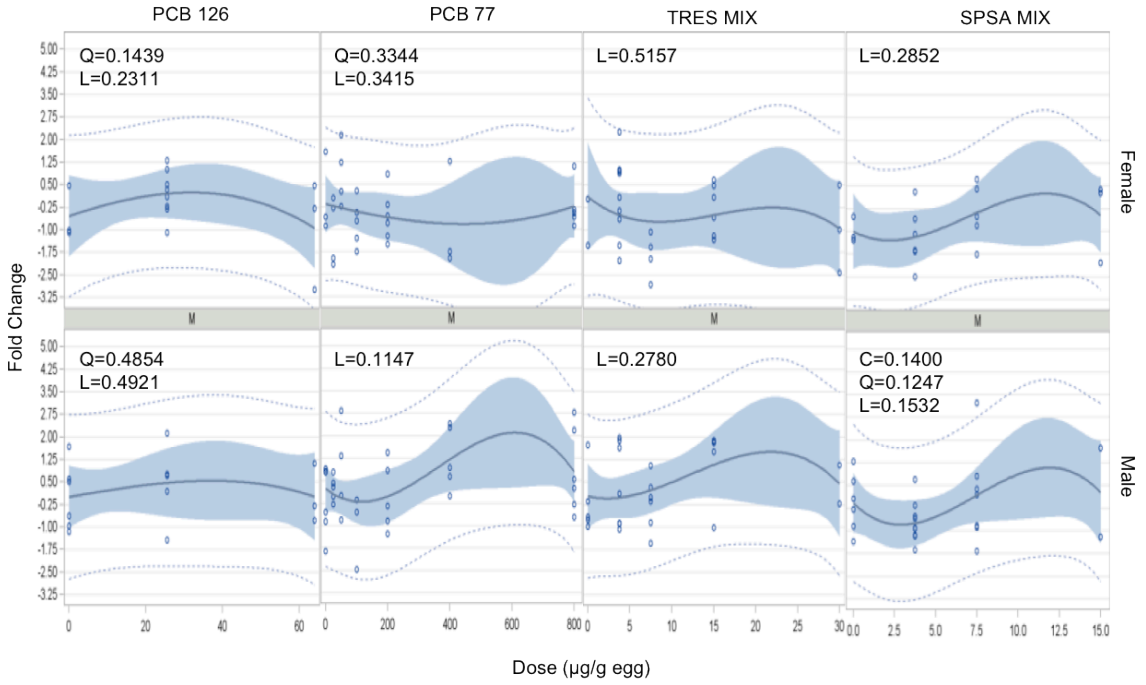


Figure 1F: HCS expression with PCB dose by compound and sex as analyzed using the GLM procedure in SAS®. See Figure 1A for pertinent details. No significance was found in any components of the 8 treatment-sex combinations.

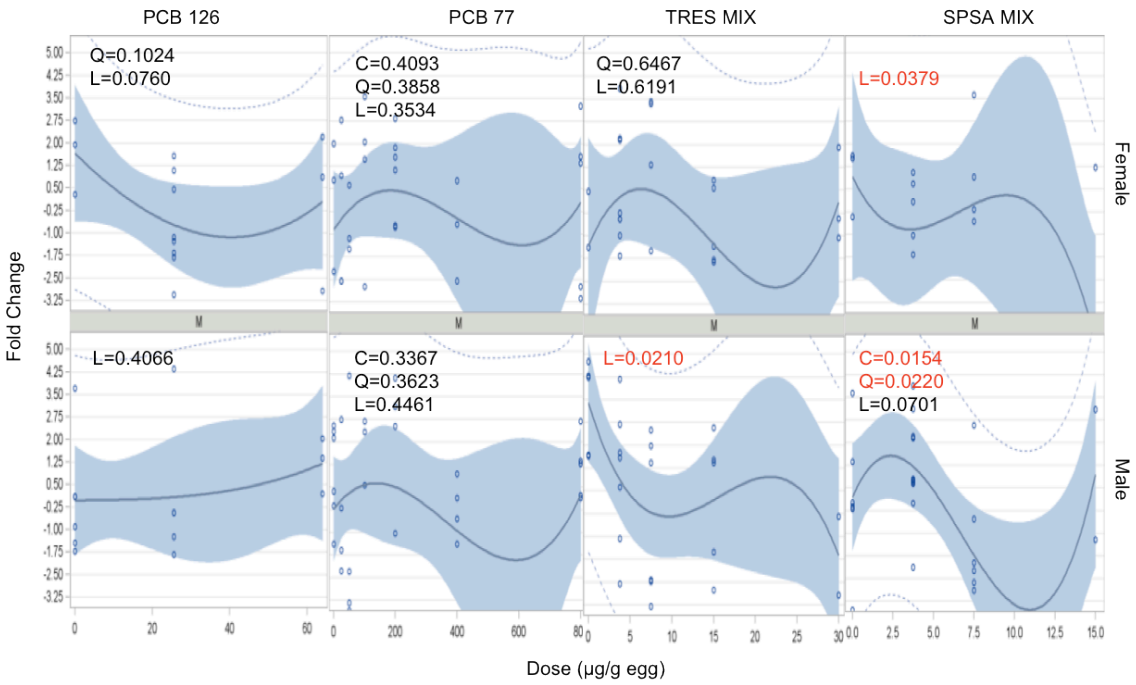


Figure 1G: DIO2 expression with PCB dose by compound and sex as analyzed using the GLM procedure in SAS®. See Figure 1A for pertinent details. No significance was found in the single congener-dosed females. The linear component was the highest order of significance in the TRES mixture-dosed males and the SPSA mixture-dosed females. The cubic component was the highest order of significance in the SPSA mixture-dosed males.

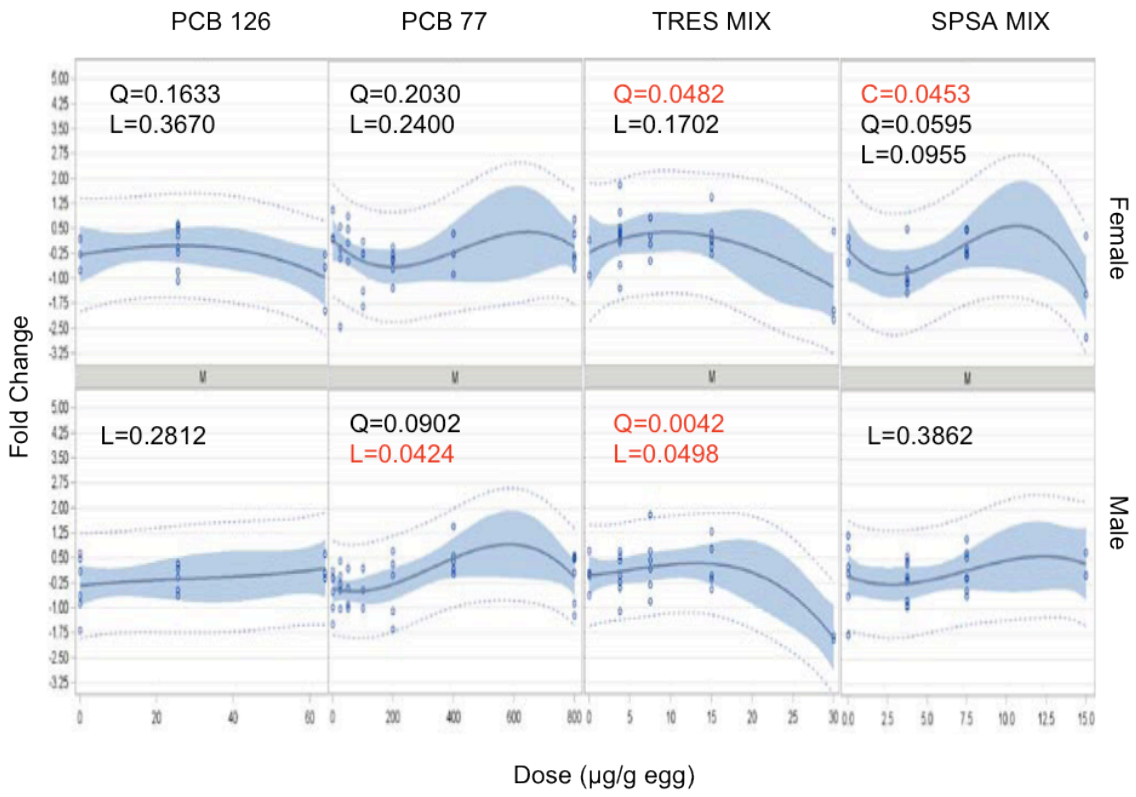


Figure 1H: CAT expression with PCB dose by compound and sex as analyzed using the GLM procedure in SAS®. See Figure 1A for pertinent details. The linear component was the highest order of signficance in the PCB 77-dosed males. The quadratic component was the highest order of significance in all TRES mix-dosed birds. The cubic component was the highest order of significance in SPSA mix-dosed females.

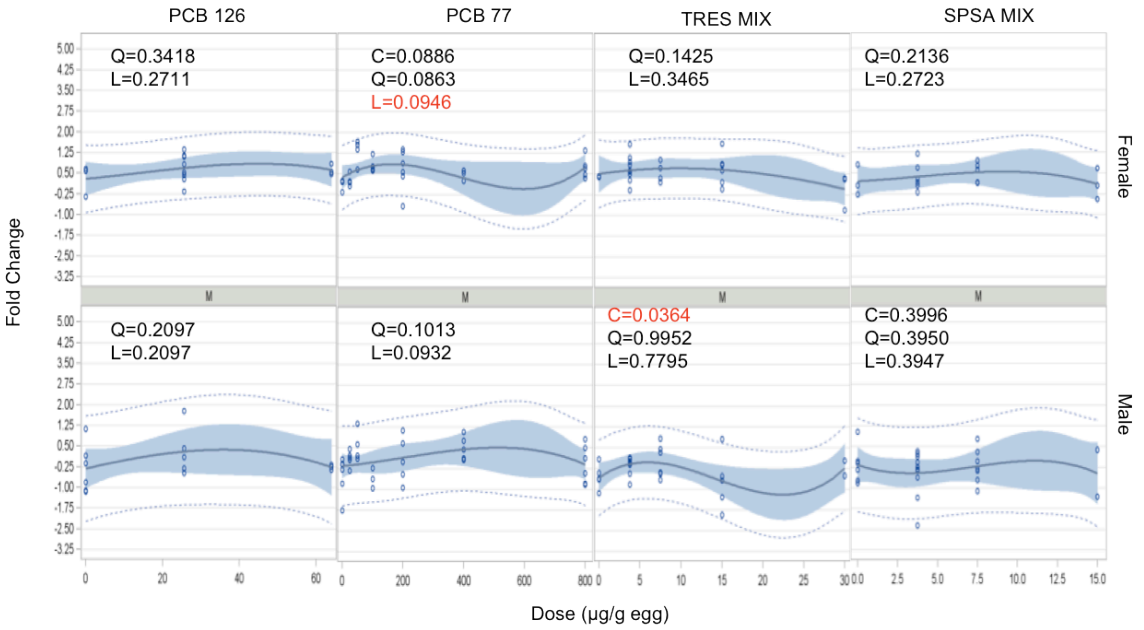


Figure 1I: GPX expression with PCB dose by compound and sex as analyzed using the GLM procedure in SAS®. See Figure 1A for pertinent details. The linear component is the highest order of significance in the PCB 77-dosed females. The cubic component was the highest order of significance in the TRES mixture-dosed males. P-values below 0.10 were found in the PCB 77-dosed males. No other significant differences were found.

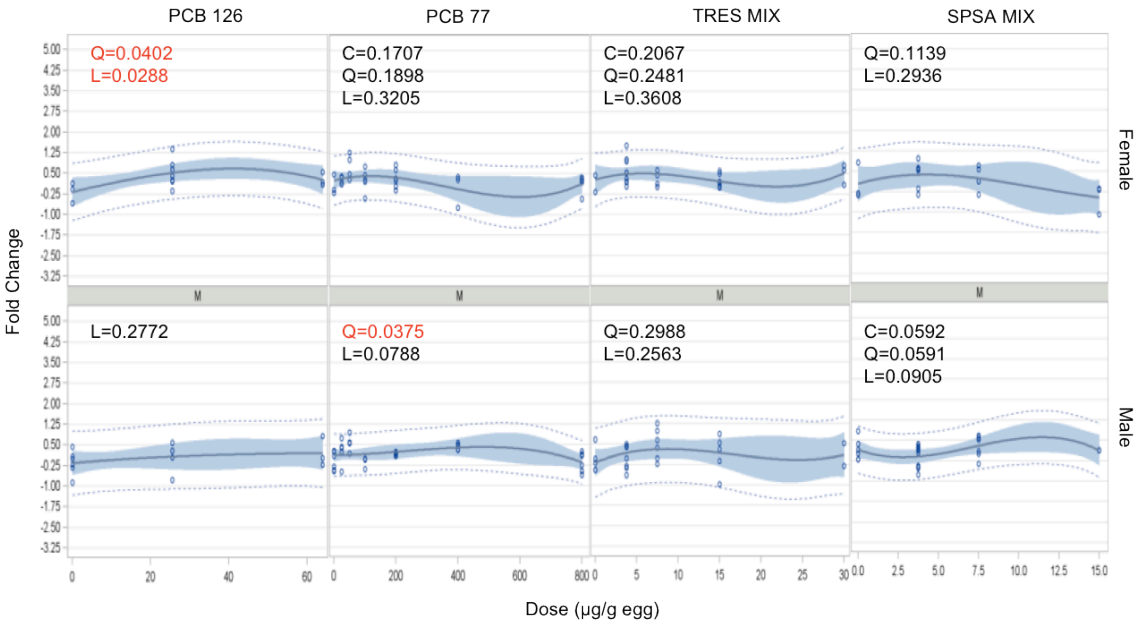


Figure 1J: GR expression with PCB dose by compound and sex as analyzed using the GLM procedure in SAS®. See Figure 1A for pertinent details. The quadratic component was the highest order of significance in the PCB 126-dosed females and the PCB 77-dosed males. P-values below 0.10 were found in the SPSA mixture-dosed males. No other significant differences were found.

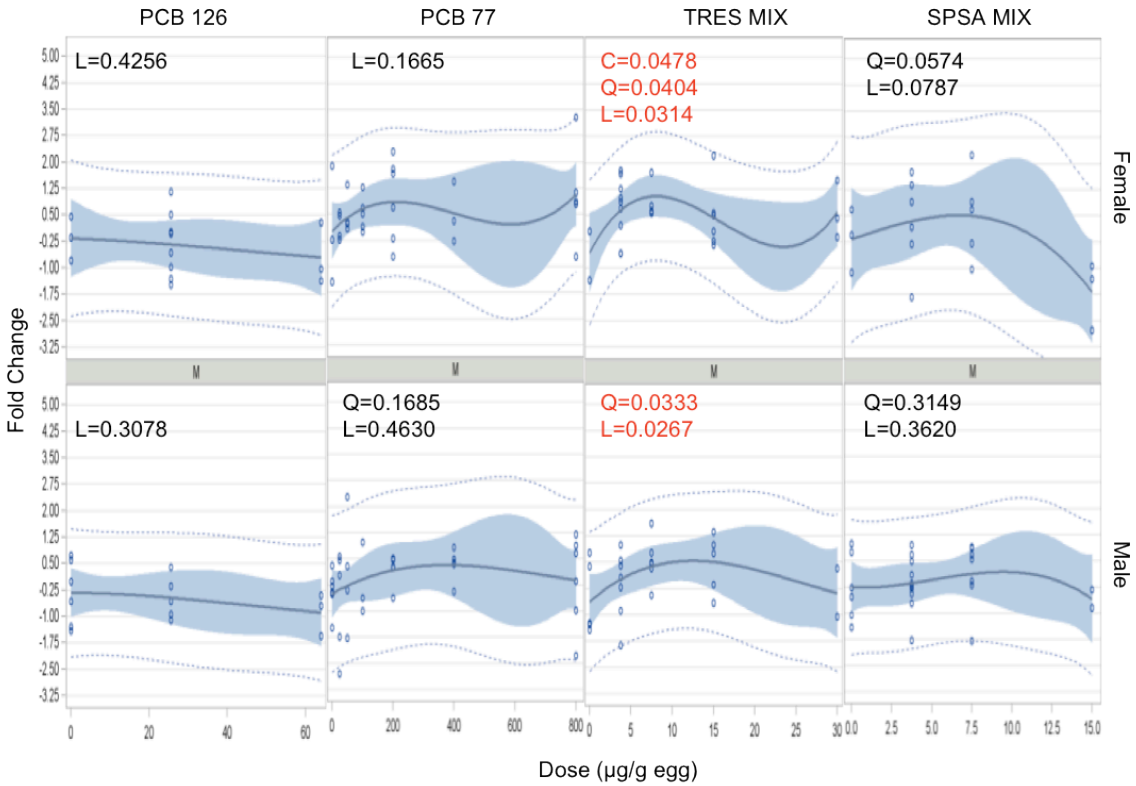


Figure 1K: GSTA expression with PCB dose by compound and sex as analyzed using the GLM procedure in SAS®. See Figure 1A for pertinent details. The quadratic component was the highest order of significance in the TRES mixture-dosed males. The cubic component was the highest order of significance in the TRES mixture-dosed females. P-values below 0.10 were found in the SPSA mixture-dosed females. No other significant differences were found.

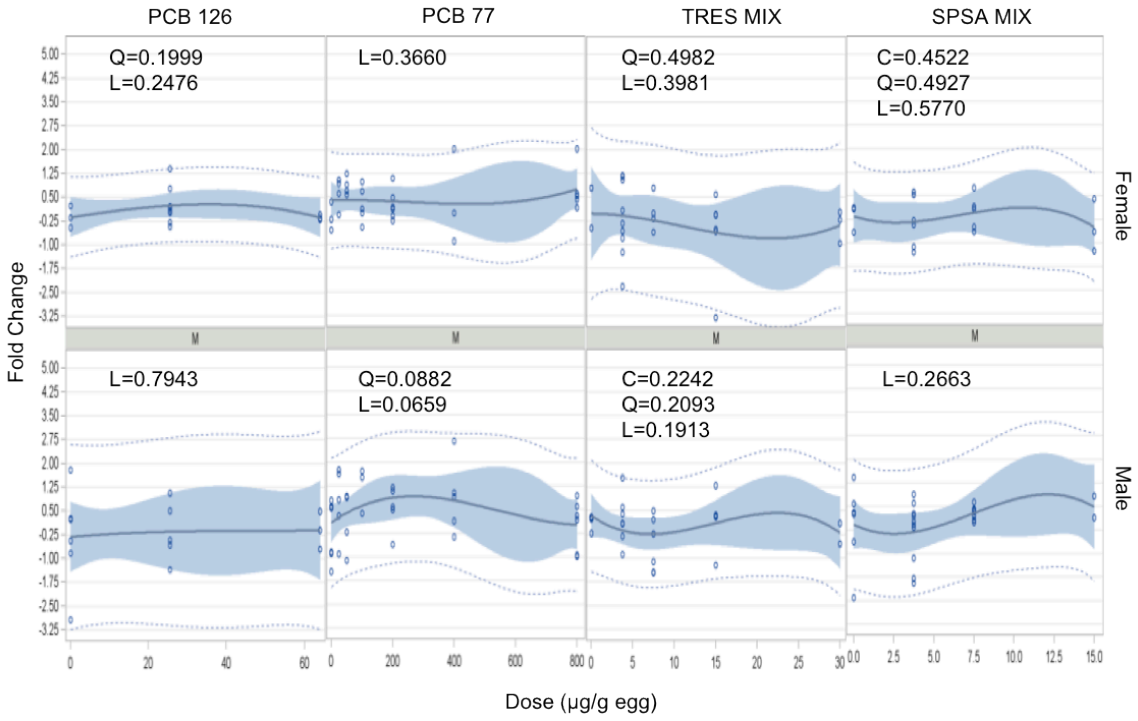


Figure 1L: TXN expression with PCB dose by compound and sex as analyzed using the GLM procedure in SAS®. See Figure 1A for pertinent details. No significant differences were seen for any of the 8 compound/sex combinatin. P-values below 0.10 were found in components of the PCB 77-dosed males.

Table 3: Principal component analysis placed genes into principal components for each sex/compound combination.

	Principal Components					
	PC 1	PC 2	PC 3	PC 4	PC 5	PC 6
PCB 126 Females	AHR1 ALDOB CAT CYB5 CYP1A4 CYP1A5 HCS TXN	DIO2	GR GPX	GSTA		
PCB 126 Males	CYP1A4 CYP1A5	GPX TXN	CAT CYB5 GR	AHR1 ALDOB HCS	GSTA	DIO2
PCB 77 Females	AHR1 ALDOB CAT HCS	GSTA TXN	DIO2	GR GPX	CYP1A4 CYP1A5 CYB5	
PCB77 Males	CYP1A5 HCS	GR GSTA TXN	AHR1 GPX	CAT CYB5	ALDOB CYP1A4	DIO2
TRES Mix Females	AHR1 CAT CYB5 GPX	DIO2 GR GSTA TXN	CYP1A4 CYP1A5	HCS	ALDOB	
TRES Mix Males	CYB5 CYP1A4 CYP1A5 GSTA	AHR1 ALDOB CAT HCS	TXN	DIO2	GPX GR	
SPSA Mix Females	CYB5 GPX GR GSTA TXN	AHR1 CAT DIO2	CYP1A4 CYP1A5	ALDOB	HCS	
SPSA Mix Males	AHR1 ALDOB CAT CYB5 HCS	GPX TXN	DIO2	CYP1A4 CYP1A5	GSTA GR	

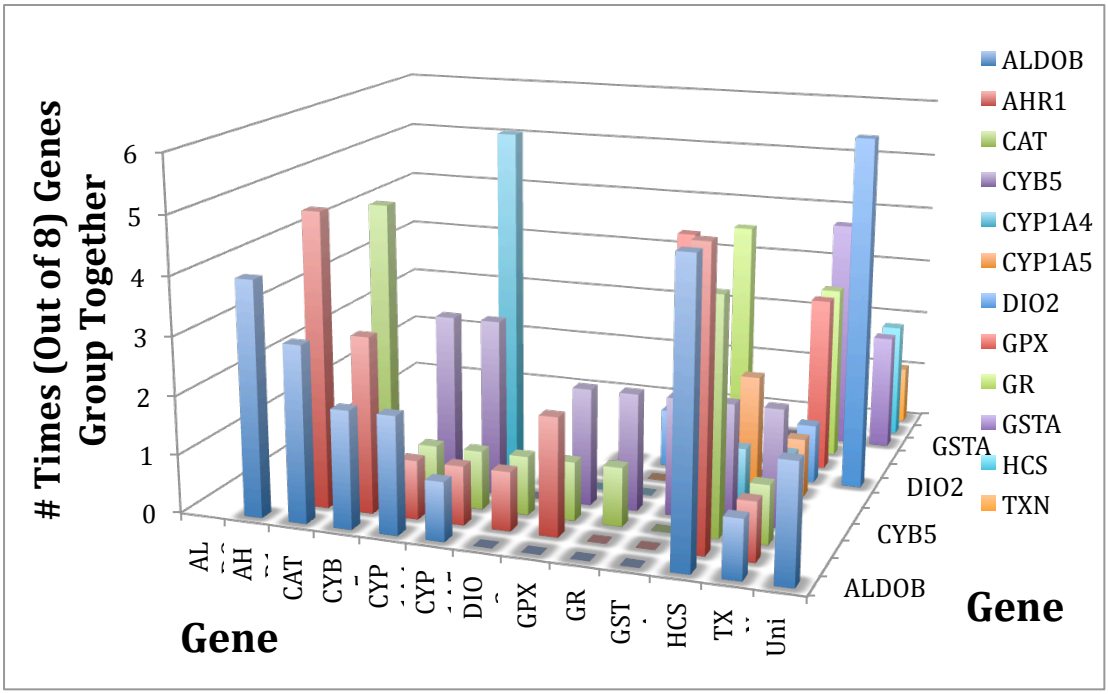


Figure 2: Principal component analysis placed genes into principal components.

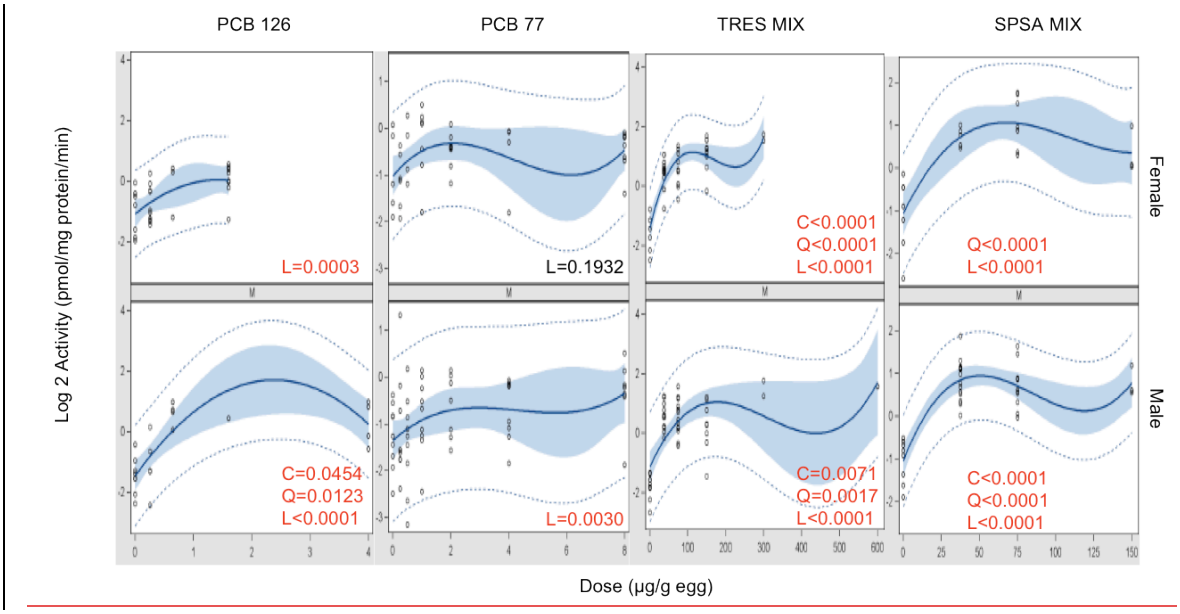


Figure 3: EROD enzyme activity with PCB dose by compound and sex as analyzed using the GENMOD procedure in SAS®. Response is the log2-transformed activity value (pmol product/mg protein/min). See Figure 1A for pertinent details. The linear component was the highest order of significance in the PCB 126-dosed females and PCB 77-dosed males. The quadratic component was the highest order of significance in the SPSA-dosed females. The cubic component was the highest order of significance in the PCB 126-dosed females, all of the TRES mixture-dosed birds, and the SPSA-dosed males.

Table 2: Number of significant differences found in compounds and sexes across 13 endpoints.

	PCB 126	PCB 126	PCB 77	PCB 77	TRES Mix	TRES Mix	SPSA Mix	SPSA Mix		
	M	**F**	**M**	**F**	**M**	**F**	**M**	**F**	**All Males**	**All Females**
Cubic	0	0	0	1	5	2	2	3	**7**	**6**
Quadratic	0	2	3	2	2	3	2	2	**7**	**9**
Linear	3	1	3	1	1	0	0	1	**7**	**3**
Total	3	3	6	4	8	5	4	6	**21**	**18**

Table 3: Number of significant differences of responses for 13 endpoints.

Endpoint	# Responses (out of 8 sex/compound combinations)
AHR1	1
ALDOB	2
CAT	4
CYB5	2
CYP1A4	7
CYP1A5	7
DIO2	3
GPX	2
GR	2
GSTA	2
HCS	0
TXN	0
EROD	7

Chapter 4: Induction of Cytochrome P450 Ethoxyresorufin-*O*-deethylase (EROD) Activity in Hatchling Tree Swallows and Bluebirds: Comparison of PCB-Dosed and Environmentally Exposed Hatchlings

Abstract

Ethoxyresorufin-*O*-deethylase (EROD) activity was measured in tree swallow and eastern bluebird livers from eggs dosed with PCBs and collected at Patuxent Research Refuge (PRR), Sacandaga Lake (SL; 2006 only), Cobleskill Reservoir (CR; 2007 and 2008 only), and the upper Hudson River (UHR) in 2006, 2007 and 2008. In 2006, eggs from PRR and SL were dosed with PCB 126. In 2007, eggs from PRR and CR were dosed with a PCB mixture present at areas of concern in the Upper Hudson River in New York State. In 2008, eggs from CR and PRR were dosed with PCB 77, a non-ortho-substituted congener thought to have a toxicity profile similar to that of PCB 126 and therefore likely to induce EROD activity through the aryl hydrocarbon receptor (AhR). Results showed that EROD activity was induced by PCB 126 at Sacandaga Lake in 2006. and by the PCB mixture at PRR. However the PCB mixture did not induce EROD at CR in 2007. PCB 77 did not affect EROD in tree swallows, but stimulated induction of EROD in bluebirds. There were no differences observed in EROD induction in the untreated eggs across all reference sites or the UHR site; no differences were observed between years with any of the reference sites. However, there was a significant difference in EROD activity across the years at UHR ($p=0.0082$ between 2006 and 2007; $p<0.0001$ between 2006 and

2008. Taken together, these data demonstrate that field relevant concentrations of the PCB mixture found at the UHR site induced EROD activity in tree swallows and bluebirds. This finding verifies EROD as a bioindicator for PCB exposure in field birds.

Introduction

The cytochrome P450 (CYP450) enzyme superfamily is involved with many metabolic processes, including detoxification of xenobiotic compounds. In birds, the CYP450 1A4 isoform has preferential specificity for ethoxyresorufin-*O*-deethylase (EROD) activity (Head and Kennedy, 2007) and is upregulated via the aryl hydrocarbon receptor (AhR) activated by dioxin-like compounds, which are known to have a strong affinity for the AhR (Brunström and Andersson, 1988; Brunström and Halldin, 1998; Head and Kennedy, 2007; Melancon, 1997; Melancon et al., 2006; Safe, 2001). EROD activity is a biomarker of exposure used to determine AhR activation by xenobiotics (Melancon et al., 2006).

The PCB congeners 126 and 77 are non-ortho-substituted dioxin-like compounds that can activate the AhR and induce EROD activity in exposed animals (Brunström et al., 1988; Brunström and Andersson, 1988; reviewed in Safe, 2001). Avian TEQs for PCBs 126 and 77 are 0.1 and 0.05 respectively rank them as two of the most toxic PCB congeners capable of activating the AhR (reported in El-Shahawi et al., 2010). Both congeners are present in the PCB mixture being tested in this study. PCBs 126 and 77 were also tested alone. It was hypothesized that all test compounds – the mixture and the single congeners – would induce EROD.

Methods and Materials

Animals and Treatments

In 2006, tree swallow eggs at two reference sites, Patuxent Research Refuge (PRR) in Greenbelt, MD, and Sacandaga Lake (SL) in upstate New York were injected with field-relevant doses of PCB 126. In 2007, tree swallow eggs at two reference sites, PRR and Cobleskill Reservoir (CR) in Cobleskill, New York, were injected with a 66-congener PCB mixture based on a congener profile found in tree swallows at the Upper Hudson River superfund site (Echols et al., 2004). In 2008, tree swallow and bluebird eggs from the same reference sites (i.e. PRR and CR) were injected with PCB 77 (see Table 1 for dosing regimes). Eggs were also sampled from the upper Hudson River site, but were not experimentally manipulated prior to hatching. Eggs were allowed to incubate in the field until ~ED10, collected, and hatched in the lab. Birds were sacrificed within 24 hours of hatching and livers were snap frozen in liquid nitrogen for later analysis (see figures for sample sizes in each treatment).

Table 1: Dosing Regimes for All Years

Year	Compound	Reference Sites	Doses (ug/g egg)	Species
2006	PCB 126	PRR	UNT, 0, 2.36, 24	TRES‡
		SL†	UNT, 0, 8, 24, 44	
2007	Mixture	PRR	UNT, 0, 6, 12, 25, 50, 100	TRES
		CR	0, 6, 12, 25, 50, 100	
2008	PCB 77	PRR	UNT, 0, 1, 10	TRES EABL‡

† PRR = Patuxent Research Refuge
SL = Sacandaga Lake
CR = Cobleskill Reservoir
‡ TRES = tree swallow; EABL = eastern bluebird

Microsomal Preparation

The frozen livers were homogenized in cold 0.2M Potassium Phosphate-Monobasic/0.2M Sodium Phosphate-Dibasic, pH 7.4 homogenization buffer. The homogenate was then centrifuged at 11,000 G at 4°C for 20 minutes. The supernatant was collected and centrifuged at 100,000 G at 4°C for 60 minutes. The supernatant was pipetted off and the pellet containing the microsomes was resuspended in 0.05M Potassium Phosphate/Sodium Phosphate/10^{-2}M EDTA resuspension buffer of pH 7.6, and frozen at -80°C for later EROD activity measurement.

Assay Validation

The assay was validated by first, ascertaining the dilution of microsomes needed for the assay was determined. Second, background fluorescence was determined for the resuspension buffer and the 0.066 M Tris-HCl assay buffer. There was no difference in fluorescence readings between the two buffers. The CV for

these two buffers used to run a standard curve was 5.1%. Third, our homogenization buffer was compared with that used by Melancon (1997). A control Japanese quail liver was used to test the two buffers; no difference was found in activity readings from resuspended sample diluted 1:50. In samples that were diluted less than 1:50, the activity curves were optimal in the samples run using our buffer. Our buffer was used with,tree swallow hatchling livers, which were roughly $1/50^{th}$ the weight of Japanese quail adult livers.

Intra- and inter-assay variability were run to test the precision and accuracy of the standard curve. The %CV for the intra-assay validation was within 15% for the replicates for each of the standards and within 20% for the blank. For the inter-assay validation, the %CV for each point on the standard curve was within 15% for five standard curves run on five plates and within 20% for the blank. The standard curve values were as follows (in uM): 0.1, 0.05. 0.01, 0.009, 0.007, 0.005, 0.003, 0.001, and 0.000. The limit of quantification or sensitivity was 0.005 uM, since <15% CV was not achieved for 0.003, 0.0001, and 0.000 uM points. All samples that fell below this limit were eliminated from the study.

Assay Procedure

Ethoxyresorufin-O-dealkylase (EROD) was assayed in triplicate on a BioTek Synergy HT fluorescence 96-well microplate reader. The assay utilized 200 μL of 1.25 μM ethoxyresorufin (Sigma-Aldrich Chemical) substrate, 10 μL of 0.125 mM NADPH (Sigma-Aldrich Chemical), and 50 μL of microsomal protein. The plate was read for a total of 12 readings over 18 minutes after incubation at 37°C. Reference Japanese quail microsomes that had not been induced were included with each plate.

Change in fluorescence units over time were converted to the rate of product formation with the use of a 9-point standard curve (0.001-0.1μM). Protein was determined using the BCA Protein Assay kit (Pierce Chemical Company, Rockford, IL, USA). Ethoxyresorufin-O-dealkylase activity was calculated as picomoles of product formed/min/mg microsomal protein.

Statistical Analysis

Wilcoxon analyses using the NPAR1WAY procedure in SAS were performed to find statistical differences between dose groups within compound, site, and species. Regression analyses using the GLM procedure in SAS were performed to test the overall slope of the dose-response curve within compound, site, and species. Untreated and 0 ug/g egg dosed samples were pooled for analysis at a significance of ≤ 0.05.

Results

PCB 126-Treated Tree Swallows at Sacandaga Lake, 2006

Wilcoxon analysis performed for treatment groups in tree swallows from Sacandaga Lake exposed to PCB 126 in 2006 showed significant differences between doses ($p<0.05$;Figure1). The control and 8 μg/g egg dosed groups were statistically different from the 24 μg/g egg dosed group ($p<0.05$). The 8 μg/g egg dosed groups was also different from the 44 μg/g egg dosed group ($p=0.0500$). The overall fold-change was 2.4.

PCB Mixture-Treated Tree Swallows and Patuxent Research Refuge and Cobleskill Reservoir, 2007

Wilcoxon analysis performed for treatment groups in tree swallows from Patuxent Research Refuge and Cobleskill Reservoir exposed to a PCB Mixture yielded significant differences between doses ($p<0.05$; Figure 2). At Patuxent Research Refuge, EROD activity was significantly increased in the 12 μg/g egg dosed group compared to controls ($p<0.05$; Figure 2A). There was a significant increase between 100 μg/g egg dosed birds and the 0, 6, and 25 μg/g egg dosed groups ($p<0.05$). Regression analysis was significant ($p=0.0002$) with the overall fold-change of 5.4.

EROD activity did not increase with in tree swallows from Cobleskill Reservoir treated with the PCB mixture as tested with a Wilcoxon analysis ($p>0.05$; Figure 2B). A regression analysis was not significant ($p=0.4113$). The overall fold-change at Cobleskill Reservoir was 2.6-fold. The field site*dose interaction between Patuxent Research Refuge and Cobleskill Reservoir was significant ($p=0.0006$).

PCB 77-Treated Tree Swallows and Bluebirds at Patuxent Research Refuge and Cobleskill Reservoir, 2008

A Wilcoxon analysis yielded increased EROD activity in tree swallows at Patuxent Research Refuge between the control group and the high-dose group (10 μg/g egg; $p=0.0282$; Figure 3A). A regression analysis was not significant ($p=0.1042$). The overall fold-change was 1.4.

EROD activity did not increase in tree swallows at Cobleskill Reservoir with a Wilcoxon analysis ($p>0.05$; Figure 3B). A regression analysis was not significant

(p=p=0.4376). The overall fold-change was 1.2. There was not a field site*dose interaction between Patuxent Research Refuge and Cobleskill Reservoir (p=0.0948).

EROD activity increased in bluebirds at Patuxent Research Refuge treated with PCB 77 as detected by a Wilcoxon analysis (Figure 3C). A significant difference was found between the 0 and 25 μg/g egg dose groups (p=0.0281), however the regression analysis was not significant (p=0.0597). The overall fold-change was 2.8.

Seasonality Data for Untreated Eggs

A two-way ANOVA for field site and year was run on all untreated eggs for all sites, including the UHR site (Figure 4). The field site*year interaction was not significant and was removed from the model. Field site was not significant, however year was ($p<0.0001$). Therefore the data for each year within each field site was tested, and the UHR data were significant between 2006 and 2007 (p=0.0082) and between 2006 and 2008 ($p<0.0001$). To account for this difference, weather data in the forms of maximum, minimum, and average temperatures, as well as precipitation and departure from normal precipitation levels during the month of May (which accounts for most of the incubation period of the eggs) in Glens Falls, NY (which was the closest weather station to Fort Edward) for 2006, 2007, and 2008 were accessed via the NOAA website. Statistics were not performed on the data, however it was found that the most visually different data were the precipitation data (Figure 5). It was found that there was more precipitation than normal in 2006, while there was less precipitation than normal in 2007 and 2008.

Discussion

EROD activity has been used as a reliable indicator of environmental contaminants, specifically of co-planar dioxin-like compounds, since enzyme induction occurs via the aryl hydrocarbon receptor. This study measured the *in ovo* EROD response to PCB compounds in wild populations of tree swallows and eastern bluebirds. The results taken together indicate that PCB 126 and environmentally relevant PCB mixture both stimulated liver EROD. PCB 77 did not stimulate EROD activity as strongly as the other treatments in tree swallows, however the response was significant in bluebirds.

PCB 126- exposed birds showed an increasing trend of EROD induction (Figure 1), which was expected as PCB 126 is a known AhR agonist (Brunström and Anderson, 1988; Brunström and Halldin, 1998; Head and Kennedy, 2007; Safe, 2001).

EROD activity also increased between several doses in tree swallow eggs from Patuxent Research Refuge. This combined with a 5.4-fold difference as well as an increasing regression of 0.0002 presents clear evidence that this environmentally relevant mixture elicits a response in EROD in wild populations (Figure 2A). This same mixture, however, did not elicit a response either between dose groups or as an overall regression in tree swallows from Cobleskill Reservoir (Figure 2B). In addition, the same doses yielded a much smaller fold-change of 2.6, compared to more than twice the fold-change at the Patuxent site. This discrepancy can be explained either because the study is underpowered at the Cobleskill site, that the

Cobleskill site is not as pristine as expected, or that there are genetic differences between populations.

PCB 77 treatment had a significant effect on EROD activity in tree swallows from Patuxent Research refuge, however no significant difference was seen at Cobleskill Reservoir (Figures 3A and 3B). However, the fold-change at the Patuxent site was a much lower 1.4-fold, compared to the 5.4 fold-change seen in the same population of birds exposed to the PCB mixture. Therefore PCB 77 did not induce EROD activity as strongly as PCB 126 or the PCB mixture. This result was unexpected since PCB 77 is a non-otho-substituted PCB congener, which is hypothesized to be a strong ligand for the Ah receptor. However, eastern bluebirds showed significant EROD activity at Patuxent Research Refuge (Figure 3C). This response indicates that bluebirds are more sensitive to PCBs via AhR activation than tree swallows. Bluebirds have been shown to be very sensitive compared to most avian species regarding the ligand-binding domain of the AhR, while there is little information about tree swallow sensitivity (Head et al., 2008). The response in tree swallows was expected, as a similar response was seen in previous work we have done in Japanese quail exposed to the same PCB mixture and PCBs 126 and 77 (Bohannon et al., unpublished data; Chapter 3). The observed difference in response between tree swallows and bluebirds illustrate species specific variation in vulnerability to PCBs across birds.

Finally, the only site to differ year to year was UHR (Figure 4), with 2006 being different from both 2007 and 2008, however 2007 and 2008 not being different from each other. Comparing this to weather data, it is seen that 2006 year was wetter

than normal, while 2007 and 2008 were drier than normal (Figure 6). It is unclear why more rainfall would lead to higher EROD activity, however this indicates that birds that nest in the contaminated site are more sensitive to stress than birds at other sites. Therefore changes in the weather will result in an elevated response. Another hypothesis is that more rainfall may stir up the sediment and soil to release more PCBs into the water and cause a higher body burden in birds that eat water insects. In total, these studies demonstrate that EROD remains a good biomarker of exposure to PCBs in wild birds as has been previously reported, however response is dependent on dose as well as species. These studies also indicate that there are species differences in response, pointing to further evidence that species sensitivity to dioxin-like compounds plays a role in biomarker utility.

Figures

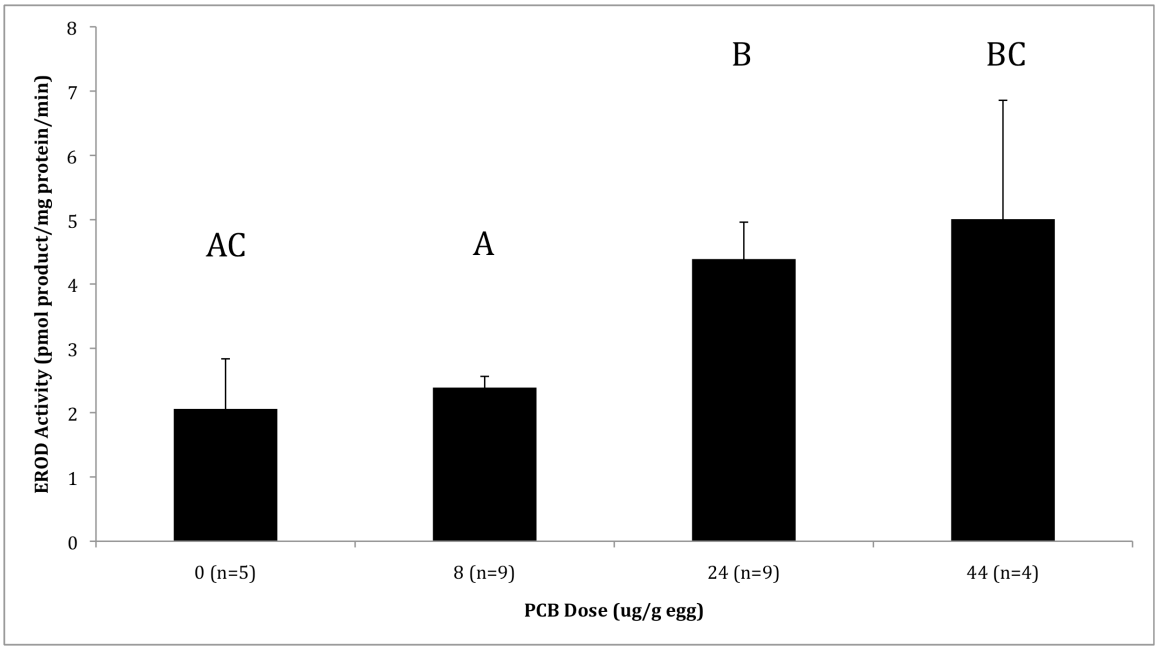


Figure 1: EROD activity increased monotonically in tree swallows at Great Sacandaga Lake experimentally exposed to PCB 126. A Wilcoxon analysis run with the NPAR1WAY procedure in SAS showed the control and low-dosed groups (8 ug/g egg) were statistically different from the medium-dosed group (24 ug/g egg; $p<0.05$), while the low dose was also different from the high-dosed (44 ug/g egg; $p=0.0500$). However the control was not significantly different from the high-dosed group due to high variance ($p=0.1542$). A regression analysis run with the GLM procedure in SAS was significant ($p=0.0216$).

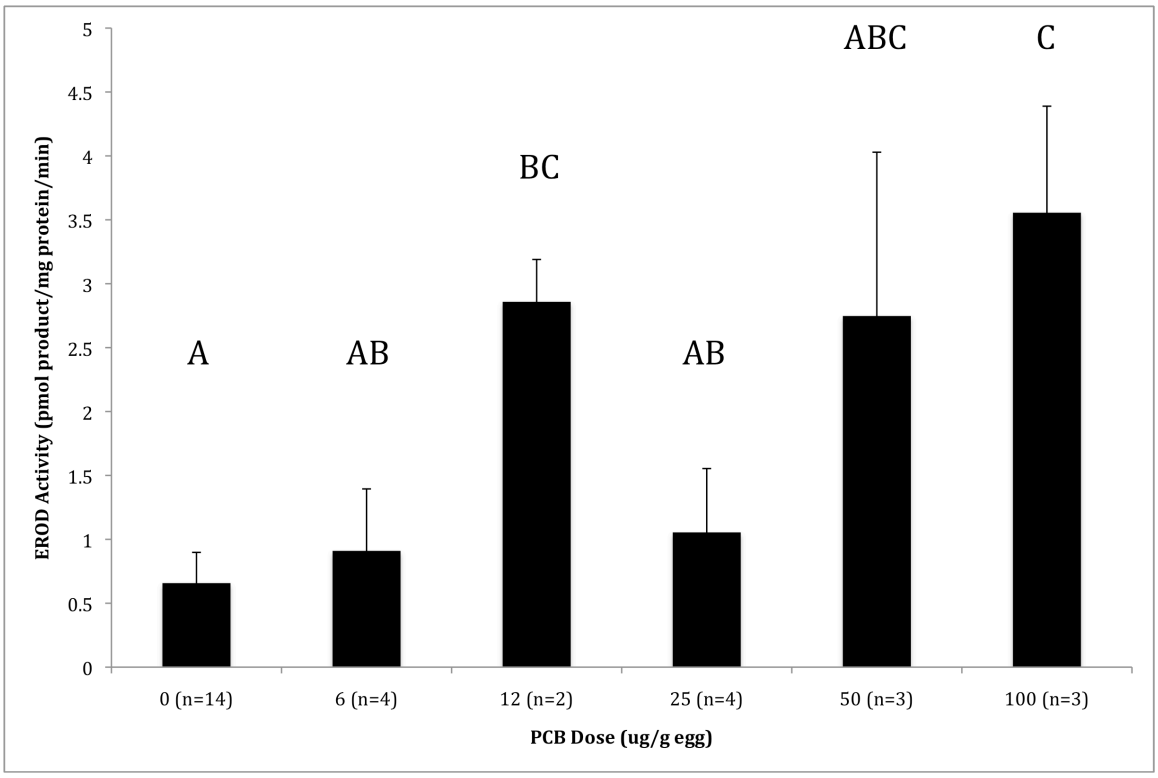


Figure 2A: EROD activity increased in tree swallows experimentally exposed to a PCB mixture at Patuxent Research Refuge. A Wilcoxon analysis using the NPAR1WAY procedure in SAS showed significant differences with higher responses seen in the 12, 50, and 100 ug/g dosed groups ($p<0.05$). A regression analysis using the GLM procedure in SAS was significant ($p=0.0002$). Untreated and 0 ug/g egg dosed samples were pooled for analysis.

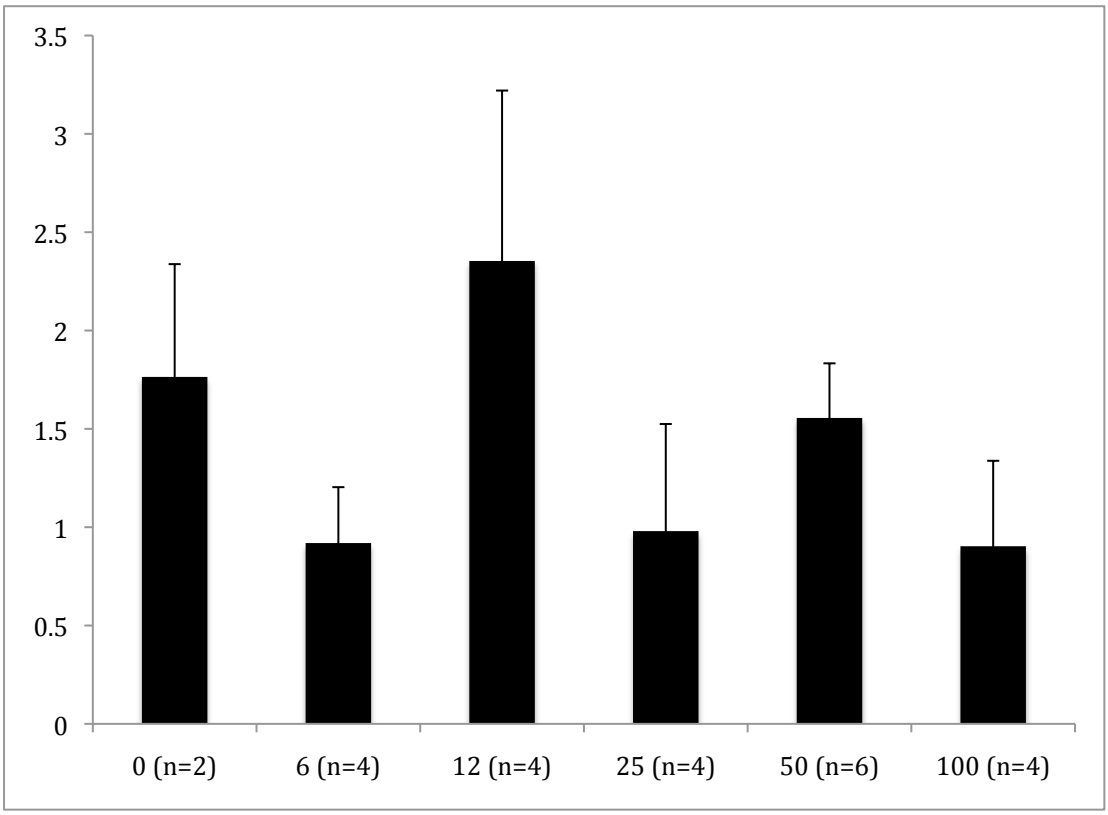


Figure 2B: EROD activity in tree swallows experimentally exposed to a PCB mixture at Cobleskill reservoir did not change with experimental exposure to a PCB mixture. A Wilcoxon analysis using the NPAR1WAY procedure in SAS did not yield any significant differences ($p>0.05$). A regression analysis using the GLM procedure in SAS was not significant ($p=0.4113$). The site-dose interaction between Patuxent Research Refuge and Cobleskil Reservoir was significant ($p=0.0006$). Untreated and 0 ug/g egg dosed samples were pooled for analysis.

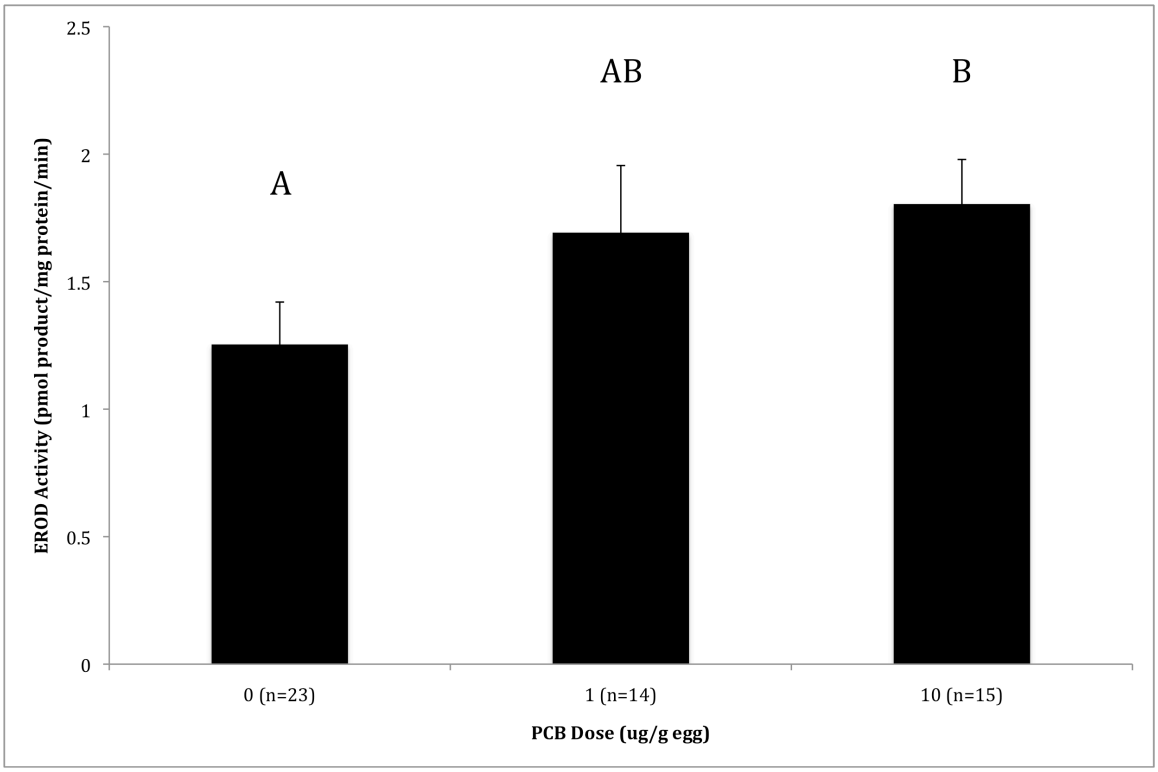


Figure 3A: EROD activity increased in tree swallows experimentally exposed to PCB 77 at Patuxent Research Refuge. A Wilcoxon analysis using the NPAR1WAY procedure in SAS yielded a significant difference between the control group and the high-dosed group (10 ug/g egg; p=0.0282). A regression analysis using the GLM procedure in SAS was not significant (p=0.1042). Untreated and 0 ug/g egg dosed samples were pooled for analysis.

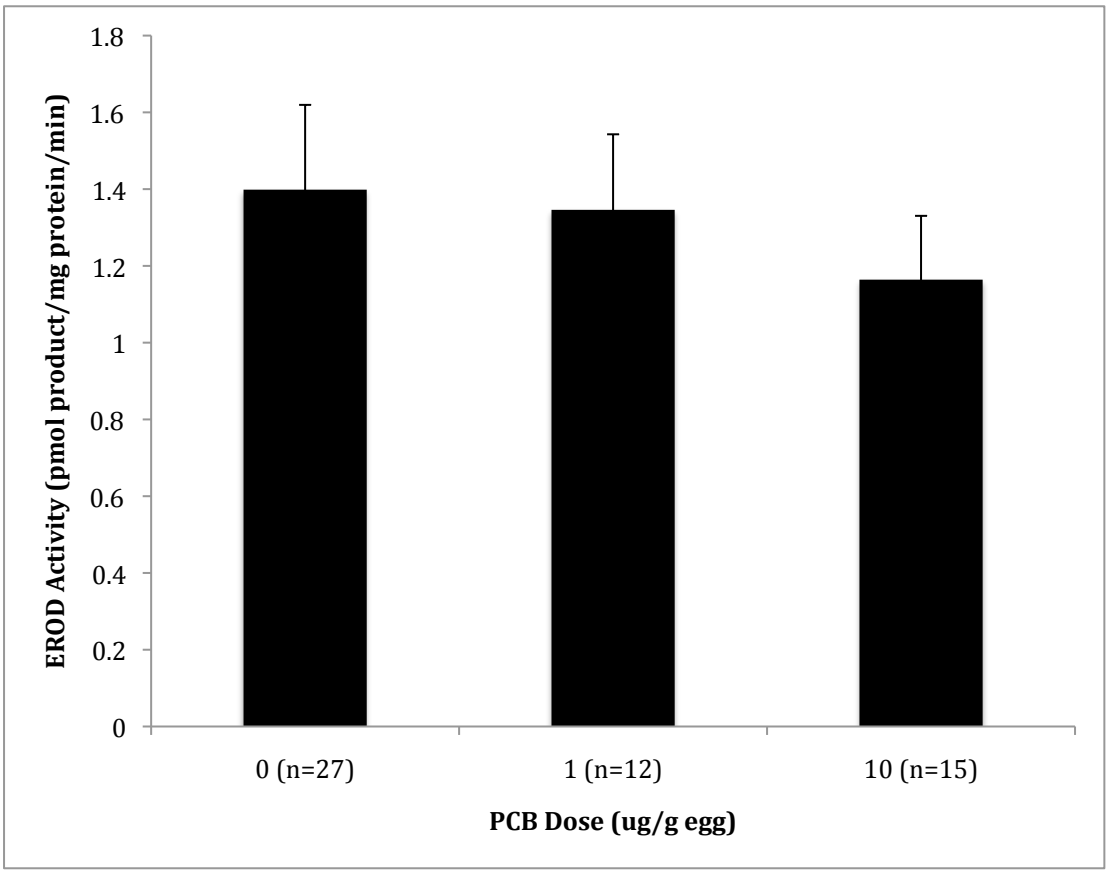


Figure 3B: EROD activity did not increase in tree swallows experimentally exposed to PCB 77 at Cobleskill Reservoir. A Wilcoxon analysis using the NPAR1WAY procedure in SAS yielded no significant differences between treatment groups ($p<0.05$). A regression analysis using the GLM procedure in SAS was not significant ($p=0.4376$). The site-dose interaction between Patuxent Research Refuge and Cobleskill Reservoir was not significant ($p=0.0948$). Untreated and 0 ug/g egg dosed samples were pooled for analysis.

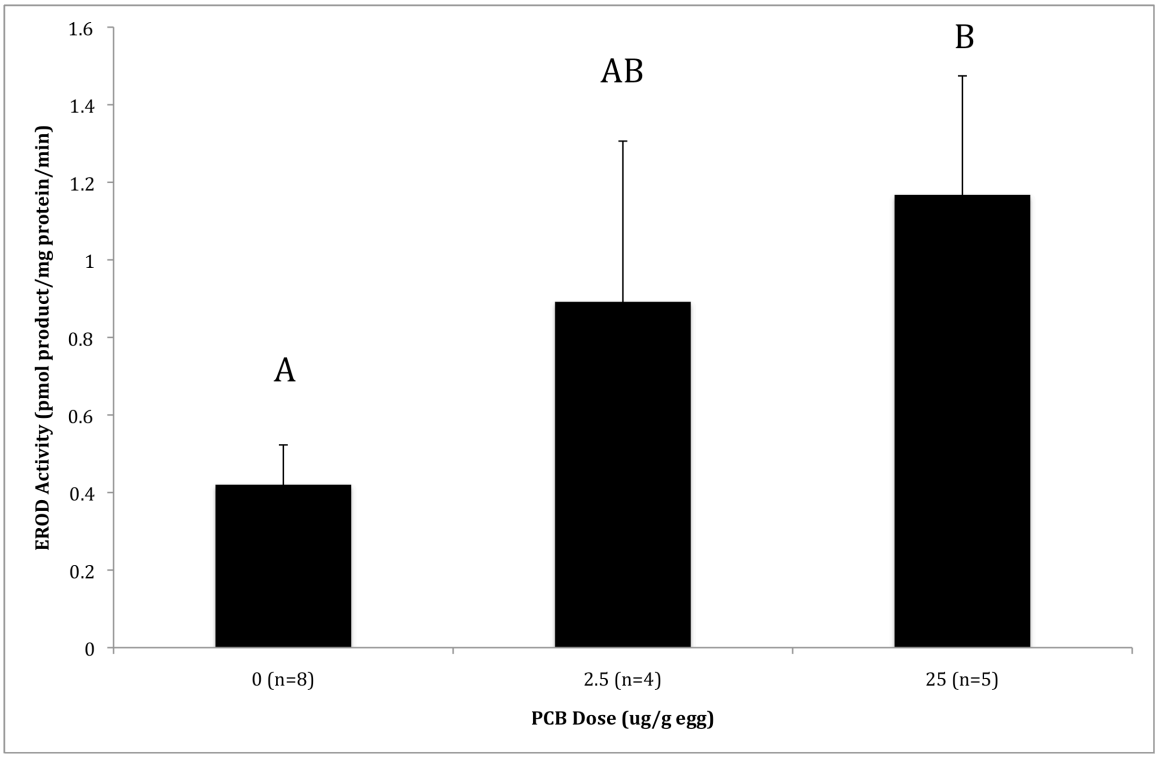


Figure 3C: EROD activity increased in bluebirds experimentally exposed to PCB 77 at Patuxent Research Refuge. A Wilcoxon analysis using the NPAR1WAY procedure in SAS yielded a significant difference between the control and high-dosed groups (10 ug/g egg; p=0.0281). A regression analysis using the GLM procedure in SAS was not significant (p=0.0597). Untreated and 0 ug/g egg dosed samples were pooled for analysis.

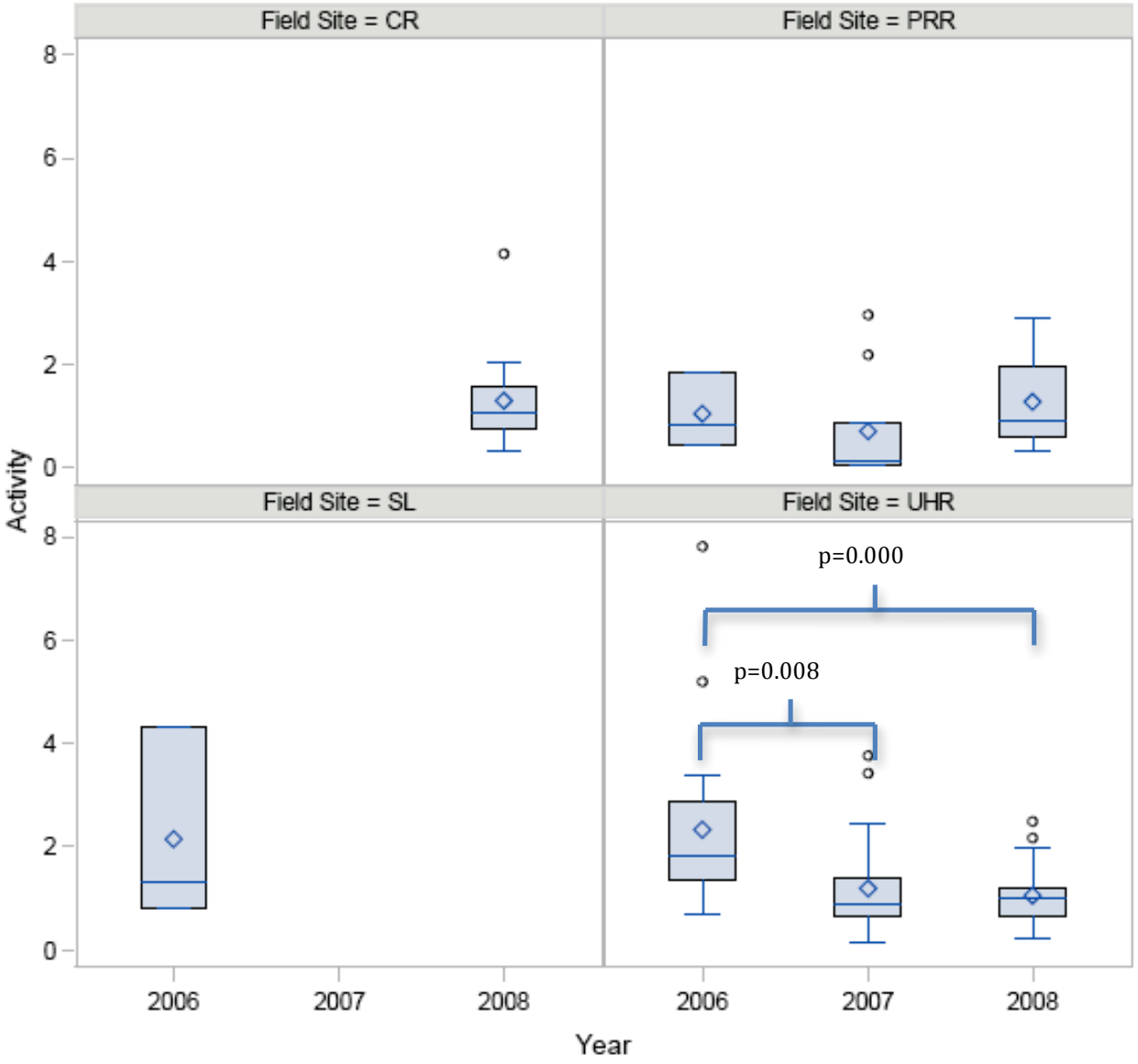


Figure 4: Two-way ANOVA for year and field site was run on untreated birds from all sites, including the UHR site. Significant differences were found between years 2006 and 2007 at Upper Hudson River, and between years 2006 and 2008 at Upper Hudson River (p=0.0082 and p<0.0001).

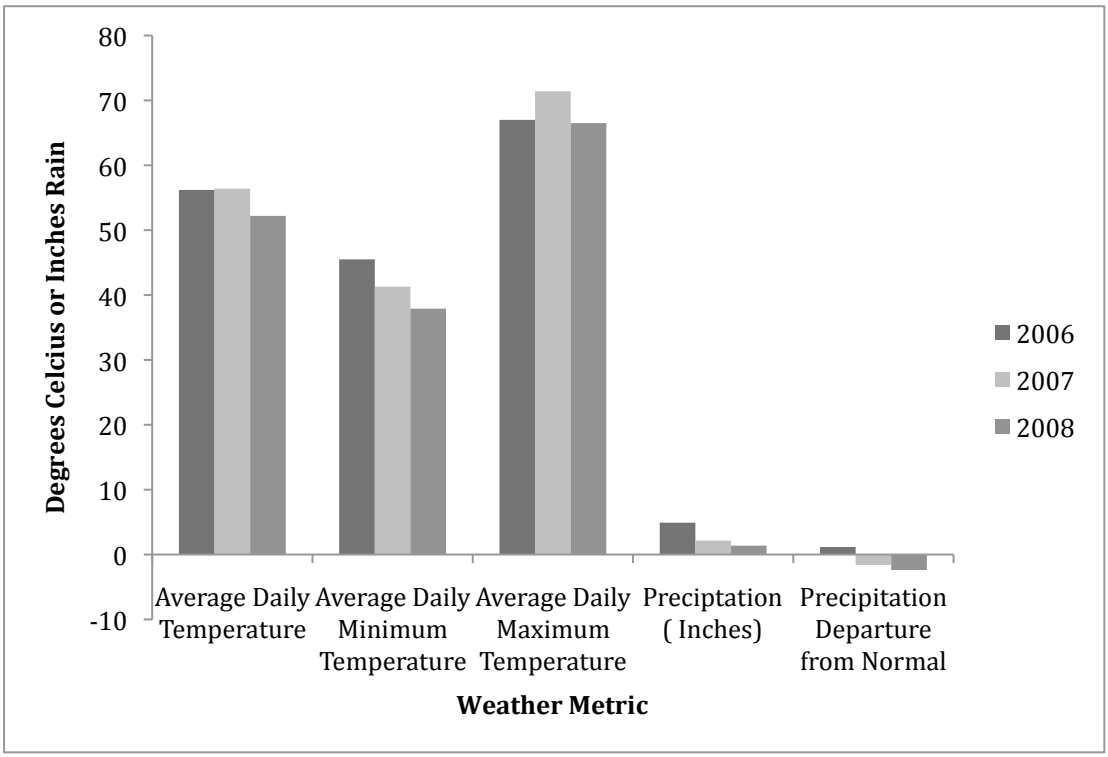


Figure 5: Weather data showed that precipitation in 2006 was higher than normal and was lower than normal in 2007 and 2008. No statistical analysis was performed on weather data.

Chapter 5: Final Discussion

The liver is an important organ in toxicity studies for its chemical processing and metabolism. Exogenous chemicals are metabolized in the liver, as in the case of xenobiotic compounds that are often toxic to the organism. Endogenous compounds are synthesized in the liver, as in the case of gluconeogenesis, lipogenesis, and glycolysis. Endogenous compounds that were synthesized and utilized in other tissues are metabolized in the liver in order to terminate their biological activity and to either recycle them for continued use or cleared from the body, as in the case with most non-protein hormones. These processes often happen cooperatively. Due to these functions, the liver has a high-energy requirement and therefore undergoes a high level of cellular respiration. Previous research has shown that liver damage and dysfunction result from exposure to environmental contaminants, therefore the liver is a key target organ during xenobiotic exposure.

The endpoints assessed in the research presented herein do not exhibit remarkable changes at the level of significance. There are several potential reasons for this. Insufficient numbers may explain this, as can be seen by many p-values, particularly in the lethality study, that approach significance ($p<0.10$). The doses of the birds that were measured for these sub-lethal endpoints were often lower than or equivalent to environmental levels, while birds dosed with higher than environmental levels did not survive. This indicates that these environmental levels are potentially not enough to produce a response. The liver could be a robust enough organ that low levels of exposure will not be enough to elicit a response.

Despite the low level of responses seen in these studies, analyses of the data provide a large amount of information about the hepatic responses to a xenobiotic challenge.

Microarray Study vs. Lethality Study

We used several powerful statistical tools to analyze the gene expression data in the microarray and lethality studies. The GLM procedure evaluated dose-response curves (DRCs) for 13 molecular and biochemical endpoints; six basic types of dose-response curves were observed from this analysis (Appendix 5). The cluster analysis from the microarray study also produced informed results for gene expression patterns. These analyses taken together lend strong evidence to varying biological effects of PCB exposure.

Positive and negative DRCs were seen in both analyses (Chapter 1, Figure 1, Panels C6, C8, and C9; Appendix 5, Panels D, E, and F). Of these, 2 of the 3 positive DRCs displayed a dramatic increase at low doses, a plateauing at medium doses, and a further increase at high doses. This indicates that a new biological set-point is possibly established with a high enough concentration of PCB exposure, but increased challenge is met with increased response, which contributes to survivability. The birds that survive at higher doses could be capable of mounting an increased response due to a genetic pre-disposition. The endpoints where this reponse was most noteable were CYP1A4, CYP1A5 (in both studies), and EROD. These results were expected, as they highlight the key role that the process of

xenobiotic metabolism has in the biological defense mechanisms against these compounds.

Another pattern seen in the DRCs are an increase at medium doses followed by a decrease that often dips below control levels at high doses. This was primarily seen in the lethality study DRCs (Appendix 5, Panels A and B). This inverted U shape is often identified as hormesis, the idea that a small challenge leads to increased response of a particular endpoint that contributes to the organism's survivability, but too much of a challenge will lead to mortality (Calabrese and Baldwin, 2001).
A third pattern of note is one where an increase is seen at low doses, a decrease is seen at medium doses, but the response returns to baseline (i.e. control levels) at high doses (Chapter 1, Figure 1, Panel C5; Appendix 5, Panel C). This points very strongly to the notion of a survivor effect. If the birds survive the initial challenge, a high challenge produces little change. This is not an adaptive response, however since the animals used in this study were hatchlings that had experienced a one-time dose. However, it is hypothesized that a chronic low dose would produce a similar effect over time.

ALDOB and CYB5 run with qPCR in the microarray study displayed a low-dose response that returned to baseline levels at high doses. This pattern was not seen in the cluster or GLM analyses.

Lethality Study vs. Wild Bird Study

Both male and female quail showed an increased dose response to PCB 126 with a slight downturn at higher doses. Tree swallows at Sacandaga Lake also had an

elevated response to PCB 126 without a downturn. However, it is important to note that the doses for the tree swallow study are in the μg/g range, while doses for the quail study are in the ng/g range, which is a 1000-fold difference. Higher mortality in quail to PCBs at a much lower concentration indicates that quail are more sensitive to PCB 126 than wild birds. However, it has been proposed that Japanese quail are less sensitive to dioxin-like compounds (such at PCB 126) than are other domesticated avian species such as chicken (*Gallus gallus*), due to an amino acid sequence difference in the ligand-binding region of the AhR that gives it a lower affinity to dioxin-like compounds (Head and Kennedy, 2007).

Tree swallows and Japanese quail were dosed with similar levels of the tree swallow PCB mixture, however there is still a discrepancy seen with mortality. There was 100% quail mortality in the high doses, however at similar doses, tree swallows were still alive and exhibiting EROD responses to the PCB challenge. Again, this supports that Japanese quail are more sensitive than tree swallows. Despite this discrepancy, this PCB mixture elicits a similar response in both species, namely that mortality does not occur until higher doses compared to PCB 126, and that the EROD response increases with increasing doses, often plateauing at higher concentrations (Figure 4 of Chapter 2 compared to Figure 2 of Chapter 3).

Tree swallows, bluebirds, and Japanese quail were dosed with PCB 77. Different concentration levels in tree swallows and Japanese quail still elicited a low to no EROD response in both species. This indicates that PCB 77 is a poor activator of the aryl hydrocarbon receptor. The avian TEQ for this congener is 0.05, which is half of that for PCB 126, and is one of the highest for all PCB congeners (Van den

Berg et al., 2005). These data suggest that this TEQ needs to be reassessed. Bluebirds, on the other hand, responded to PCB 77, which indicates that this species is more sensitive than the other two.

Pathway Responses

It is clear from these data that xenobiotic metabolism remains one of the most responsive pathways to contaminant exposure, based on the expression profiles of CYP1A4 and 1A5 and EROD activity levels in the lethality study. CYP1A5 was the most responsive gene on the microarray, at 2.5 fold response over controls.
It had been hypothesized that oxidative damage would be highly responsive. However, this system in the lethality study demonstrated low responsiveness at the significance level. Of the antioxidative genes (CAT, GPX, GR, GSTA, and TXN), the most remarkable responses exhibit mild increases at low or medium doses with a dramatic decrease at high doses. An example of this is seen with GSTA in SPSA-mixture-dosed females (Chapter 3, Figure 1K). This indicates that PCBs do not cause an excess of ROS production that would elicit a drastic physiological response. However, as the challenge increases, the body is unable to perform at normal levels.

The genes involved with energy balance and endocrine disruption (i.e. ALDOB, HCS, DIO2) did not show clear expression responses to PCBs in the lethality study, though they were both identified as being responsive on the microarray. An explanation for this might be that the responses detected on the microarray were very high compared to the rest of the genes on the array. It should be noted that the SPSA mixture doses measured in the microarray study were 0, 6, 12,

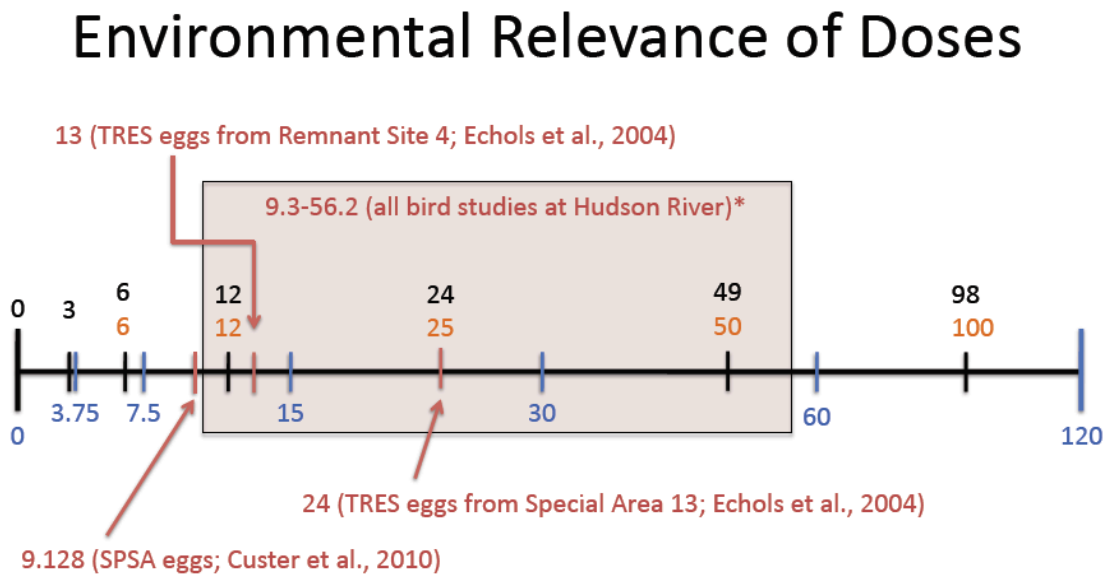
and 49 μg/g egg, while the doses measured in the lethality study only went up to 30 μg/g egg for the same mixture. Therefore, perhaps birds that survive to higher doses also mount higher responses.

These results contribute to the adverse outcome pathways (AOP) model of toxicity testing and risk assessment (Ankley et al., 2010; Kramer et al., 2011). AOP constructs rely on multiple species, compounds, biological levels of organization, and compound-biochemical interactions to create informative analyses for risk assessment while relying on less toxicity testing. These data show responses at the cellular level that point to organ responses such as homeostasis disruption as seen in endocrine function and energy balance (Chapter 2, Table 4; Ankley et al., 2010). Therefore these data confirm previous suspicions that biochemical pathways other than xenobiotic metabolism are affected by xenobiotic exposure, which is informative to the AOP model.

These data are also informative at the population level. Birds at the Hudson River show differential EROD activity seasonally, which may be linked to weather and precipitation patterns (Chapter 4, Figure 4), while birds at cleaner sites do not. In addition, birds at Patuxent Research Refuge exhibited a higher response to a PCB mixture and to PCB 77 than birds at Cobleskill Reservoir (Chapter 4, Figures 2 and 3). These data will also be informative to the AOP model.

Appendices

Appendix 1: Environmental Relevance of spotted sandpiper and tree swallow mixture doses



* Secord et al., 1999; HRNR Trustees, 2005; Custer et al., 2003; Henny and Elliott, 2007
Black/Orange = SPSA/TRES mix for microarray/wild bird studies
Blue = SPSA and TRES mix for lethality study
Units in ug/g egg or wet weight

Appendix 2: PCB Mixture TEQs

Table 1: Tree Swallow Mixture Congener Concentrations and TEQs

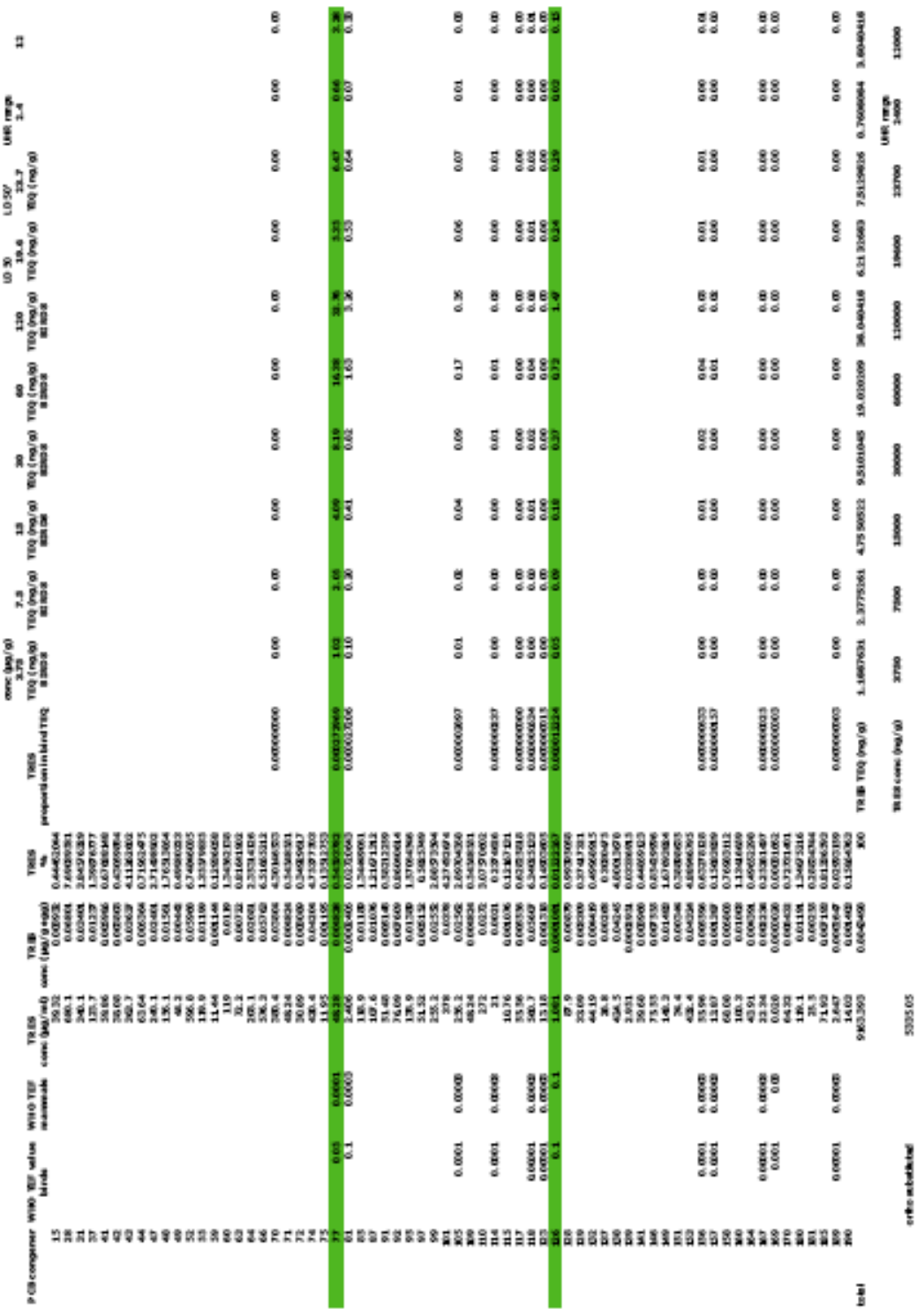


Table 2: Spotted Sandpiper Mixture Congener Concentrations and TEQs

Appendix 3: Primer Information for Chapters 2 and 3

Table 1: Primer Information for Chapter 2

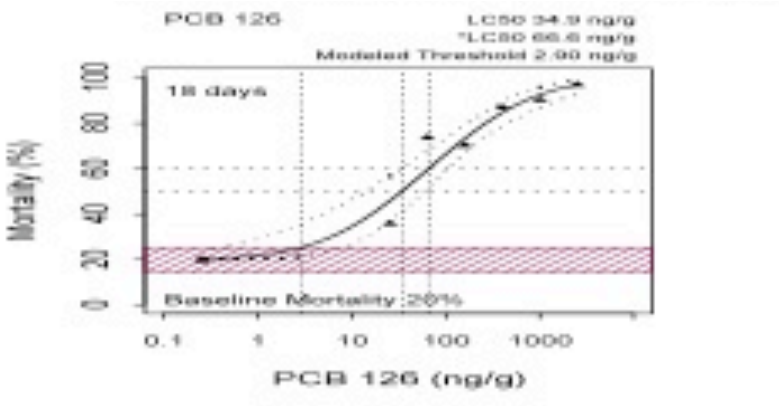
Gene Name	Abbreviation	Forward Primer	Reverse Primer	Primer Concentration (uM)	Tm	Efficiency
Fructose Bisphosphate Aldolase B	ALDOB	GCGGGCGCAGATTAATAGTTTG	GTGGCAGCTGTGTCAGTCTT	0.8	62.0	1.02
Cytochrome P450 1A5	CYP1A5	CAGCTGAGGCTGATTGACAA	CCCCAGGCCAAAAGTCATCA	0.8	62.0	1.03
Glutathione S-Transferase Alpha	GSTA	GCGAGTTTCCTGTCTTGCAGAG	TTTTCCTCTAGGCGTGGTTT	0.8	55.5	1.03
Cytochrome B5	CYB5	TCCGGGGACCGGCATAGCA	TCACTCTGACATGTAGGAMCGATA	0.8	57.5	1.05
Glyceraldehyde 3-phosphate dehydrogenase	GAPDH	AAGGAGTGAGCCAAGCACACA	TCACTGCAGGATGCAGAACTG	0.8	62.0	0.956
Beta Actin	Beta Actin	CAGGATGCAGAAGGAGATCACA	TAGAGCCTCCAATCCAGACAGAGTA	0.8	62.0	1

Table 2: Primer Information for Chapter 3

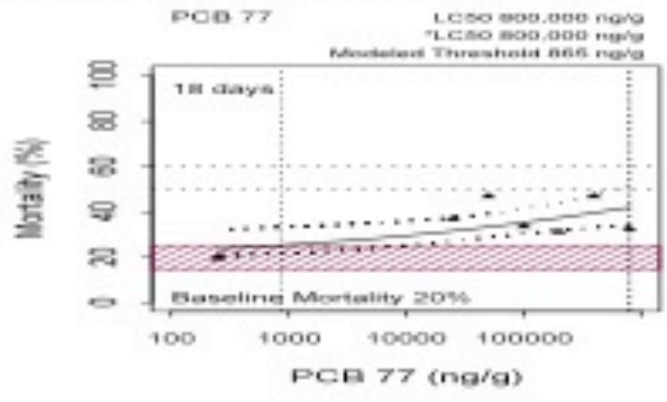
Gene Name	Abbreviation	Forward Primer	Reverse Primer	Primer Concentration (uM)	Tm (°C)	Efficiency	
						Male	Female
Aryl Hydrocarbon Receptor 1	AHR1	CCTACTGGCTGTGATGCAAA	GCATATCAGTGCTGAACG	2.5	67.3	0.975	0.977
Fructose Bisphosphate Aldolase B	ALDOB	GCGGGCGCAGATTAATAGTTTG	GTGGCAGCTGTGTCAGTCTT	0.8	62	0.932	0.965
Beta Actin	BA	CAGGATGCAGAAGGAGATCACA	TAGAGCCTCCAATCCAGACAGAGTA	0.8	62	1.00	1.05
Catalase	CAT	CCTCGAGGCTTTGCAATGAA	GAAACAACATTGCATCCCGAA	1.0	60.1	0.973	0.936
Cytochrome B5	CYB5	TCCGGGGGACCGGCATAGCA	TCACTCTGACATGTAGGAACGATA	0.8	57.5	1.05	1.03
Cytochrome P450 1A4	CYP1A4	TGGGAGGTCTTCCTCTTCCT	GCGTTTCTTCATCTGGAAGC	1.0	60.1	1.01	0.949
Cytochrome P450 1A5	CYP1A5	TTCAACCCAGAGCGGTTCC	TTTTCCCCAATGCACCTCCT	2.75	70	0.96	0.98
Deiodinase Type 2	DIO2	ACGCCTACAAGCAGGTCAAA	TTCCACACTTGCCACCAACA	1.5	60.1	1.01	1.03
Glyceraldehyde 3-phosphate dehydrogenase	GAPDH	AAGGAGTGAGCCAAGCACACA	TCACTGCAGGATGCAGAACTG	0.8	62	0.982	1.02
Glutathione Peroxidase	GPX	CCAAGGAGGCTCTGCAATAA	TTCGAGGATTTGGAAGATGC	2.75	63	0.98	0.913
Glutathione Reductase	GR	TGAGTTCCAGAACACCACCA	TGGTTACCAAAGAGCCGAAG	1.0	64	0.956	1.05
Glutathione S-Transferase Alpha	GSTA	GCGAGTTTCCTGTCTTGCAGAG	TTTTCCTCTAGGCGTGGTTT	0.8	55.5	0.976	0.978
HMG-CoA Synthase	HCS	ACGCCTTCAGTCTACGGTTG	CGTAGCAGCAAAACCAGAGC	1.0	60.2	0.988	1.00
Thioredoxin	TXN	ATCGATGTGGATGATGCCCA	CCCCAGAGAATTCCTGCACC	1.0	60.1	0.936	0.919

Appendix 4: Lethality curves from the Probit analysis (conducted by Dave Cacela) of the dose related mortality of quail embryos for (A) PCB 126, (B) PCB 77, (C) TRES mix and (D) SPSA mix. The shaded area represents control mortality.

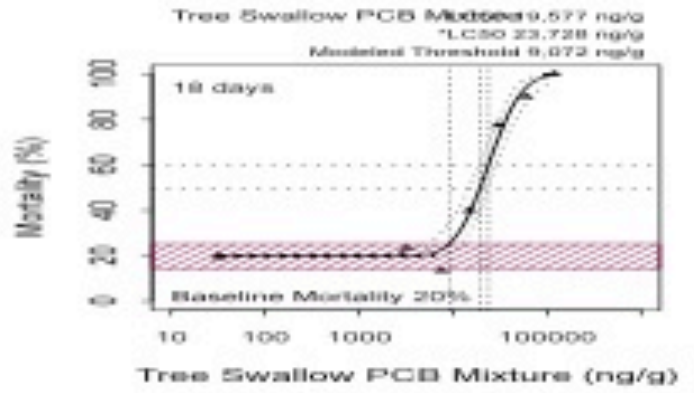
A)



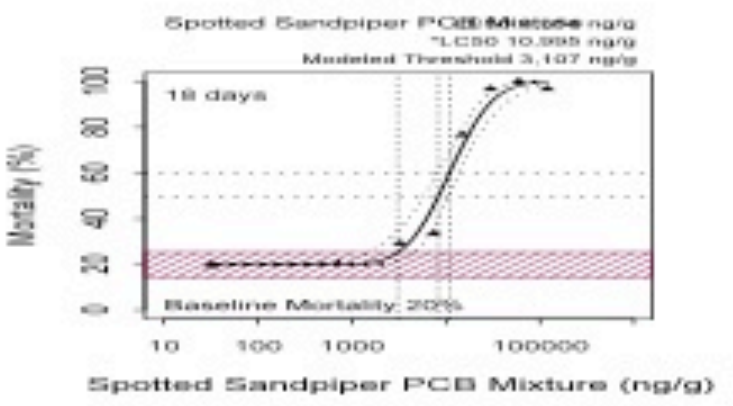
B)



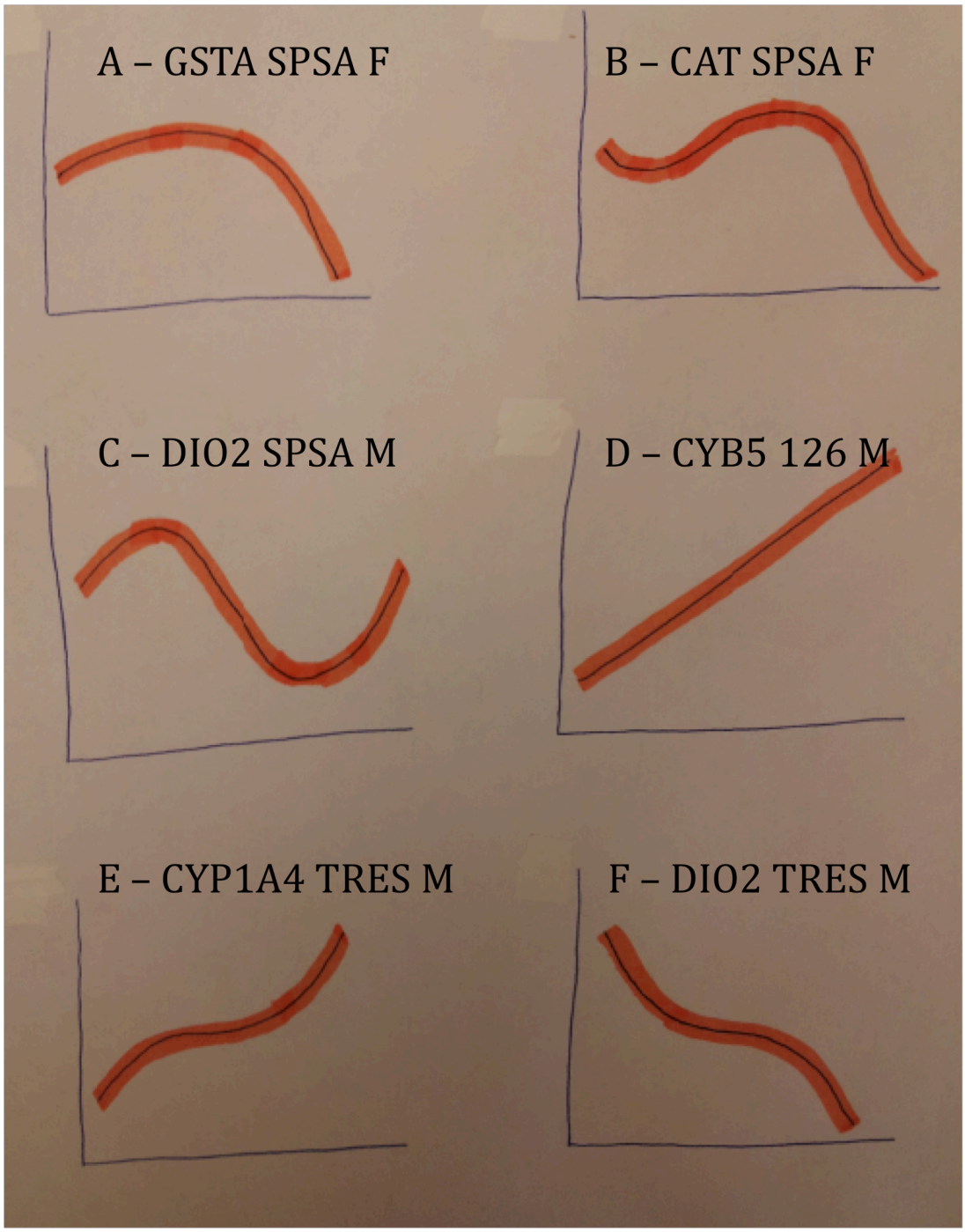
C)



D)


Spotted Sandpiper PCB Mixture ng/g
*LC50 10,995 ng/g
Modeled Threshold 3,107 ng/g
18 days
Mortality (%)
0
20
40
60
80
100
Baseline Mortality: 20%
10
100
1000
100000
Spotted Sandpiper PCB Mixture (ng/g)

Appendix 5: Dose-response curves differed with sex, compound, and gene. Six basic types of dose-response curves were observed in 13 endpoints (Chapter 3)



Bibliography

Aly, H.A.A. and Domènech, O. 2009. Aroclor 1254 induced cytotoxicity and mitochondrial dysfunction in isolated rat hepatocytes. Toxicology 262: 175-183.

Ames, B.N., Shigenaga, M.K., and Hagen, T.M. 1993. Oxidants, antioxidants, and the degenerative diseases of aging. Proceeding of the National Academy of Sciences, USA 90: 7915-7922.

Andreyev, A.Y., Kushnareva, Y.E., and Starkov, A.A. 2005. Mitochondrial Metabolism of Reactive Oxygen Species. Biochemistry (Moscow) 70(2): 246-264.

Ankley, G.T., Bennett, R.S., Erickson, R.J., Hoff, D.J., Hornung, M.W., Johnson, R.D., Mount, D.R., Nichols, J.W., Russom, C.L., Schmieder, P.K., Serrano, J.A., Tietge, J.E., and Villeneuve, D.L. 2010. Adverse outcome pathways: a conceptual framework to support ecotoxicology research and risk assessment. Environmental Toxicology and Chemistry 29(3): 730-741.

Anway, M.D., A.S. Cupp, M. Uzumcu, and M.K. Skinner. 2005. Epigenetic transgenerational actions of endocrine disruptors and male fertility. Science 308: 1466-1469.

Bak, M., Kicinska-Krogulska, M., Czerniak, P., Michowicz, A., Krakowiak, A. 2011. Toxic Liver Injuries: A Current View on Pathogenesis, Part II. Medycyna Pracy 62(2): 203-210.

Basu, N, Scheuhammer, A.M., Bursian, S.J., Elliott, J., Rouvinen-Watt, K., Chan, H.M. 2007. Review: mink as a sentinel species in environmental health. Environmental Research 103: 130-144.

Beland, P., Vezina, A., and Martineau, D. 1988. Potential for growth of the St. Lawrence (Quebec, Canada) Beluga whale (Delphinapterus leucas) population based on modeling. ICES Journal of Marine Science 45(1): 22-32.

Biessmann, A. 1982. Effects of PCBs on gonads, sex hormone balance and reproduction process of Japanese quail Coturnix coturnix japonica after ingestion during sexual maturaltion. Environmental Pollution (Series A) 27(1): 15-30.

Bremer, K.E. Dealing with multi-agency and multi-state regulation of polychlorinated biphenyls, in PCBs: Human and Environmental Hazards, D'Itri, F.M., and Kamrin, M.A., Eds., Butterworths, Woburn, MA, 1983, 367.

Brown, J.F., Bedard, D.L., Brennan, M.J., Carnahan, J.C., Feng, H., and Wagner R.E. 1987. Polychlorinated biphenyls dechlorination in aquatic sediments. Science 236(4802): 709-712.

Brown, J.F., Jr., Mayes, B.A., Silkworth, J.B., and Hamilton, S.B. 2007. Polychlorinated biphenyls-modulated turmorigenesis in Sprague-Dawley rats: correlation with mixed function oxidase activities and superoxide (O_2^-) formation potentials and implied mode of action. Toxicological Sciences 98(2): 375-394.

Brunström, B. 1988. Sensitivity of embryos from duck, goose, herring gull, and various chicken breeds to 3,3',4,4'-tetrachlorobiphenyl. Poultry Science 67(1): 52-57.

Brunström, B. 1989. Toxicity of coplanar polychlorinated biphenyls in avian embryos. Chemosphere 19(1-6): 765-768.

Brunström, B. and Halldin, K. 1998. EROD induction by environmental contaminants in avian embryo livers. Comparative Biochemistry and Physiology, Part C, 121: 213-219.

Bustnes, J.O., Tveraa, T., Henden, J.A., Varpe, O., Janssen, K., and Skaare, J.U. 2006. Organochlorines in Antarctic and Arctic avian top predators: a comparison between the south polar skua and two species of northern hemisphere gulls. Environmental Science and Technology 40: 2826-2831.

Cadenas E. and Davies K.J. 2000. Mitochondrial free radical generation, oxidative stress, and aging. Journal of Free Radicals in Biology and Medicine 29: 222-230.

Calabrese, E.J. and Baldwin, L.A. 2001. Hormesis: U-shaped Dose Responses and Their Centrality in Toxicology. Trends in Pharmacological Sciences 22(6): 285-291.

Cesh, L.S., Elliott, K.H., Quade, S., McKinney, M.A., Maisoneuve, F., Garcelon, D.K., Sandau, C.D., Letcher, R.J., Williams, T.D., and Elliott, J.E. 2010. Polyhalogenated aromatic hydrocarbons and metabolites: Relation to circulating thyroid hormone and retinol in nestling bald eagles (Haliaeetus leucocephalus). Environmental Toxicology and Chemistry 29 (6): 1301-1310.
Capel, P.D., and Eisenreich, S.J. 1985. PCBs in Lake Superior, 1978-1980. Journal of Great Lakes Research 11: 447-461.

Carlson, E.A., Roy, N.K., and Wirgin, I.I. 2009. Microarray analysis of polychlorinated biphenyl mixture-induced changes in gene expression among Atlantic tomcod populations displaying differential sensitivity to halogenated aromatic hydrocarbons. Environmental Toxicology and Chemistry 28(4): 759-771.

Chiu, A., Chiu, N., Beaubier, N.T., Beaubier, J., Nalesnik, R., Singh, D., Hill, W.R., Lau, C., and Riebow, J. 2000. Effects and mechanisms of PCB ecotoxicity in food chains: algae ð fish ð seal ð polar bear. Journal of Environmental Science and Health, Part C: Environmental Carcinogenesis and Ecotoxicology Reviews 18(2):127-152.

Cogburn, L.A., Wang, X., Carre, W., Rejto, L., Aggrey, S.E., Duclos, M.J., Simon, J., and Porter, T.E. 2004. Functional genomics in chickens: development of integrated-systems microarrays for transcriptional profiling and discovery of regulatory pathways. Comparative and Functional Genomics 5: 253-261.

Colborn, T. 1991. Epidemiology of Great-Lakes bald eagles. Journal of Toxicology and Environmental Health 33(4): 395-453.

Crews, D. and J.A. McLachlan. 2006. Epigenetics, evolution, endocrine disruption, health, and disease. Endocrinology 147 (6) (Supplement): S4-S10.

Custer, T.W., Custer, C.M., Hines, R.K., Sparks, D.W., Melancon, M.J., Hoffman, D.J., Bickham, J.W., Wickliffe, J.K. 2000. Mixed function oxygenases, oxidative stress, and chromosomal damage in lesser scaup wintering on the Indiana Harbor Canal. Archives of Environmental Contamination and Toxicology 38(4): 522-529.

Custer, T.W., Custer, C.M., Gray, B.R. 2010. Polychlorinated biphenyls, dioxins, furans, and organochlorine pesticides in spotted sandpiper eggs from the upper Hudson River basin, New York. Ecotoxicology 19(2): 391-404.

Dean, K.M., Marcell, A.M., Carpenter, L.D., Lipton, J., Cacela, D., Carro, T., Bohannon, M.E.B., and Ottinger, M.A. 2010. Comparison of lethality following *in ovo* exposure to PCB 126, PCB 77, and two environmentally relevant PCB mixtures in the Japanese quail (*Coturnix japonica*). Manuscript in review.

Durfee, R.L., Contos, G., Whitmore, F.C., Barden, J.D., Hackman, E.E., III, and Westin, R.A., PCBs in the United States – Industrial Use and Environmental Distribution, Report No. EPA 56016-16-005, Versar, Inc., Springfield, VA, 1976, 254.

Echols, K.R., Tillitt, D.E., Nichols, J.W., Secord, A.L., and McCarty, J.P. 2004. Accumulation of PCB congeners in nestling tree swallows (*Tachycineta bicolor*) on the Hudson River, New York. Environmental Science and Technology 38: 6240-6246.

Elliott, J.E., Kennedy, S.W., Peakall, D.B., and Won, H. 1990. Polychlorinated biphenyl (PCB) effects on hepatic mixed function oxidases and porphyria in birds. I. Japanese quail. Comparative Biochemistry and Physiology, Part C, 96: 205-210.

El-Shahawi, M.S., A. Hamza, A.S. Bashammakh, W.T. Al-Saggaf. 2010. An overview on the accumulation, distribution, transformations, toxicity, and analytical methods for the monitoring of persistent organic pollutants. Talanta 80: 1587-1597.

Faust, D., Vondráček, J., Krčmář, P., Šmerdová, L., Procházková, J., Hrubá,E., Hulinková, P., Kaina, B., Dietrich, C., Machala, M. 2013. AhR-mediated changes in

global gene expression in rat liver progenitor cells. Archives of Toxicology 87: 681-698.

Feng, H., Cochran, J.K., Lwiza, H., Brownawell, B.J., and Hirschberg, D.J. 1998. Distribution of heavy metal and PCB contaminants in the sediments of an urban estuary: the Hudson River. Marine Environmental Research 45(1): 69-88.

Fernie, K., and Bortolotti, G. 2003. Reproductive abnormalities, teratogenicity, and developmental problems in American kestrels (Falco sparverius) exposed to polychlorinated biphenyls. Journal of Toxicology and Environmental Health, Part A, 66: 2089-2103.

Fernie, K., Bortolotti, G., Drouillard, K., Smits, J., and Marchant, T. 2003a. Developmental toxicity of *in ovo* exposure polychlorinated biphenyls: II. Effects of maternal or paternal exposure on second-generation nestling American kestrels. Environmental Toxicology and Chemistry 22(11): 2688-2694.

Fernie, K., Smits, J., and Bortolotti, G. 2003b. Developmental toxicity of in ovo exposure to polychlorinated biphenyls: I. Immediate and subsequent effects on first-generation nestling American kestrels (Falco sparverius). Environmental Toxicology and Chemistry 22(3): 554-560.

Fischer, L.J., Seegal, R.F., Ganey, P.E., Pessah, I.N., Kodavanti, P.R. 1998. Symposium overview: toxicity of non-coplanar PCBs. Toxicological Sciences 41(1): 49-61.

Frame, G.M., Cochran, J.W. and Bowad, S.S. 1996. Complete PCB congener distributions for 17 Aroclor mixtures determined by 3 HRGC systems optimized for comprehensive, quantitative, congener-specific analysis. Journal of High Resolution Chromatography 19: 657-668.

Frame, G.M., Wagner, R.E., Carnahan, J.C., Brown, J.F., Jr., May, R.J., Smullen, L.A., and Bedard, D.L. 1996. Comprehensive, quantitative, congener-specific analyses of eight Aroclors and complete PCB congener assignments on DB-1 capillary GC columns. Chemosphere 33(4): 603-623.

Geneious Ver.6.16 created by Biomatters. Available from http://www.geneious.com/.

Genex Ver. 5.4.4, MultiD Analyses AB, Gothenburg, Sweden.

Giesy, J.P., Ludwig, J.P., and Tillitt, D.E. 1994. Deformities in birds of the Great Lakes region: assigning causality. Environmental Science and Technology 28(3): A128-A135.

Gilday, D., Gannon, M., Yutzey, K., Bader, D., and Rifkind, A.B. 1996. Molecular cloning and expression of two novel avian cytochrome P450 1A enzymes induced by

2,3,7,8-tetrachlorodibenzo-p-dioxin. Journal of Biological Chemistry 271: 33054-33059.

Gilbertson, M., Kubiak, T., Ludwig, J., and Fox, G. 1991. Great Lakes embryo mortality, edema, and deformities syndrome (GLEMEDS) in colonial fish-eating birds: similarity to chick-edema disease. Journal of Toxicology and Environmental Health 33(4): 455-520.

Glauert, H.P., Tharappel, J.C., Lu, Z., Stemm, D., Baneriee, S., Chan, L.S., Lee, E.Y., Lehmler, H.J., Robertson, L.W., and Spear, B.T. 2008. Role of oxidative stress in the promoting activities of PCBs. Environmental Toxicology and Pharmacology 25(2):247-50.

Grasman, K.A., Fox, G.A., Scanlon, P.F., Ludwig, J.P. 1996. Organochlorine-associated immunosuppression in prefledgling Caspian terns and herring gulls from the Great Lakes: an ecoepidemiological study. Environmental Health Perspectives 104, Supplement 4: 829-842.

Grasman, K.A, Scanlon, P.F., and Fox, G.A. 1998. Reproductive and physiological effects of environmental contaminants in fish-eating birds of the Great Lakes: a review of historical trends. Environmental Monitoring and Assessment 53:117-145.

Grove, R.A., C.J. Henny, J.L. Kaiser. 2009. Osprey: worldwide sentinel species for assessing and monitoring environmental contamination in rivers, lakes, reservoirs, and estuaries. Journal of Toxicology and Environmental Health, Part B 12: 25-44.

Gust. K.A., M. Pirooznia, M.J. Quinn, Jr., M.S. Johnson, L. Escalon, K.J. Indest, X. Guan, J. Clarke. Y. Deng, P. Gong, and E.J. Perkins. 2009. Neurotoxicogenomic investigations to assess mechanisms of action of the munitions constituents RDX and 2,6-DNT in Northern bobwhite (*Colinus virginianus*). Toxicological Sciences 110(1): 168-180.

Gutierrez, P.L. 2000. The metabolism of quinone-containing alkylating agents: free radical production and measurement. Frontiers in Bioscience 5 (d629-638).

Halldin, K., Bergman, Å., Brandt, I., Brunström, B. 2005. Developmental toxicity in Japanese quail exposed to hydroxylated metabolites of PCBs *in ovo*. Avian and Poultry Biology Reviews 16(1): 11-17.

Harding, L.E., Harris, M.L., Stephen, C.R., and Elliott, J.E. 1999. Reproductive and morphological condition of wild mink (Mustela vison) and river otters (Lutra canadensis) in relation to chlorinated hydrocarbon contamination. Environmental Health Perspectives 107(2): 141-147.

Hargrave, B.T., Harding, G.C., Vass, W.P., Erickson, P.E., Fowler, B.R., and Scott, V. 1992. Organochlorine pesticides and polychlorinated biphenyls in the Arctic

Ocean food web. Archives of Environmental Contamination and Toxicology 22: 41-54.

Head, J.A., Hahn, M.E. Hahn, and Kennedy, S.W. 2008. Key amino acids in the aryl hydrocarbon receptor predict dioxin sensitivity in avian species. Environmental Science and Technology 42: 7535-7541.

Head, J.A. and Kennedy, S.W. 2007. Same-sample analysis of ethoxyresorufin-*O*-deethylase activity and cytochrome P450 1A mRNA abundance in chicken embryo hepatocytes. Analytical Biochemistry 360: 294-302.

Hervé, J.C., Crump, D., Jones, S.P., Mundy, L.J., Giesy, J.P., Zwiernik, M.J., Bursian, S.J., Jones, P.D., Wiseman, S.B., Wan, Y., and Kennedy, S.W. 2010. Cytochrome P4501A induction 2,3,7,8-tetrachlorodibenzo-*p*-dioxin and two chlorinated dibenzofurans in primary hepatocyte cultures of three avian species. Toxicological Science 113(2): 380-391.

Hestermann, E.V., Stegeman, J.J., and Hahn, M.E. 2000. Relative contributions of affinity and intrinsic efficacy to aryl hydrocarbon receptor ligand potency. Toxicology and Applied Pharmacology 168(2): 160-172.

Hettling, L.J., Horn, E.G., and Tofflemeier, T.J. 1978. Summary of Hudson River PCB Study Results, Technical paper 51, Department of Environmental Conservation, Albany, NY.

Hoffman, D.J., Melancon, M.J., Klein, P.N., Eisemann, J.D., and Spann, J.W. 1998. Comparative developmental toxicity of planar polychlorinated biphenyl congeners in chickens, American kestrels, and common terns. Environmental Toxicology and Chemistry 17(4): 747-757.

Hoffman, D.J., Rice, C.P., and Kubiak, T.J. PCBs and Dioxins in Birds. In *Environmental Contaminants in Wildlife: Interpreting Tissue Concentrations*, eds. Beyer, W.N., Heinz, G.A., and Redmon-Norwood, A.W. Boca Raton: Lewis Publishers, 1996. Chapter 7, pp. 165-207.

Hoogesteijn, A.L., Kollias, G.V., Quimby, F.W., De Caprio, A.P., Winkler, D.W., and DeVoogd, T.J. 2008. Development of a brain nucleus involved in song production in zebra finches (Taeniopygia guttata) is disrupted by Aroclor 1248. Environmental Toxicology and Chemistry 27(10): 2071-2075.

Hudson River Natural Resources Trustees. 2013. PCB Contamination Of The Hudson River Ecosystem: Compilation Of Contamination Data Through 2008. January.

Hutzinger, O., Nash, D., Safe, S., Norstrom, R.J., Wildish, D.J., and Zitko, V. 1972. Polychlorinated biphenyls: metabolic behavior of pure isomers in pigeons, rats, and brook trout. Science 178: 312.

Iguchi, T., H. Watanabe, and Y. Katsu. 2007. Minireview: Toxicogenomics and ecotoxicogenomics for studying endocrine disruption and basic biology. General and Comparative Endocrinology 153: 25-29.

Jancova, P., Anzenbacher, P., Anzenbacherova, E. 2010. Phase II drug metabolizing enzymes. Biomedical Papers of the Medical Faculty of the University Palacký, Olomouc, Czechoslovakia 154(2): 103-16.
Jensen, S. 1972. The PCB Story. Ambio 1(4): 123-131.

Johnson BL, Hicks HE, Cibulas W, Faroon O, Ashizawa AE, DeRosa CT, Cogliano J, and Clark M. 2000. Public health implications of exposure to polychlorinated biphenyls (PCBs). Agency for Toxic Substances and Disease Registry, Div of Toxicol, Atlanta, GA, USA.

Knerr S. and Schrenk D. 2006. Carcinogenicity of "non-dioxinlike" polychlorinated biphenyls. Critical Reviews in Toxicology 36 (9): 663-694.

Kramer, V.J., Etterson, M.A., Hecker, M., Murphy, C.A., Roesijadi, G., Spade, D.J., Spromberg, J.A., Wang, M., Ankley, G.A. 2011. Adverse outcome pathways and ecological risk assessment: bridging to population-level effects. Environmental Toxicology and Chemistry 30(1): 64-76.

Kubota, A., Iwata, H., Goldstone, H.M.H., Kim, E.-Y., Stegeman, J.J., and Tanabe, S. 2006. Cytochrome P450 1A4 and 1A5 in common cormorant (Phalacrocorax carbo): evolutionary relationships and functional implications associated with dioxin and related compounds. Toxicological Sciences 92(2):394-408.

Lavoie, E.T., and K.A. Grasman. 2007. Effects of in ovo exposure to PCBs 126 and 77 on mortality, deformities, and post-hatch immune function in chickens. Journal of Toxicology and Environmental Health, Part A, 70: 547-558.

Letcher, R.J., Bustnes, J.O., Dietz, R. Jenssen, B.M., Jorgensen, E.H., Sonne, C., Verreault, J., Vijayan, M.M., and Gabrielsen, G.W. 2010. Exposure and effects assessment of persistent organohalogen contaminants in arctic wildlife and fish. Science of the Total Environment. In press.

Mahajan, S.S. and Rifkind, A.B. 1999. Transcriptional activation of avian CYP1A4 and CYP1A5 by 2,3,7,8-tetrachlorondibenzo-*p*-dioxin: differences in gene expression and regulation compared to mammalian CYP1A1 and CYP1A2. Toxicology and Applied Pharmacology 155: 96-106.

Manning, G.E., Mundy L.K., Crump, D., Jones, S.P., Chiu, S., Klein, J., Konstantinov, A., Potter, D., and Kennedy, S.W. 2013. Cytochrome P4501A induction in avian hepatocyte cultures exposed to polychlorinated biphenyls: Comparisons with AHR1-mediated reporter gene activity and in ovo toxicity. Toxicology and Applied Pharmacology 266: 38-47.

Mariussen, E. and Fonnum, F. 2006. Neurochemical targets and behavioral effects of organohalogen compounds: an update. Critical Reviews in Toxicology 36: 253-289.

McKernan, M.A., Rattner, B.A., Hale, R.C., and Ottinger, M.A. 2009. Toxicity of polybrominated diphenyl ethers (DE-71) in chicken (*Gallus gallus*), mallard (*Anas platyrhynchos*), and American kestrel (*Falco sparverius*) embryos and hatchlings. Environmental Toxicology and Chemistry 28(5):1007-1017.

McKernan, M.A., Rattner, B.A., Hatfield, J.S., Hale, R.C., and Ottinger, M.A. 2010. Absorption and biotransformation of polybrominated diphenyl ethers DE-71 and DE-79 in chicken (*Gallus gallus*), mallard (*Anas platyrhynchos*), American kestrel (*Falco sparverius*), and black-crowned night heron (*Nycticorax nycticorax*) eggs. Chemosphere 79(1): 100-109.

McNabb, F.M.A. 2005. Biomarkers for the assessment of avian thyroid disruption by chemical contaminants. Avian and Poultry Biology Reviews 16(1): 3-10.

Melancon, M.J. Bioindicators of contaminant exposure and effect in aquatic and terrestrial monitoring. In *Handbook of Toxicology 2nd Edition*, eds. Hoffman, D.J., Rattner, B.A., Burton, Jr., G.A., and Cairns, Jr., J. Boca Raton: Lewis Publishers, 2003. Chapter 11, pp. 257-278.

Melancon, M. J., Development of Cytochromes P450 in Avian Species as a Biomarker for Environmental Contaminant Exposure and Effect: Procedures and Baseline Values, Environmental Toxicology and Risk Assessment: Biomarkers and Risk Assessment (5th. Volume), ASTM STP 1306*, David A. Bengston and Diane S. Henshel, Eds., American Society for Testing and Materials, Philadelphia, 1997.

Melancon, M., Kutay, A.L., Woodin, B.R., and Stegeman, J.J. 2006. Evaluating cytochrome P450 in lesser scaup (*Aythya affinis*) and tree swallow (*Tachycineta bicolor*) by monooxygenase activity and immunohistochemistry: possible nonlethal assessment by skin immunohistochemistry. Environmental Toxicology and Chemistry 25(10): 2613-2617.

Menzel, R., H.L. Yeo, S. Rienau, S. Li, C.E.W. Steinberg, and S.R. Sturzenbaum. 2007. Cytochrome P450s and short-chain dehydrogenases mediate the toxicogenomic response of PCB52 in the nematode *Caenorhabditis elegans*. Journal of Molecular Biology 370: 1-13.

Mitchelmore, C.L., Birmelin, C., Chipman, J.K., and Livingstone, D.R. 1998. Evidence for cytochrome P450 catalysis and free radical involvement in the production of DNA strand breaks by benzo[a]pyrene and nitroaromatics in mussel (*Mytilus edulis* L.) digestive gland cells. Aquatic Toxicology 41: 193-212.

National Oceanographic and Atmospheric Agency website.
http://www.erh.noaa.gov/aly/Climate/GlensFalls/2006/gfllcdmay06.html
http://www.erh.noaa.gov/aly/Climate/GlensFalls/2007/gfllcdmay07.html
http://www.erh.noaa.gov/aly/Climate/GlensFalls/2008/gfllcdmay08.html

New York State Department of Environmental Conservation Website. Preassessment Screen Determination for the Hudson River.
http://www.dec.ny.gov/lands/32758.html

Niimi, A.J. PCBs in Aquatic Organisms. In *Environmental Contaminants in Wildlife: Interpreting Tissue Concentrations*, eds. Beyer, W.N., Heinz, G.A., and Redmon-Norwood, A.W. Boca Raton: Lewis Publishers, 1996. Chapter 5, pp. 117-152.

Nikolaidis, E. Brunström, B., and Dencker, L. 1989. Effects of TCDD and its congeners 3,3',4,4'-tetrachloroazoxybenzene and 3,3',4,4'-tetrachlorobiphenyl on lymphoid development in the bursa of Fabricius and thymus of the avian embryo. Chemosphere 19(1-6): 817-822.

Norstrom, R.J., and Hebert, C.E. 2006. Comprehensive re-analysis of archived herring gull eggs reconstructs historical temporal trends in chlorinated hydrocarbon contamination in Lake Ontario and Green Bay, Lake Michigan, 1971-1982. Journal of Environmental Monitoring 8: 835-847.

Ohtake F, Baba A, Takada I, Okada M, Iwasaki K, Miki H, Takahashi S, Kouzmenko A, Nohara K, Chiba T, Fujii-Kuriyama Y, Kato S. 2006. Dioxin receptor is a ligand-dependent E3 ubiquitin ligase. Nature 446: 562-566.

Ohtake, F., Takeyama, K., Matsumoto, T., Kitagawa, H., Yamamoto, Y., Nohara, K., Tohyama, C., Krust, A., Mimura, J., Chambon, P., Yanagisawa, J., Fujii-Kuriyama, Y., and Kato, S. 2003. Modulation of oestrogen receptor signaling by association with the activated dioxin receptor. Nature 423: 545-550.

Ojeda, S.R. Female Reproductive Function. In *Textbook of Endocrine Physiology, 5th Edition*, eds. Griffin, J.E. and Ojeda, S.R. Oxford: Oxford University Press, 2004. 186-225.

Oliver, B.G. and Niimi, A.J. 1988. Trophodynamic analysis of polychlorinated biphenyl congeners and other chlorinated hydrocarbons in the Lake Ontario ecosystem. Environmental Science and Technology 22: 388-397.

Ottinger, M.A., Carro, T., Bohannon, M., Baltos, L., Marcell, A. M., McKernan, M., Dean, K.M., Lavoie, E., Abdelnabi, M. 2013. Assessing effects of environmental chemicals on neuroendocrine systems: potential mechanisms and functional outcomes. General and Comparative Endocrinology 190: 194-202.

Ottinger, M.A., Lavoie, E.T. , Abdelnabi, M. , Quinn, Jr., M.J., Marcell, A., and Dean, K.. 2009a. An overview of dioxin-like compounds, PCB, and pesticide exposures associated with sexual differentiation of neuroendocrine systems, fluctuating asymmetry, and behavioral effects in birds. Journal of Environmental Science and Health Part C, 27: 286-300.

Ottinger, M.A., Lavoie, E.T., Thomspon, N., Bohannon, M., Dean, K, and Quinn, Jr., M.J. 2009b. Is the gonadotropin releasing hormone system vulnerable to endocrine disruption in birds? General and Comparative Endocrinology 163: 104-108.

Padhi, B.K., Pelletier, G., Williams, A., Berndt-Weis, L., Yauk, C., Bowers, W.J., and Chu, I. 2008. Gene expression profiling in rat cerebellum following in utero and lactational exposure to mixtures of methylmercury, polychlorinated biphenyls, and organochlorine pesticides. Toxicology Letters 176: 93-103.

Porter TE, and Ellestad LE. *Gene expression profiling in the developing neuroendocrine system of the chick. In: Functional Avian Endocrinology, edited by Dawson A, Sharp PJ. New Delhi: Narosa, 2005, p. 45a, 2*

Rattner, B.A., Melancon, M.J., Rice, C.P., Riley, Jr., W., Eisemann, J., and Hines, R.K. 1997. Cytochrome P450 and organochlorine contaminants in black-crowned night herons from the Chesapeake Bay region, USA. Environmental Toxicology and Chemistry 16(11): 2315-2322.

Rattner, B.A., Melancon, M.J., Custer, T.W., and Hothem, R.L. 1996. Cytochrome P450 and contaminant concentrations in nestling black-crowned night-herons and their interrelation with sibling embryos. Environmental Toxicology and Chemistry 15(5): 715-721.

Rattner, B.A., Hatfield, J.S., Melancon, M.J., Custer, T.W., and Tillitt, D.E. 1994. Relation among cytochrome-P450, AH active PCB congeners and dioxin equivalents in pipping black-crowned night-heron embryos. Environmental Toxicology and Chemistry 13(11): 1805-1812.

Rattner, B.A., Hoffman, D.J., and Marn, C.M. 1989. Use of mixed-function oxygenases to monitor contaminant exposure in wildlife. Environmental Toxicology and Chemistry 8:1093-1102.

Reeves, R. and Mitchell, E. 1984. Catch history and initial population of white whales (Delphinapterus leucas) in the River and Gulf of St. Lawrence, Eastern Canada. Naturaliste Canadien 111(1):63-121.

Reich, M., Ohm, K., Tamayo, P., Angelo, M., and Mesirov, JP. 2004. GeneCluster 2.0: an advanced toolset for bioarray analysis. Bioinformatics 20: 1797-1798.

Rice, C.P., P.W. O'Keefe, and T.J. Kubiak. Sources, Pathways, and Effects of PCBs, Dioxins, and Dibenzofurans. In *Handbook of Toxicology 2nd Edition*, eds. Hoffman, D.J., Rattner, B.A., Burton, Jr., G.A., and Cairns, Jr., J. Boca Raton: Lewis Publishers, 2003. Chapter 18, pp. 501-573.

Ryan, D.E., Thomas, P.E., and Levin, W. 1977. Properties of purified liver microsomal cytochrome P-450 from rats treated with the polychlorinated biphenyl mixture Aroclor 1254. Molecular Pharmacology 13: 521.

Ryan, D.E., Thomas, P.E., Korzeniowski, D., and Levin, W. 1979. Separation and charaterization of highly purified forms of liver microsomal cytochrome P-450 from rats treated with polychlorinated biphenyls, Phenobarbital, and 3-methylcholanthrene. Journal of Biological Chemistry 254: 1365.

Saeed, A.I., Sharov, V., White, J., Li, J., Liang, W., Bhagabati, N., Braisted, J., Klapa, M., Currier, T., Thiagarajan, M., Sturn, A., Snuffin, M., Rezantsev, A., Popov, D., Ryltsov, A., Kostukovich, E., Borisovsky, I., Liu, Z., Vinsavich, A., Trush, V., and Quackenbush, J. 2003. TM4: a free, open-source system for microarray data management and analysis. Biotechniques 34: 374-378.

Safe, S. 2001. PCBs as aryl hydrocarbon receptor agonists: implications for risk assessment. In Robertson, L.W. and Hansen, L.G. *PCBs: Recent Advances in Environmental Toxicology and Health Effects*. Louisville, KY: University Press of Kentucky. 171-177.

Safe, S. 1990. Polychlorinated biphenyls (PCBs), dibenzo-p-dioxins (PCDDs), dibenzofurans (PCDFs) and related compounds: environmental and mechanistic considerations which support development of toxic equivalency factors (TEFs). Critical Reviews in Toxicology 21: 51-88.

Safe, S. 1984. Polychlorinated biphenyls (PCBs) and polybrominated biphenyls (PBBs): biochemistry, toxicology, and mechanism of action. CRC Critical Reviews in Toxicology 13(4): 319-395).

Safe, S. Metabolism, uptake, storage, and bioaccumulation. In *Halogenated Biphenyls, Terphenyls, Naphthalenes, Dibenzodioxins, and Related Products*, eds. Kimbrough. R.D. and A.A. Jensen. New York: Elsevier/North Holland Biomedical Press, 1980. Chapter 5.

Sanders, J.E. 1989. PCB-pollution problem in the Upper Hudson River: from environmental disaster to environmental gridlock. Northeastern Environmental Science 8(1):1-86.

Sanderson, J.T., Kennedy, S.W., and Giesy, J.P. 1998. In vitro induction of ethoxyresorufin-O-deethylase and porphyrins by halogenated aromatic hydrocarbons in avian primary hepatocytes. Environmental Toxicology and Chemistry 17: 2006-2018.

SAS software version 9.3, copyright© 2011, Cary, NC.

Scandalios, J.G. 2005. Oxidative Stress: Molecular Perception and Transduction of Signals Triggering Antioxidant Gene Defenses. Brazilian Journal of Medical and Biological Research 38: 995-1014.

Scanes, C.G., and McNabb, F.M.A. 2003. Avian models for research in toxicology and endocrine disruption. Avian and Poultry Biology Reviews 14(1): 21-52.

Schlezinger, J.J., Keller, J., Verbrugge, L.A., Stegeman, J.J. 2000. 3,3`,4,4`-tetrachlorobiphenyl oxidation in fish, bird, and reptile species: relationship to cytochrome P450 1A inactivation and reactive oxygen species. Comparative Biochemistry and Physicology Part C: Pharmacology, Toxicology, and Endocrinology 125 (3): 273-286.

Secord A.L., McCarty, J.P., Echols, K.R., Meadows J.C., Gale R.W., Tillitt, D.E. 1999. Polychlorinated biphenyls and 2,3,7,8-tetrachlorodibenzo-p-dioxin equivalents in tree swallows from the Upper Hudson River, New York State, USA. Environmental Toxicology and Chemistry 18: 2519-2525.

Shertzer, H.G., Genter, M.B., Shen, D., Nebert, D.W., Chen Y., and Dalton, T.P. 2006. TCDD decreases ATP levels and increases reactive oxygen production through changes in mitochondrial F0F1-ATP synthase and ubiquinone. Toxicology and Applied Pharmacology 217(3): 363-374.

Shimada, M., Kameo, S., Sugawara, N., Yaginuma-Sakurai, K., Kurokawa, N., Mizukami-Murata, S., Nakai, K., Iwahashi, H., Satoh, H. 2009. Gene expression profiles in the brain of the neonate mouse perinatally exposed to methylmercury and./or polychlorinated biphenyls. Archives of Toxicology 84(4): 271-286.

Silkworth, J.B., E.A. Carlson, C. McCulloch, K. Illouz, S. Goodwin, and T.R. Sutter. 2008. Toxicogenomic analysis of gender, chemical, and dose effects in livers of TCDD- or Aroclor 1254-exposed rats using a multifactor linear model. Toxicological Sciences 102(2): 291-301.

Sloan, R.J., Simpson, K.W., Schroeder, R.A., and Barnes, C.R. 1983. Temporal trends toward stability of Hudson River PCB contamination. Bulletin for Environmental Contamination and Toxicology 31: 377-385.

Stefani, G. and F.J. Slack. 2008. Small non-coding RNAs in animal development. Nature Reviews Molecular Cell Biology 9: 219- 230.

Swackhamer, D.L. and Armstrong, D.E. 1987. Distribution and characterization of PCBs in Lake Michigan waters. Journal of Great Lakes Research 13: 24-36.

Tanabe, S., Hidaka, H., and Tatsukawa, R. 1983. PCBs and chlorinated pesticides in Antarctic atmosphere and hydrosphere. Chemosphere 12: 277-288.

Tanabe, S., Tanaka, H., and Tatsukawa, R. 1984. Polychlorobipheyls, ΣDDT, and hexachlorocyclohexane isomers in the western North Pacific ecosystem. Archives of Environmental Contamination and Toxicology 13: 731-738.

Tanaka, T., Nishiyama, Y., Okada, K., Hirota, K., Matsui, M., Yodoi, J., Hiai, H., and Toyokuni, S. 1997. Induction and nuclear translocation of thioredoxin by oxidative damage in the mouse kidney: independence of tubular necrosis and sulfhydryl depletion. Laboratory Investigations 77(2):145-55.

Toschik, P.C., Rattner, B.A., McGowan, P.C., Christman, M.C., Carter, D.B., Hale, R.C., Matson, C.W., and Ottinger, M.A. 2005. Effects of contaminant exposure on reproductive success of ospreys (*Pandion haliaetus*) nesting in Delaware River and Bay, USA. Environmental Toxicology and Chemistry 24(3): 617-628.

Tsankova, N., W. Renthal, A. Kumar, and E.J. Nestler. 2007. Epigenetic regulation in psychiatric disorders. Nature Reviews Neuroscience 8: 355-367.

USEPA Website. Summary of TSCA | Laws and Regulations | US EPA. Last updated on 10/21/2010.
http://www.epa.gov/lawsregs/laws/tsca.html

Van den Berg, M., Craane. B.L.H.J., Sinnige, T., van Mourik, S., Dirksen, S., Boudewijn, T., Van der Gaag, M., Lutke-Schipholt, I., Spenkelink, B., and Brouwer, A. 1994. Biochemical and toxic effects of polychlorinated biphenyls (PCBs), dibenzo-*p*-dioxins (PCDDs), and dibenzofurans (PCDFs) in the cormorant (Phalacrocorax carbo) after *in ovo* exposure. Environmental Toxicology and Chemistry 13(5): 803-816.

Van den Berg, M., Birnbaum, L., Bosveld, A.T.C., Brunström, B., Cook, P., Feeley, M., Giesy, J.P., Hanberg, A., Hasegawa, R., Kennedy, S.W., Kubiak, T., Larsen, J.C., van Leeuwen, F.X.R., Liem. A.K.D., Nolt, C., Peterson, R.E. Poellinger, L., Safe,S., Schrenk, D., Tillitt, D., Tysklind, M., Waern, R., and Zacharewski, T. 1998. Toxic equivalency factors (TEQs) for PCBs, PCDDs, PCDFs for humans and wildlife. Environmental Health Perspectives 106(12): 775-792.

Van den Berg, M., Birnbaum, L., Denison, M., De Vito, M., Farland, W., Feeley, M., Fiedler, H., Hakansson, H., Hanberg, A., Haws, L., Rose, M., Safe, S., Schrenk, D.,

Tohyama, C., Tritscher, A., Tuomisto, J., Tysklind, M., Walker, N., and Peterson, R.E. 2006. The 2005 World Health Organization reevaluation of human and mammalian toxic equivalency factors for dioxins and dioxin-like compounds. Toxicological Sciences 93(2): 223-241.

Vezina, C.M., Walker, N.J., Olson, J.R. 2004. Subchronic exposure to TCDD, PeCDF, PCB126, and PCB153: effects on hepatic gene expression. Environmental Health Perspectives 114(16) Toxicogenomics: 1636-1644.

Wan, Y., Jones, P.D., Holem, R.R., Khim, J.S., Chang, H., Kay, D.P., Roark, S.A., Newsted, J.L., Patterson, W.P., and Giesy, J.P. 2010. Bioaccumulation of polychlorinated dibenzo-*p*-dioxins, dibenzofurans, and dioxin-like polychlorinated biphenyls from the Tittabawassee and Saginaw Rivers, Michigan, USA. Science of the Total Environment 408: 2394-2401.

Webb, C.M. and McNabb, F.M.A. 2008. Polychlorinated biphenyl effects on avian hepatic enzyme induction and thyroid function. General and Comparative Endocrinology 155: 650-657.

Weseloh, D.V., Ewins, P.J., Struger, J., Mineau, P., Bishop, C.A., Postupalsky, S., Ludwig, J.P. 1995. Double-crested cormorants of the Great Lakes: changes in population size, breeding distribution, and reproductive output between 1913 and 1991. Colonial Waterbirds 18, Special Publication 1: The Double-Crested Cormorant: Biology, Conservation, and Management: 48-59.

Wirgin I, Roy NK, Loftus M, Chambers RC, Franks DG, and Hahn ME. 2011. Mechanistic Basis of Resistance to PCBs in Atlantic Tomcod from the Hudson River. Science 331 (6022): 1322-1325.

Wright, D.A. and Welbourn, P. Organic Compounds. In *Cambridge Environmental Chemistry Series, Volume 11: Environmental Toxicology*. Cambridge: Cambridge University Press, 2002. Chapter 7, pp. 349-407.

Yoshimura, T. 2003. Yusho in Japan. Industrial Health 41(3): 139-148.

You, L., Sar, M., Bartolucci, E., Ploch, S., Whitt, M. 2001. Induction of hepatic aromatase by *p,pp*-DDE in adult male rats. Molecular and Cellular Endocrinology 178: 207-214.

I can do all things in Christ, who strengthens me.
- Phillipians 4:13