Health Impacts of Polychlorinated Biphenyls (PCBs) in Aquatic Organisms

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HEALTH IMPACTS OF POLYCHLORINATED BIPHENYLS (PCBs)
IN AQUATIC ORGANISMS

by

Marla Ann Fisher

Advisor: Charles F. Ide, Ph.D.

A Dissertation
Submitted to the
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Marla Ann Fisher
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CHAPTER 1

OVERVIEW OF DISSERTATION RESEARCH

Toxic contaminants are ubiquitous in the environment and in wildlife, with new xenobiotics both intentionally and non-intentionally introduced into the ecosystem. Currently, over 10,000 chemicals are produced on an industrial scale, with several thousands of new chemicals introduced each year. Via runoff, atmospheric fate and transport, and other geophysical processes, xenobiotics cycle through the environment, eventually accumulating in water reservoirs such as oceans, lakes, and rivers resulting in contamination of resident wildlife species. In addition to the “global background” contamination patterns present in tissue compartments of examined species, certain xenobiotics have been shown to be correlated with health impacts when present at high environmental concentrations. For example, lead, mercury, and polychlorinated biphenyls in the Great Lakes watershed have been associated with adverse health effects in wildlife and in human populations, and restrictions and/or bans have been placed on these and many other chemicals in order to mitigate the observed adverse health impacts. However, despite contamination at high doses, the health risks to aquatic species in many watersheds are not known. In this research I attempt to understand the relationship between environmental contamination and health of aquatic organisms, with the objective of providing
biological information and research tools that can be used to aid in environmental risk assessment.

In this research, I focus on a class of synthetically produced compounds, polychlorinated biphenyls (PCBs). PCBs were produced from about 1930 until 1977, when mounting toxicological studies in human and in wildlife populations instigated a ban on their production (ATSDR, 2000). PCBs have many desirable properties for industrial and electrical applications, such as low flammability, low solubility, low vapor pressure, and resistance to breakdown by other chemicals. PCBs were consequently widely used for industrial applications, with about 2.2 to 3.3 million tons produced worldwide. However, due to their resistance to breakdown, PCBs persist in the environment as part of the “global background”. Additionally, there are many watersheds worldwide that are areas of concern due to high PCB contamination levels. Since PCBs are highly lipophilic, PCBs bioaccumulate in fat tissue, rendering aquatic organisms and especially organisms high on the food chain greatly enriched in PCBs compared to environmental levels.

I examined the relationship between environmental exposure and potential health effects of PCBs to aquatic organisms in 3 ways. First, I wanted to rapidly dose a laboratory model aquatic animal, *Xenopus laevis* tadpoles (African clawed-frog tadpoles), to high concentrations of PCBs to test for observable health effects. Tadpoles were exposed from day 5 through day 9 postfertilization to the PCB mixture Aroclor 1254 via uptake through the gills with a DMSO (dimethylsulfoxide) solvent carrier. I found significantly reduced survival and body size at the highest doses used.
In addition, I observed several histological indicators of health effects, including aberrant tail tip, myotomal, and melanocyte morphologies (Chapter 2).

After these experiments showed that PCBs affected aquatic organisms, I shifted my attention to health effects in a real-world organism exposed to PCBs in a local PCB contaminated superfund site. Due to PCB disposal in the Kalamazoo River by several paper companies as part of the recycling of carbonless copying paper, an 80 mile stretch of the Kalamazoo river was designated a Superfund site. PCB concentrations present in sediments exceed the U.S. Environmental Protection Agency (USEPA) interim sediment quality criteria of 2.47 ug/g (parts per million) and the Michigan Water Quality Standards for the protection of aquatic life, and PCBs present in fish exceed the U.S. Food and Drug Administration’s (USDA’s) criteria of 2 ug/g. Due to these high PCB concentrations, the Michigan Department of Community Health (MDCH) currently issues an advisory restricting consumption of fish species in the Kalamazoo River.

To examine the health effects of PCBs in an environmentally relevant context, I secondly did parallel laboratory and field experiments utilizing the common carp, *Cyprinus carpio*, the most PCB-contaminated fish found in the Kalamazoo River Superfund area. Currently, the MDCH promotes a no consumption advisory for carp for every segment of the Kalamazoo River covered in the Superfund site. To investigate the effects of PCBs on carp, I fed PCBs to a cohort of juvenile carp (purchased from a commercial fish supplier) that were raised in the laboratory to establish a dose-response relationship. Additionally, I caught carp from PCB contaminated sites in the Superfund area and from upstream reference Kalamazoo
River sites. To test for possible health effects, I used real time RT-PCR to examine mRNA expression levels of several genes hypothesized to be differentially affected by PCBs in carp. Real time RT-PCR is a technique that allows quantification of the amount of messenger RNA present for a specific gene by the addition of a dually-labeled fluorescent probe to the PCR amplification reaction. I found that both laboratory PCB fed carp and carp caught from PCB contaminated superfund sites in the Kalamazoo River were responding to PCB exposure via induction of a drug metabolizing gene (CYP1A1) in the hepatopancreas. Real time RT-PCR was also applied to compare mRNA expression levels of other potential health bioindicator genes, validating a promising new technique to use in environmental risk assessment analysis (Chapter 3).

Thirdly, to follow up on potential health effects I examined histopathology in Kalamazoo River carp to see if there was a difference in macrophage aggregate densities in carp hepatopancreas from reference and PCB contaminated sites. Macrophage aggregates function in the body’s defense against injurious agents and in sequestration and detoxification of effete cellular materials, and have previously been shown to increase in association with exposure to environmental toxins. I found that carp from PCB contaminated sites have a higher macrophage aggregate percentage area and number per area than carp from reference sites. Furthermore, the number of macrophage aggregates per area was higher in carp from Lake Allegan compared to carp from Trowbridge and both reference sites, and macrophage number was significantly correlated with PCB levels in these carp. The macrophage aggregates consisted primarily of lipofuscin/ ceroid formed from effete cellular lipid material,
suggesting an increased need for sequestration of these products in carp chronically exposed to PCBs (Chapter 4).

In summary, I found that both in laboratory raised *Xenopus laevis* tadpoles and in juvenile carp, as well as in field collected carp, PCBs affect select biological endpoints and raise questions about health implications. In *Xenopus*, health effects that would be predicted to affect fitness (i.e., altered body size and tail morphologies, diminished survivorship) were evident. However, the PCB levels measured in the tadpoles were higher than that found in real world exposures. In carp exposed to PCBs in the laboratory and in PCB contaminated river sites, there was evidence of a biological response to PCB exposure, indicated by elevated CYP1A1 mRNA expression levels and additionally increased macrophage aggregate densities in PCB exposed river carp.

Although I showed that Kalamazoo River carp from PCB contaminated sites are responding to exposure at both the molecular and histological level, evidence of adverse health effects was not seen at the molecular level. Using real time RT-PCR, it is only possible to examine mRNA expression levels of single genes. Thus, although I chose to examine genes that I hypothesized to have a high probability of being differentially regulated by PCBs, these genes are a small sample size of genes that are part of a larger, complex regulatory pathway. For example, even though I saw no effect in relative expression levels of 2 metabolic genes, pepck and nucleolin, it is possible that expression levels of preceding or successive genes on the same metabolic pathway are altered. Also, it is possible that fish species, such as carp, modulate levels of different metabolic genes than mammalian models, from which our
understanding of metabolism is predominantly based. Therefore, it is possible that
testing a limited sample of genes could overlook adverse health effects in response to
PCBs, whereas testing expression levels of additional genes might reveal adverse
health effects. However, this research demonstrates that real time RT-PCR could be a
promising tool for quantifying gene-level effects indicative of organismal health in
environmental risk assessment analysis.

In this research, mRNA gene expression level changes and histological
pathologies in carp were examined to characterize health effects resulting from PCB
exposure. Both methods corroborated on finding a biological response to PCB
exposure. Interestingly, elevated CYP1A1 mRNA (Chapter 3) and elevated
macrophage aggregate densities (Chapter 4) in carp hepatopancreas could be linked.
An increase of macrophage aggregate densities in carp from PCB sites may be a result
from increased lipid peroxidation generated by elevated cyp1A1 protein metabolism.
It is also possible that PCBs act similar to cationic amphiphilic drugs (CADs) to bind
lipids or lipid metabolizing enzymes directly, interfering with cellular lipid uptake and
clearance. If the removal and sequestration in macrophage aggregates of PCB
induced lipid degradation products is beneficial in mitigating adverse health effects,
and/or if PCBs are sequestered in macrophage aggregates are not known. Further
research investigating the relationship between PCBs and lipid metabolism,
investigating macrophage aggregate function at the gene expression level to examine
activity, and investigating the relationships among PCB exposure, macrophage
aggregate densities, and fitness could be instrumental in defining health impacts in
PCB exposed carp and other fishes in the Kalamazoo River Watershed.
CHAPTER 2

HEALTH EFFECTS OF *XENOPUS LAEVIS* TADPOLES EXPOSED IN THE LABORATORY TO THE PCB MIXTURE AROCLOR 1254

Abstract

Polychlorinated biphenyls (PCBs) are ubiquitous environmental contaminants that have damaging effects on both ecosystems and human health. Numerous studies have shown that exposure to PCBs can alter growth and development of aquatic organisms, including frogs. In this study, developing *Xenopus laevis* tadpoles were exposed to the PCB mixture Aroclor 1254. Tadpoles were exposed from 5 through 9 days post-fertilization to either 0, 1, 10, 50 or 100 ppm Aroclor 1254. Exposure to an acute, high concentration of Aroclor 1254 (10, 50 and 100 ppm) caused statistically significant reductions in survival and body size. In addition, tadpoles exposed to these higher concentrations showed histological abnormalities, including aberrant tail tip, myotomal and melanocyte morphologies. Described adverse health effects associated with PCBs exposure of developing tadpoles will serve as useful health endpoints in ongoing and future molecular-based studies that correlate health effects with changes in gene expression.

Introduction

Polychlorinated biphenyls are widespread environmental contaminants present in the air, water and soil. They were manufactured starting in the 1920’s for use in electrical insulators, plasticizers and carbonless copy paper (Mayes et al., 1988; Safe,
1994). Although their manufacture was banned in the 1970's, they are resistant to degradation and persist in the environment. There are 209 PCB congeners, each containing a unique configuration of chlorine molecules attached to a biphenyl ring (Safe, 1994; Connell et al., 1997). Polychlorinated biphenyls can elicit biological effects through different mechanisms (Tilson et al., 1998). For example, some congeners bind to the Ah receptor, which causes activation of cytochrome p450 enzymes (Denison et al., 1995; Nebert et al., 1990) whereas other congeners elicit damaging effects by altering calcium homeostasis and calcium-mediated mechanisms (Shama et al., 2000). Most environmental PCBs are present as mixtures of congeners that elicit both types of biological effects.

PCBs cause a variety of adverse health effects in humans and wildlife. Studies with humans who were exposed accidentally or through the consumption of contaminated fish have shown that PCBs alter endocrine, immune and nervous system functions, particularly in developing children (Patandin et al., 1998; Gore, 2001; Aoki, 2001). In wildlife, including amphibians, fish and birds, PCBs reduce survival and alter reproductive system functions (Burkhart, 1998; Mac and Edsall, 1991; Bowerman, 2000). In addition, studies have shown that PCBs decrease growth and cause developmental malformations (Powell et al., 1996; Fernie et al., 2003). Coletti et al. (2001) demonstrated that exposure to Aroclor 1254 reduces skeletal muscle differentiation, suggesting that PCBs induced growth deficits may include decreased muscle development.

The present study was designed to identify health endpoints resulting from an acute, laboratory based exposure of *Xenopus laevis* tadpoles to Aroclor 1254 that will
serve as guidelines for ongoing and future molecular-based studies to define bioindicators of exposure to PCBs. In this report, developing *Xenopus laevis* tadpoles were exposed to the commercial PCB mixture Aroclor 1254. Tadpoles were observed for survivorship, growth, and tail morphological and histological health endpoints. In addition, tissue levels of PCBs were measured using GC/MS (gas chromatograph/mass spectrometry).

Materials and Methods

**Animals**

*Xenopus laevis* tadpoles were obtained by injecting adult breeder frogs with human chorionic gonadotropin (Sigma, St.Louis, MO USA). Tadpoles were reared in spring water (Absopure, Plymouth, MI USA) and staged according to Nieuwkoop and Faber (1994). Tadpoles were fed commercially available frog food ad libitum and changed into fresh water every 2 days. Animals were treated in accordance with an animal use protocol approved by the Institutional Animal Care and Use Committee at Western Michigan University, Kalamazoo, MI USA (Appendix 5).

**Chemical exposures**

Tadpoles were exposed to the PCB mixture Aroclor 1254, found in both the Hudson River and Kalamazoo River superfund sites (Tams Consultants, 1999; Blasland, Bouck & Lee, 2000). Five day post-fertilization (approximately stage 42/43) tadpoles were placed in glass bowls (20 or 25 tadpoles per bowl) containing
Aroclor 1254 (AccuStandard, New Haven, CT USA). Aroclor 1254 was suspended/dissolved in dimethylsulfoxide (DMSO, final concentration of 0.25% in each bowl). Experimental treatment bowls contained either 1, 10, 50 or 100 parts per million (ppm) Aroclor 1254. A spring water control group and a DMSO control group (0.25% DMSO) were included for each exposure experiment. Tadpoles were exposed from day 5 through day 9 post-fertilization (approximately stage 46).

**Gross tail morphology and histology**

Tadpoles were placed in treatment bowls (25 tadpoles per bowl) as described above. After the start of chemical exposures (5 days post-fertilization), on each successive day 4 tadpoles from each treatment were randomly sampled for possible histological analysis. Tadpoles were anesthetized with tricaine methanesulfonate (MS-222, diluted 1:2000), and fixed overnight at 4°C in Bouin's solution. Examination of gross tail morphology indicated that 4 days of exposure was sufficient to document abnormally bent tails and other health effects. The exposure experiment was repeated 3 times with tadpoles obtained from different pairs of breeder frogs.

**Survivorship**

Four additional spawnings were observed for assessment of the effects of Aroclor 1254 on survivorship. The chemical exposures were the same as above, with 20 tadpoles per bowl. The percentage of tadpoles surviving after 4 days of exposure was averaged for the 4 spawnings for each of the chemical treatments. One-way analysis of variance (ANOVA) followed by Fisher's post-hoc test was calculated to
compare differences between treatment groups. Statistics were computed with StatView software (Abacus Concepts, Berkeley, CA USA).

Analysis of PCBs

One additional exposure experiment was carried out to obtain tissue samples for gas chromatograph/mass spectrometer (GC/MS) analysis of levels of PCBs and for calculating bioaccumulation factors in tadpole tissues. The chemical exposure was the same as above (1, 10, 50 or 100 ppm Aroclor 1254 plus a spring water control group and a DMSO control group) with 20 tadpoles per treatment bowl. Tadpoles were anesthetized with MS-222 then frozen (-20C) until processing for GC/MS.

Measurements of body size / melanocyte area

Images of control and exposed tadpoles were taken with a Nikon SMZ-U dissecting microscope (Tokyo, Japan) and a Javelin Chromachip II (Japan) camera system. Images of 4 tadpoles chosen randomly from each treatment from each of the 3 spawnings (12 total) were used for quantitative image analysis. For 50 ppm tail area measurement, 11 images were analysed. Head, tail and melanocyte areas were measured with computer assisted image analysis using NIH Image v1.62 software. Total melanocyte area was measured in the area of skin covering the forebrain and between the otic vesicles. One way ANOVA statistics were computed using StatView software (SAS).
Histology

After imaging, 2 tadpoles from each treatment group for each of the 3 spawnings ($n=6$) were prepared for paraffin embedding and histological analysis. Briefly, the tadpoles were dehydrated in a series of graded ethanols, cleared with methyl salicylate and infiltrated with paraffin. Twenty micrometer ($\mu$m) horizontal tissue sections were cut with a Zeiss HM35 microtome and floated onto gelatin subbed slides.

Tissue sections were double stained with picrO' ponceau and hematoxylin according to a modified protocol from Humason's Animal Tissue Techniques (Presnell and Schreibman, 1997). The tissue sections were deparaffinized in xylene and rehydrated through a series of graded ethanols, rinsed briefly with distilled H$_2$O (dH$_2$O) and stained with Gill's hematoxylin for 5 min., and then stained with picro-ponceau (0.014% ponceau S, 86% saturated picric acid, 0.7% acetic acid) for 10 min. Tissue sections were rinsed with dH$_2$O, dehydrated through a series of graded ethanols and coverslipped with Permount (Fisher Scientific, Pittsburgh, PA USA).

Tissue sections were analyzed with a Nikon Eclipse E600 microscope. Images were taken with a digital Spot camera (Diagnostic Instruments, McHenry, IL USA), and imported into Adobe Photoshop to compare muscle disposition among treatment groups. The presence of continuous and evenly packed versus disorganized muscle cells in the third myotome distal to the large intestine (in the same plane of section as the pronephros and IXth cranial ganglia) was charted in treated and control tadpoles. Cellular disposition at the tip of the tail was also noted in each animal.

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Morphological and histological differences among treatments were compared using a 2-way data contingency test using StatView software (Abacus Concepts, Berkeley, CA USA).

PCBs GC/MS Analysis

Reagents

PCBs standards and internal standards were purchased from AccuStandard (New Haven, CT USA) and Ultra Scientific (North Kingstown, RI USA). Table 1 lists the congeners measured individually in this study, as well as the chlorination groups quantified. An internal standard, 4,4'-dibromo-octafluoro biphenyl (DBOFP) was purchased from Ultra Scientific (North Kingstown, RI USA). Five deuterated aromatic hydrocarbon surrogate standards (Ultra Scientific) used are also listed. Solvents utilized in the extraction process were pesticide grade hexane and dichloromethane obtained from Baker Scientific (Phillipsburg, NJ USA). Solid phase C-18 used for matrix solid phase dispersion extraction was purchased from Varian Scientific (Cary, NC USA).

Extraction

Pooled groups (30-50 organisms/dose level) of whole tadpoles ranging in wet weight mass from 0.4 to 0.9 g were extracted for PCBs using a matrix solid phase dispersion method (Barker et al., 1993). Briefly, the tissue was weighed and combined with 5 g of dry, pre-cleaned C-18 solid phase material in a glass mortar, spiked with 25 µl of a solution containing the five deuterated surrogate standards (40 µg/ml each) and then thoroughly ground into a homogeneous powder with a glass
Table 1. PCB (polychlorinated biphenyl) congeners or chlorination groups quantified and surrogates used for recovery corrections.

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<td>2,2',4,4',5-penta-CL BP (99)</td>
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<td>CL3-PCB (2,4,5-tri-BP)(29)</td>
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<td>CL4-PCB (2,2',4,6-tetra-BP)(50)</td>
<td>2,3,3',4,4'-penta-CL BP (105)</td>
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<td>2,3',4,4',5-penta-CL BP (118)</td>
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<td>CL6-PCB</td>
<td>CL6-PCB (2,2',4,4',5,6'-hexa-BP)(154)</td>
<td>3,3',4,4',5,5'-hexa-CL BP (169)</td>
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<tr>
<td>CL7-PCB</td>
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<td>2,2',3,4,4',5,6'-hepta-CL BP (180)</td>
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<td>CL8-PCB</td>
<td>CL8-PCB (2,2',3,3',4,5,6,6'-octa-BP)(205)</td>
<td>2,2',3,3',4,4',5,5'-octa-CL BP (194)</td>
</tr>
<tr>
<td>CL9-PCB</td>
<td>CL9-PCB</td>
<td></td>
</tr>
<tr>
<td>CL10-PCB</td>
<td>CL10-PCB (perchloro-BP)(209)</td>
<td></td>
</tr>
</tbody>
</table>

*Surrogates used were d8-naphthene, d10-acenaphthene, d10-phenanthrene, d12-chrysene, and d12-pyrene.

The average detection limit (ng/g) was 9 ng/g for all PCB congeners. Congeners listed in bold type are the congeners utilized to quantify the associated chlorination groups. Additional individual congeners quantified are listed by chlorination groups. Congener numbers are in parentheses.

The C18 was then transferred quantitatively into the barrel of a disposable 10 ml plastic syringe plugged at the end with a 4 mm glass fiber filter and a small amount of inactivated glass wool. The matrix solid phase dispersion columns were then eluted as follows: the column bed was first saturated with approximately 2 ml of...
dichloromethane and allowed to stand for 20 minutes. An additional 8 ml of dichloromethane was passed through each matrix solid phase dispersion column and allowed to elute by gravity into a 40 ml glass vial. In some cases, the solvent was slowly forced through the column using a syringe.

The solvent eluent was evaporated to approximately 2 ml under a stream of pure nitrogen, transferred quantitatively to a 4 ml amber glass vial and further evaporated under nitrogen to a final volume of 200 µl. Samples were stored at -20°C until GC/MS analysis.

**GC/MS Analysis of PCB congeners**

The PCB congeners listed individually in Table 1 as well as chlorination group sums were quantified by the multiple selected ion monitoring GC/MS method adapted from Means (1998) and McMillin and Means (1996). Analysis was performed on 2µl samples of the extracts using an Agilent Technologies (Palo, Alto, CA USA) 6890A gas chromatograph equipped with the capillary column (AG DB-5MS)(30m X 0.025mm i.d.), which was directly interfaced to an AG 5973N Mass Selective Detector, equipped with a 7683 autosampler. A temperature program for the GC oven using a series of linear temperature ramps from 50°C to 300°C in order to optimally separate the analytes was developed. The mass spectrometer was tuned daily and/or after each 16 hours of analysis using perfluorotributylamine (PFTBA). The instrument autotune was then manually fine-tuned to achieve at least 50-75% abundance of m/z ion 219 and at least 5% abundance of m/z ion 502 relative to the m/z 69 base peak intensity. Injector septa and injector glass liners were routinely inspected and replaced (after approximately 25 injections and every analysis day,
respectively), to minimize variations in retention times of analytes and to optimize
peak shapes. An initial calibration curve was prepared (described below) and
continuing calibrations for all analytes were run at the beginning and end of each
analysis group.

A multiple selected ion monitoring method was developed for monitoring up
to 18 ions in each of 16 retention time windows. This method included ions selected
to allow for estimating the total amounts of CL1 to CL 10 congener groups in the
appropriate retention window(s). The congener groups were quantified using a
calibration standard containing a representative congener from each chlorination
group (AccuStandard, New Haven, CT USA). Individual congeners were quantified
using authentic individual standards or mixtures. This was also done routinely after
major instrument repairs, after changing columns or any column conditions.

**Determination of method detection limits**

Detection limits for each analyte in the sample matrix type were estimated
from statistical information derived from standard calibration curves used to
determine instrumental detection limits, corrected for the type sample concentration
factor. Triplicate analyses of a 5-point standard calibration curve were used to obtain
a mean standard deviation for each analyte. This value was then multiplied by a factor
of 3 (Taylor, 1997), to obtain an instrumental detection limit in units of ng on-column.
The use of the 3X multiplier has the advantage of maximizing the amount of
numerical values reported in data sets obtained in research projects. The instrument
detection limits were then corrected for average concentration factors for each matrix
to obtain a sample detection limit for that matrix. For the tadpole samples, this limit
was typically approximately 5-12 ng/g wet wt. (ppb) with a mean value of 9 ng/g. Samples were spiked immediately before injection with 10 µl of a 100ug/ml solution of DBOFP as an internal standard.

Results

Survival

After 4 days of exposure to Aroclor 1254, statistically significant decreases in survival occurred in tadpoles exposed to 10, 50 and 100 ppm ($p<0.05$; one way ANOVA followed by Fisher's post hoc test) compared to DMSO controls (Figure 1).

Figure 1. Exposure to 10, 50 and 100 ppm Aroclor 1254 caused statistically significant decreases in survival (expressed as % group survivorship) of tadpoles exposed from 5 through 9 days post-fertilization compared to the DMSO (dimethylsulfoxide) control (n=80/treatment, $p<0.05$). Error bars in all figures are standard error of the mean.

Compared to untreated controls, only the 50 and 100 ppm treatments showed

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significant differences. Exposure to 1 ppm Aroclor 1254 had no significant effect on survival. In addition, survival rates (98.8%) for DMSO treated controls were not significantly different from untreated controls (96.3%).

**Gross tail morphology**

Tadpoles exposed to 1 ppm Aroclor 1254 were not different from untreated and DMSO controls with respect to gross tail morphology (0 out of 12 animals examined, Table 2 and Figure 2 A-C). One of the tadpoles (1 out of 12) exposed to 10 ppm Aroclor 1254 showed a bent tail (Table 2). Tadpoles exposed to 50 ppm

<table>
<thead>
<tr>
<th>Table 2. Number of tadpoles displaying aberrant tail morphology, disorganized tail muscle histology, and abnormal tail tip morphology.</th>
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<tr>
<td></td>
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<tr>
<td>-----------------------------</td>
</tr>
<tr>
<td>Control</td>
</tr>
<tr>
<td>DMSO(^c)</td>
</tr>
<tr>
<td>1 ppm A1254</td>
</tr>
<tr>
<td>10 ppm A1254</td>
</tr>
<tr>
<td>50 ppm A1254</td>
</tr>
<tr>
<td>100 ppm A1254</td>
</tr>
</tbody>
</table>

\(^a\)2-way data contingency tests were performed to assess whether the frequency of observed abnormalities were different among treatments. Differences among treatments for aberrant tail morphology (chi-square= 20.45, Cramer’s V= 0.775, p= 0.001), and cell disposition at the tail tip (chi-square=032.07, Cramer’s V=0.944, and p<0.001) were all significant.

\(^b\)in the third myotome distal to the large intestine; see text for details

\(^c\)DMSO = dimethylsulfoxide
(11/12 animals) and 100 ppm (10/11 animals) Aroclor 1254 exhibited more pronounced tail bends (e.g., bent dorsally (lordosis) or bent laterally (scoliosis); Table 2 and Figure 2 E,F).

Body size

Exposure to Aroclor 1254 reduced the overall size of developing *Xenopus laevis* tadpoles. Compared to DMSO treated controls, statistically significant decreases in head area occurred after 4 days of exposure to 1 ppm and greater concentrations of Aroclor 1254 (one way ANOVA, p<0.05; Figure 3); statistically significant reductions in tail area occurred after exposure to 10 ppm and greater

![Figure 2. Xenopus laevis tadpoles exposed to Aroclor 1254 from 5 though 9 days post-fertilization. A: untreated control, B: DMSO (dimethylsulfoxide) only control, C: tadpole exposed to 1 ppm Aroclor 1254, D: tadpole exposed to 10 ppm, E: tadpole exposed to 50 ppm, and F: tadpole exposed to 100 ppm. Scale bar =1 mm.](image-url)
concentrations of Aroclor 1254 (one way ANOVA, \(p<0.05\); Fig. 3).

Dimethylsulfoxide (DMSO) treatment had no significant effect on tail area, but increased head area compared to untreated controls.

**Melanocyte area and morphology**

Quantitative image analysis demonstrated a PCB induced decrease in total melanocyte area in the region of skin overlying the forebrain from its anterior aspect to the posterior margins of the otic vesicles. One way ANOVA showed statistically significant reductions in melanocyte area for tadpoles exposed to 1 ppm and greater concentrations of Aroclor 1254 compared to DMSO treated control animals (\(p<0.05\); Figure 3). DMSO treatment alone caused significant increases in head area and decreases in melanocyte area compared to untreated controls.

![Graph showing the effect of Aroclor 1254 concentration on melanocyte area](image.png)

Figure 3. Mean tail area (diagonal bars), mean head area (cross-hatched bars) and mean melanocyte areas (black bars) in mm² for *Xenopus* tadpoles treated with Aroclor 1254. Statistically significant decreases occurred in treated tadpoles compared to DMSO controls (n=12/treatment except 50 ppm tail area, n= 11, *p<0.01; **p<0.05). DMSO treatment alone caused significant increases in head area and decreases in melanocyte area compared to untreated controls.
Figure 3). Also, DMSO treatment significantly reduced melanocyte area compared to untreated control animals ($p<0.05$; Figure 3).

Aroclor 1254 exposure induced changes in melanocyte area appear to be due to altered melanocyte morphology (Figure 4). Tadpoles exposed to Aroclor 1254 showed punctate melanocytes with little or no dendritic arborizations (Figure 4 C-F) compared to untreated and DMSO control tadpoles, although DMSO only treated controls showed melanocytes with somewhat reduced dendritic arbors compared to untreated controls (Figure 4 A-F).

Figure 4. Morphology of melanocytes in the skin covering the brain was altered by exposure to Aroclor 1254. A: untreated control, B: DMSO (dimethylsulfoxide) only control, C: 1 ppm Aroclor 1254, D: 10 ppm, E: 50 ppm, and F: 100 ppm. Tadpoles treated with Aroclor 1254 are devoid of dendritic arborizations compared to both untreated controls and DMSO treated controls. Scale bar =100 μm.
Histological organization of the tail

Corresponding tissue sections of the tail were analyzed from each treatment group \((n=6)\). Histological analysis showed that exposure to Aroclor 1254 altered tail muscle structure in a dose-dependent manner (Figure 5). Tail muscle fibers in the untreated and DMSO control tadpoles were tightly packed and organized into clear myotomal structures (Figure 5 A,B). After exposure to 10 ppm and greater Aroclor 1254, the muscle cells appeared increasingly disorganized (Figure 5 D-F).

Figure 5. Histological analysis of tail muscle in tadpoles treated with Aroclor 1254. Myotomes of untreated control (A) and DMSO (dimethylsulfoxide) only control (B) showed contiguous and evenly packed muscle cells (black arrow) across myotomal boundaries (white arrow). Treatment with 1 ppm Aroclor 1254 (C). Treatment with 10 ppm (D), 50 ppm (E), and 100 ppm (F) Aroclor 1254 caused apparent increases in extracellular space between muscle cells and produced disorganization of muscle cells which obscured myotomal boundaries. Scale bar = 50 μm.

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Disorganized muscle cells with obscured or absent myotomal boundaries were present in 2/6 tadpoles treated with 10 ppm Aroclor 1254, 5/6 tadpoles treated with 50 ppm and 5/6 tadpoles treated with 100 ppm Aroclor 1254 (Table 2). A 2-way data contingency test showed significant differences among frequencies of disorganized tail muscle (chi-square = 20.45, Cramer’s V = 0.775, p = 0.001).

In addition, tadpoles exposed to higher concentrations of Aroclor 1254 (50 and 100 ppm) showed abnormal cell morphology at the tip of the tail (Table 2 and Figure 6). The untreated and DMSO only control tadpoles and the tadpoles exposed to 1 and 10 ppm Aroclor 1254 had small, elongate cells at the tip of their tails (Fig. 6 A-D), whereas tadpoles exposed to 50 and 100 ppm Aroclor 1254 had larger, rounded cells that formed a clump at the tail tip (Fig. 6 E,F). 5/6 tadpoles treated with 50 ppm Aroclor 1254 and 6/6 tadpoles treated with 100 ppm displayed this effect (Table 2). A 2-way data contingency test showed significant differences among frequencies of cell proliferation at the tail tip (chi-square = 32.07, Cramer’s V = 0.944, p < 0.001).

**PCBs Analysis**

The concentrations of PCBs that were present in pooled tadpole tissues are presented in Table 3. Untreated and DMSO control tadpoles showed low levels of PCBs in their tissues (1.18 and 0.85 ppm, respectively). This is most likely due to the presence of PCBs in the tadpole food that was purchased from a commercial source. The commercial tadpole food was analyzed using GC/MS and showed PCBs levels of 0.7 ppm.
Figure 6. Cell morphology at the tip of the tail was altered by exposure to Aroclor 1254. Control (A), DMSO (dimethylsulfoxide) only control (B), 1 ppm Aroclor 1254 (C), and 10 ppm (D) show elongated tail tips with cells oriented end to end. Treatment with 50 ppm (E) and 100 ppm (F) causes a ball of rounded cells to appear in place of an elongated tail tip. Scale bar = 20 μm.

Exposure to Aroclor 1254 caused dose-dependent increases in PCBs in pooled whole organism tissues (Table 3). Tadpoles (30-50 pooled individuals) exposed to a low concentration of Aroclor 1254 (1 ppm) contained 114 ppm (wet weight values) and up to 4163 ppm for pooled tadpoles exposed to 100 ppm. The relative concentrations of chlorinated congener groups measured in pooled tadpole tissues were very similar to those in the PCB 1254 exposure mixture.
Table 3. Total tissue levels of PCBs (polychlorinated biphenyls, µg/g wet weight) and bioaccumulation factors (BAF) for tadpoles exposed to Aroclor 1254.

<table>
<thead>
<tr>
<th>Sample</th>
<th>PPM Wet Weight</th>
<th>BAF</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>1.18</td>
<td>1.69</td>
</tr>
<tr>
<td>DMSO</td>
<td>0.85</td>
<td>1.21</td>
</tr>
<tr>
<td>1 ppm A1254</td>
<td>114.09</td>
<td>114.09</td>
</tr>
<tr>
<td>10 ppm A1254</td>
<td>956.44</td>
<td>95.64</td>
</tr>
<tr>
<td>50 ppm A1254</td>
<td>1660.28</td>
<td>33.21</td>
</tr>
<tr>
<td>100 ppm A1254</td>
<td>4163.84</td>
<td>41.63</td>
</tr>
</tbody>
</table>

*DMSO = dimethylsulfoxide

Bioaccumulation factor (BAF) values were calculated by dividing the tissue level of PCBs by the exposure concentration for each treatment group (Table 3). Bioaccumulation factor values for untreated and DMSO control tadpoles were calculated by dividing the tissue level concentration of PCBs by the concentration of PCBs found in the tadpole food (0.7 ppm). BAF values were elevated in the low dose exposure groups, 1 and 10 ppm (114 and 96, respectively) compared to untreated and DMSO controls (1.67 and 1.14). Exposure to higher concentrations of Aroclor 1254 (50 and 100 ppm) decreased BAF values (33.2 and 41.63, respectively) compared to 1 and 10 ppm exposure groups.
Discussion

**Changes in survivorship induced by Aroclor 1254**

Numerous studies have shown that PCBs decrease survival of developing organisms, including chicks, mink and zebrafish (Powell et al., 1996; Brunstom et al., 2001; Orn et al., 1998). In *Xenopus laevis* and *Rana temporaria*, Gutleb et al. (2000) showed that exposure through diet to 2 ppm and 200 ppm of the PCB mixture Clophen A50 decreased overall survival from stage 50/51 (*X. laevis*) and stage 25 (*R. temporaria*) up to metamorphosis.

In studies where tadpoles were exposed through PCBs dissolved in DMSO added to water, decreases in survival were seen in developing *X. laevis* tadpoles exposed from 11 to 13 days post-fertilization (approximately stage 47) to 25 ppm and 50 ppm of the PCB mixture Aroclor 1254 (Jelaso et al., 2002). Tadpoles exposed from 5 to 7 days post-fertilization (approximately stage 46) showed no decreases in survival. However, in the present study, exposure to 10 ppm, 50 ppm and 100 ppm Aroclor 1254 (also in 0.25% DMSO) decreased survival in *Xenopus laevis* tadpoles exposed from day 5 through day 9 post-fertilization (after an additional 2 days of exposure compared to that used by Jelaso et al.).

**Changes in growth and gross tail morphology induced by Aroclor 1254**

Exposure to PCBs and related chlorinated hydrocarbons is associated with alterations in growth and development in animals and in humans (Gutleb et al., 1999; Kakeyama and Tohyama, 2003). For example, Powell et al. (1996) showed that
exposure to 6.67 ppm Aroclor 1254 reduced growth and body weight in developing
chicks and Henry et al. (1997) showed that exposure to 2,3,7,8-tetrachlorodibenzo-p-
dioxin (TCDD) decreased growth in developing zebrafish. In the current report,
exposure to Aroclor 1254 decreased both head area (at an exposure of 1 ppm and
greater) and tail area (10 ppm and greater) in developing tadpoles.

Field and laboratory studies have shown that PCBs and related compounds
cause a wide range of developmental deformities in a variety of organisms. For
example, nestling bald eagles in PCB contaminated regions of the Great Lakes have a
high incidence of bill deformities (Bowerman, 2000) and chick embryos exposed to
TCDD developed abnormal beak, limb and skull/brain deformities (Powell et al.,
1996). In developing *Xenopus laevis* tadpoles, Gutleb et al. reported an increase in
developmental malformations, including tail and eye deformities after exposure to
PCBs (Gutleb et al., 1999; Gutleb et al., 2000). In the current report, exposure to
Aroclor 1254 (10 ppm and greater) induced dose-dependent alterations in gross
morphology of the tail in tadpoles exposed from 5 through 9 days post-fertilization.

**Changes in cellular disposition that underlie gross morphological changes**

The current report describes Aroclor 1254 induced histological changes in cell
structure/organization that underlie gross morphological changes. Histological
analysis of the bent tail regions showed a dose-dependent effect on tail muscle
organization. Tadpoles exposed to Aroclor 1254 had disorganized muscle cells,
which correlated with the appearance of scoliosis and lordosis. This effect occurred
in tadpoles exposed to low concentrations of Aroclor 1254 (10 ppm) and was more
pronounced after exposure to higher concentrations (50 and 100 ppm). Coletti et al. (2001) showed that exposure of a rat myogenic cell line to Aroclor 1254 decreased skeletal muscle differentiation and fusion of myoblasts into myotubes, indicating that, in vivo, developing muscle is a potential target for PCBs.

Tail muscle organization and decreased tail area (seen in tadpoles exposed to 10 ppm Aroclor 1254 and above) may be also be associated with the appearance of rounded up cells at the tail tip (seen in tadpoles exposed to 50 and 100 ppm Aroclor 1254). In this case, PCBs may be altering internal calcium concentrations that regulate cytoskeletal disposition and/or cell surface adhesion properties. Interestingly, the growing tip of the tail in Xenopus laevis has been shown to retain Spemann's organizer and associated genes (i.e., Xnot2, Xbra) until late stages of development (Gont et al., 1993). Gene expression studies on organizer associated genes could be carried out on tadpoles with normal versus PCB induced tail tip abnormalities to ascertain basic mechanisms that might underlie tail tip malformations.

Aroclor 1254 induced reductions in pigmentation/melanocyte area are due to changes in melanocyte shape. This is consistent with studies by Gutleb et al., who observed a decrease in gross pigment levels in developing Xenopus laevis tadpoles after exposure to PCBs. In the present study, exposure to Aroclor 1254 decreased gross pigment levels by inhibiting melanocyte arborization in the skin covering the brain.

Melanocytes are important for protection from ultraviolet radiation damage, which has been implicated in the worldwide decline of amphibian populations (Wake, 1991; Pechmann and Wilbur, 1994). Alterations in protective melanocyte responses to
ultraviolet light due to exposure to PCBs and possibly other chlorinated hydrocarbon contaminants may heighten amphibian risk to increases in incident ultraviolet radiation.

Bioaccumulation of PCBs in tadpole tissues

To accomplish rapid loading of tadpole tissues, animals were exposed to PCBs that were dissolved in DMSO/spring water because uptake through the gills is more efficient than through dietary uptake. Dimethylsulfoxide (DMSO, 0.25%) was used as a carrier solvent to facilitate solubilization/suspension of the PCBs in water. Dimethylsulfoxide treatment alone caused significant increases in head area and decreases in melanocyte area compared to untreated controls. Still, exposure to PCBs caused significant decreases in head, tail and melanocyte area compared to DMSO only treated animals.

Due to the presence of small amounts of PCBs in the tadpole food, there were trace amounts of PCBs found in untreated controls and DMSO control tadpole tissues. BAF values for these tadpoles are consistent with dietary exposure studies that show that uptake occurs according to an approximate 1:1 ratio if lipid content of the food and the lipid content of the animal are approximately equal (estimated 5% for both) (Means and McElroy, 1997). The unanticipated exposure due to the commercial (fish meal based) food did not significantly affect overall BAF values in exposure groups, nor did the added PCB body burdens from dietary exposure contribute significantly in tadpoles showing positive responses as reflected by the large changes showed relative to control groups.
Bioaccumulation factors were reduced at higher Aroclor 1254 doses compared to lower doses. Tadpoles exposed to 1 and 10 ppm Aroclor 1254 had elevated BAF values (114 and 96) compared to untreated and DMSO controls (1.7 and 1.14). These values compare well with values reported in the literature for larval organisms and certain invertebrates (Hawker and Connell, 1986). Tadpoles exposed to 50 and 100 ppm Aroclor 1254 had decreased BAF values (33.2 and 41.6) compared to the lower dose tadpole groups. This type of reduction in relative bioaccumulation has been observed in *Xenopus laevis* tadpoles exposed to PCBs at 5 days and 11 days post-fertilization for 2 days (Jelaso et al., 2003), and other species when the organisms are severely stressed and/or intoxicated with PCBs (Means and McElroy, 1997).

**Correlation of health effects with changes in gene expression patterns**

A previous study by Jelaso et al. (2002) showed that in *Xenopus laevis* tadpoles exposed from 11 to 13 days post-fertilization, changes in survivorship, gross tail morphology, and behavior correlated with changes in specific gene expression. In their study, changes in gene expression for 11 physiologically relevant genes (D2 Rt, NGF, CPP32β, ICE, Thy Rt, POMC, RAR-α, p53, β-actin, GAPDH, and p4501A1) were quantified by real time reverse transcriptase polymerase chain reaction (RT-PCR). They showed that a decrease in gene expression for 9 out of 11 genes was associated with mortality at high doses of PCBs (50 and 100 ppm), and predicted future mortality at lower doses (1 ppm and 10 ppm). Also in their study, though, tadpoles exposed from 5 to 7 days post-fertilization showed no health effects or
correlated gene expression changes for select genes. This present study extended the exposure time from 5 to 9 days post-fertilization. The addition of 2 exposure days produced changes in morphology and survivorship and further defined health effects to include changes in head, tail, and melanocyte area, histological changes in tail muscle, and changes in morphology of cells at the growing tip of the tail. The physiologic and morphometric health endpoints described here will serve as guideposts for additional molecular based studies that will correlate PCB exposure with changes in gene expression signatures that help define underlying mechanisms of disruption.
CHAPTER 3

MOLECULAR ANALYSIS OF HEALTH BIOINDICATORS IN CARP (CYPRINUS CARPIO) EXPOSED TO PCBS IN THE KALAMAZOO RIVER AND IN THE LABORATORY

Abstract

The Kalamazoo River, MI, USA, is a Superfund site due to the presence of polychlorinated biphenyls (PCBs) from recycling of carbonless copying paper before the production and use of PCBs were banned. To characterize health impacts in a resident fish population, I utilized real time RT-PCR to examine mRNA expression levels of multiple genes in carp (Cyprinus carpio) hepatopancreas from PCB contaminated and reference sites in the Kalamazoo River. In an additional laboratory experiment, I exposed juvenile carp to PCBs through the diet for 4 months. Significantly elevated levels of CYP1A1 mRNA was found in PCB contaminated Kalamazoo River carp (t-test, t= 0.0302) and in carp fed PCBs in the Laboratory (ANOVA, p= 0.0012). No significant effects were found for specific oxidative stress genes (gamma-glutamyl cystein synthetase and magnesium superoxide dismutase) and specific metabolic genes (phosphoenolpyruvate carboxykinase and nucleolin) examined. Defining gene expression signatures in wildlife indicative of PCB contamination would be a useful tool in risk based studies, and would aid decisions regarding clean-up in the Kalamazoo River Superfund site.
Introduction

The Kalamazoo River Superfund site (MI, USA) is impacted by environmental contamination by a variety of industrial, municipal, and agricultural sources. Notably, polychlorinated biphenyls (PCBs), commercially synthesized compounds with hepatotoxic, immunotoxic, and neurotoxic activities, were heavily discharged into the river from several paper companies as part of the de-inking process in the 1950’s-1970’s. The Environmental Protection Agency (EPA) estimates that over 1.5 billion pounds of PCBs were manufactured in the US prior to their ban in 1977, and it is estimated that there are approximately 230,000 pounds in Kalamazoo River sediments (Blasland, Bouck & Lee, 2000). Due to PCB contamination, approximately an 80 mile stretch of the Kalamazoo River, from Morrow Pond to Lake Michigan, was designated as a Superfund Site. Despite known PCB contamination of aquatic organisms in these sites, the health impacts of PCBs on these organisms are not well defined.

To assess health of an organism traditional methods employ examination involving quantitation of overt health indicies, either in gross morphology or in histology. However, these methods are often non-specific indicators and are not able to relate effect to probable cause. Furthermore, health effects can manifest at the cellular and molecular level, often resulting in physiological changes to the organism before overt indicies are apparent. In order to examine potential health impacts of PCBs in aquatic organisms, I therefore wanted to examine bioindicators to indicate exposure and ultimately, overt and cryptic health effects.

To test if molecular tools can be applied to define bioindicators of exposure
and/or health effects in a risk-assessment setting, I employed real time RT-PCR, using a Taqman assay (Applied Biosystems, Foster City, CA). Taqman real time RT-PCR uses the 5' exonuclease activity of Taq polymerase to cleave a reporter dye from proximity to a quencher molecule on a single hybridized probe during the polymerase extension step of PCR (Livak et al., 1995). Fluorescence level of the reporter dye is directly proportional to PCR product, and is monitored in real time. Real time RT-PCR is increasingly used in molecular medicine and drug development to rapidly and accurately quantify the expression of target mRNAs.

For this research, real time RT-PCR was used in an environmental risk assessment context in order to define mRNA expression levels of multiple genes as bioindicators of PCB exposure and health effects in the common carp *Cyprinus carpio*. Carp are found throughout the Kalamazoo River and feed benthically in heavily PCB-contaminated sediment. Carp are considered the most contaminated fish in the river. Additionally, many carp genes have been sequenced and are publicly available. I raised juvenile, hatchery bred carp in the laboratory, and exposed these carp through the diet to the PCB mixture Aroclor 1242 (A1242), the mixture most commonly found in the Kalamazoo River (Blasland, Bouck & Lee, 2000). Additionally, I compared wild carp caught from PCB contaminated sites in the Kalamazoo River Superfund area to carp caught upstream of the Superfund area, in relatively non-contaminated PCB sites.

I quantified mRNA expression levels in hepatopancreas in laboratory raised carp and in field caught carp of health indicator, oxidative stress, and metabolic genes hypothesized to be differentially regulated by PCB exposure. I first tested for an
exposure-related health indicator by determining the quantitative biological response to PCB exposure using induction of cytochrome P450 1A1 (CYP1A1) in hepatopancreas.

CYP 1A1 is a phase I drug metabolizing enzyme induced by a broad range of environmental contaminants, including halogenated dibenzo-p-dioxin, dibenzofuran, azo(xy)benzene, naphthalene and planar polychlorinated biphenyl congeners (Goldstein and Safe, 1989). Cytochrome 1A induction has been used as a bioindicator for exposure to Ah receptor ligands in a wide variety of animals (Peakall, 1992). Field studies have additionally used increased cyp 1A1 activity in fish liver as an environmental bioindicator in polluted water (Blanchard et al., 1999; Campbell et al., 1996; Haasch et al., 1993; and reviewed in Payne et al., 1987). Recently it has been shown, however, that in Superfund sites and in other PAH/HAH contaminated sites populations of resident fish are refractory to CYP1A1 induction (reviewed in Hahn, 1998; Elskus, 2001), questioning the utilization of CYP1A1 as a robust bioindicator for exposure at highly contaminated sites. Thus I wanted to test for CYP1A1 induction as a bioindicator of exposure in the PCB contaminated Kalamazoo River Superfund site.

In addition to examining CYP1A1 mRNA expression, I examined expression levels of oxidative stress genes and metabolism genes as bioindicators of health effects. Exposure to planar aromatic hydrocarbons including PCBs have been shown to shift the balance between prooxidant and antioxidant forces in various tissues (Otto and Moon, 1995) and consequently are associated with adverse health effects such as DNA damage, protein damage, and lipid peroxidation. Reduced cellular glutathione
(L-γ-glutamyl-L-cysteinylglycine, GSH) is a major cellular antioxidant involved in detoxification of electrophiles, and has been shown to be affected by PCB exposure in rainbow trout (Otto and Moon, 1995). Superoxide dismutase functions to remove O$_2^-$ electrophiles, which are formed from certain xenobiotics including aromatic nitro compounds, quinones, and PCBs by NAD(P)H-dependent reductases followed by redox cycling (Di Giulio et al., 1989; Otto and Moon, 1995). Therefore, also using real time RT-PCR, I measured mRNA expression levels of 2 genes involved in an oxidative stress response: γ-glutamyl cysteinylsynthetase (g-gcs), the rate limiting enzyme in glutathione synthesis, and magnesium superoxide dismutase (mnsod), involved in dismutation of the superoxide anion.

To examine bioindicators of health effects that can be altered independently of CYP1A1 but also likely to be affected by PCBs, I examined the expression of metabolic genes. Depletion of hepatic glycogen is commonly observed in response to toxicants, including organochlorinated xenobiotics such as DDT, TCDD (2,3,7,8-tetrachlorodibenzo-p-dioxin), and PCBs (Hugla and Thome, 1999; Hacking et al., 1977). Aryl hydrocarbon receptor (AhR) agonists, such as TCDD and the PCB congener 3,3',4,4',5-pentachlorobiphenyl, have also been shown to alter enzyme activity levels involved in gluconeogenesis (Weber et al., 1991; Ishii et al., 2001). Recently, small mouth bass inhabiting a PCB contaminated site in the Kalamazoo River were shown to be glycogen depleted (Anderson et al., 2003). In addition to altered glycogen, altered hepatic nuclear and nucleolar profiles are seen in response to dioxin-like compounds including PCBs (Wu et al., 1999). I used real time RT-PCR
to test mRNA expression levels in 2 genes involved in metabolism, phosphoenolpyruvate carboxykinase (pepck) and nucleolin. Pepck is the rate limiting enzyme in the gluconeogenesis pathway and has been shown to be regulated in carp (Panserat et al., 2002). Nucleolin is a nucleolar protein which represses ribosomal RNA synthesis, and is up-regulated in cold adapted carp (Alvarez et al., 2003).

In this study, I use real time RT-PCR as a diagnostic tool of exposure and health effects for bioindicator evaluation in carp. Developing gene expression signatures in wildlife indicative of PCB contamination would be a useful tool in risk based studies, and would aid decisions regarding clean up in the Kalamazoo River Superfund site.

Materials and Methods

Laboratory Raised Carp

Maintenance

Juvenile carp were purchased from Aquatic Research Organisms fish hatchery (NH, USA) and reared in the WMU Animal Care Facility. Carp were allowed to acclimatize for 2 months prior to the start of the experiment. Light/dark cycles were set for 12 hours light/12 hours dark, and water temperature was maintained at 18 ± 2 C. Carp were fed 0.6% of their body weight 2x/day. Health checks, feed checks, oxygenation check, and tank siphoning was performed daily, and aquaria NH₃, NO₂, NO₃ levels were monitored every 2 weeks to ensure that all levels were below toxic or
0. There was no fish mortality during the acclimatization period or during the experiment.

**PCB exposures in diet**

Eighty carp (8.02 ± 0.38 g) were placed in ten-10 gallon aquaria. Carp were fed a standard diet (Laguna Carp and Koi Chow, medium floating pellets) either mixed with the commercial PCB mixture Aroclor 1242 (AccuStandard, New Haven, CT) or vehicle only. 5 tanks were fed food mixed with PCBs, and 5 tanks were fed food with vehicle only. A target value of 12 ug/g (ppm) PCB concentration for the PCB treatment food was chosen for this laboratory feeding experiment, since 12 ug/g is thought to reflect source PCB inputs for carp via lower trophic organisms and in sediments present in the Kalamazoo River Superfund site areas.

Fish food was prepared twice for the duration of the experiment. First, treatment food was made by adding 7.2 ml of a 500 ppm stock solution of A1242 in hexane to 343 ml of 95% ethanol for a calculated concentration of 12 ppm A1242. The control food was made by adding 7.2 ml of hexane only, without A1242, to 343 ml 95% ethanol. The treatment and control solutions were each added to 300 g of fish food and left to mix on a rotary shaker in a ventilated fume hood for 48 hrs. The second batch of food was made from the same stock solution of 500 ppm A1242 in hexane for treatment food, but scaled to add to 150 g of fish food for the remainder of the experiment. Control food was made similarly, adding hexane alone to 95% ethanol. Control food had a low but detectable level of PCB contamination (0.7 ug/g.
dry weight and 0.6 ug/g dry weight, respectively). Treatment food had a PCB concentration of 14.9 ug/g dry weight and 18.1 ug/g dry weight, respectively.

**Sampling procedure for real-time RT-PCR and PCB analysis**

After 1, 2, 3, and 4 months of feeding, 1 carp from each of 10 tanks was sampled for real-time RT-PCR and PCB analysis, and 1 carp from each of 10 tanks was sampled for future histological examination. For real-time RT-PCR and PCB analysis, carp were randomly chosen for sacrifice, netted, euthanized, and completely processed within 30 minutes. Carp were euthanized with 1:500 tricaine methansulfonate (MS-222) for approximately 1 min, the weight and length was measured, and the viscera and brain were removed, immediately frozen in liquid N\textsubscript{2}, and stored at -80°C until use. Muscle filets from these carp were stored at -20°C until PCB analysis.

**Kalamazoo River Carp**

**Sampling for real-time RT-PCR processing and PCB analysis**

A total of 18 carp were caught from the Kalamazoo River in the late spring and summer, 2001. Carp were sampled from 2 upstream Kalamazoo River sites that have low, background levels of PCB contamination (Ceresco and just upstream of Morrow Lake) and from 2 Kalamazoo River sites known to be contaminated with PCBs (above Trowbridge Dam and Lake Allegan) (Figure 7). Average PCB values \pm standard error of 0-2" sediment cores below Morrow Lake, above Trowbridge Dam,
and Lake Allegan are 0.9 ± 2.7 ppm, 12.3 ± 4.0 ppm, and 3.4 ± 3.0 ppm, respectively (Blasland, Bouck & Lee, 2000).

Figure 7. Map of the Kalamazoo River in MI, USA. Carp were sampled from reference sites (Ceresco, upstream of Morrow Pond; black arrow) located upstream of the superfund site and PCB contaminated sites within the superfund sites (Lake Allegan, Trowbridge; light arrows). Date of capture and number of carp caught are shown.

![Map of the Kalamazoo River in MI, USA](image)

Carp were caught with a seine net (Trowbridge Dam, n=4), fishing rods (Lake Allegan, n=8), upstream of Morrow Lake, n=2), or electroshocked (Ceresco, n=4). Carp were immediately bought back to the laboratory live in holding pens, euthanized with MS-222 (1:2000) for approximately 1 min., measured, and dissected. For real time RT-PCR analysis, liver and brains were removed, immediately frozen with liquid...
N₂, and stored at -80°C until use. Both liver tissue and muscle tissue was sampled and stored at -20°C for PCB analysis. Muscle tissue was sampled posterior to the pectoral fin for each carp.

**RNA Extraction**

Total RNA was extracted using Qiagen RNeasy® midipreps from either 150(±30) mg (Kalamazoo River carp, and Laboratory carp, 30, 60, 90 days of feeding), or 240 (±30) mg (laboratory carp, after 120 days of feeding) of liver tissue. Sample preparation included placing dissected liver tissue for each animal in plastic bags pre-dipped in liquid N₂ and grinding with a pestil over dry ice. Samples were homogenized by passing through an 18-gauge needle. In addition, 50 ng/µl of yeast mRNA (Clonetech) was spiked into each total RNA sample prior to binding of RNA to the RNeasy membrane as an exogenous internal control for extraction and PCR efficiency. Total RNA integrity for extractions was verified with denaturing 1.25% pre-cast agarose gels (Sigma) and quantified using a GeneQuant pro Spectrophotometer (Pharmacia Biotech, Cambridge, England). After total RNA extraction, each sample was extracted for messenger RNA using oligo d(T) cellulose (Ambion MicroPoly (A) Pure™), following the recommended guidelines. Up to 0.13 mg of Total RNA was used if available for mRNA extraction. Glycogen was not added to the eluted mRNA, and samples were dried in a VacDry prior to re-suspending. Messenger RNA recovery for each carp liver was quantified using a GeneQuant pro Spectrophotometer and stored at -80°C until use.
Real-Time RT-PCR

Operation

Real-time RT-PCR (Reverse Transcriptase-Polymerase Chain Reaction) involves the reverse transcription step of a target mRNA to cDNA followed by the use of PCR to amplify DNA. Amplification allows quantification of specific PCR products in real time. The method utilizes the 5’-3’ nuclease activity of AmpliTaq Gold™ polymerase to cleave a fluorogenic reporter dye from a gene-specific probe bound to amplified cDNAs. Cleavage of the reporter dye frees it from close proximity to a quencher on the probe. Increase fluorescence levels of the reporter dye with cleavage is directly proportional to PCR product concentration, as a direct consequence of target amplification during PCR. Non-specific detection is avoided, since probe binding to the target gene sequence and cleavage by AmpliTaq Gold™ polymerase are both required for reporter fluorescence. Real-time RT-PCR is therefore a sensitive, accurate, and rapid technique, capable of quantifying a wide range of existing mRNA levels.

Primer and Fluorogenic Probe Design

Gene sequences used for primer and probe design were obtained from GenBank. Primers and dual-labeled fluorogenic probes were designed using Primer Express™ software (ver. 1.1: Applied Biosystems, Foster City, CA). Sequences and concentrations for forward primers and reverse primers are listed in Table 4. Briefly, primers were chosen as close as possible to the probe without overlapping, with an
amplicon length less than 100 base pairs. Using Primer Express software, the Tm was in the range of 58-60C.

Table 4. Primer and probe sequences and volumes used for real time RT-PCR. Primer and probe concentrations were 10μM. Sequences were designed using Primer Express ver. 1.1. Sequences used are publically available (NCBI).

<table>
<thead>
<tr>
<th>Gene*</th>
<th>Sequence</th>
<th>Amount Used</th>
</tr>
</thead>
<tbody>
<tr>
<td>CYP1A1</td>
<td>forward primer: CTGTGGCCAACGTGATCTGT</td>
<td>300 nM</td>
</tr>
<tr>
<td></td>
<td>reverse primer: CGAACTCGTGCTCAAATTG</td>
<td>300 nM</td>
</tr>
<tr>
<td></td>
<td>probe: 6FAM-TGCTTCGGCCGGCGCTACA-TAMRA</td>
<td>200 nM</td>
</tr>
<tr>
<td></td>
<td>mnsod</td>
<td>forward primer: GGCITTTGATAAGGACAGTGGAAAGA</td>
</tr>
<tr>
<td></td>
<td>reverse primer: GAAATGGGACGAGCCTGTAGTG</td>
<td>300 nM</td>
</tr>
<tr>
<td></td>
<td>probe: 6FAM-TCTTGGTTCCCACATGCAGCAATCCT-TAMRA</td>
<td>200 nM</td>
</tr>
<tr>
<td></td>
<td>g-gcs</td>
<td>forward primer: AGACTCTTTTCACAGTGTTACATCC</td>
</tr>
<tr>
<td></td>
<td>reverse primer: CAACTGGTTGGGTTGATTCTG</td>
<td>300 nM</td>
</tr>
<tr>
<td></td>
<td>probe: 6FAM-AGGCTGGCCAGGCTCACTCAGC-TAMRA</td>
<td>200 nM</td>
</tr>
<tr>
<td></td>
<td>pepck</td>
<td>forward primer: TCACATCTTCCTGACACCAAGACACA</td>
</tr>
<tr>
<td></td>
<td>reverse primer: GCAAATGGTGAAGCGATAC</td>
<td>300 nM</td>
</tr>
<tr>
<td></td>
<td>probe: 6FAM-CCGTAACCGCTGCGGAGGACA-TAMRA</td>
<td>200 nM</td>
</tr>
<tr>
<td></td>
<td>nucleolin</td>
<td>forward primer: TGCACGCACGTTGTTCGT</td>
</tr>
<tr>
<td></td>
<td>reverse primer: TAAAGACTCCAGGTATGGATTCC</td>
<td>300 nM</td>
</tr>
<tr>
<td></td>
<td>probe: 6FAM-AAGAACCTGCCCTACTCCATAACGCAGG</td>
<td>200 nM</td>
</tr>
<tr>
<td></td>
<td>yeast actin</td>
<td>forward primer: TGGAATCCCGGTGATGGGTGT</td>
</tr>
<tr>
<td></td>
<td>reverse primer: TCAAAAATGGCCGTAGGTTAGAGA</td>
<td>300 nM</td>
</tr>
<tr>
<td></td>
<td>probe: 6FAM-CTCACAGTCGATTTACCTACGCGG</td>
<td>200 nM</td>
</tr>
</tbody>
</table>
Abbreviations used: cytochrome P4501A1 (CYP1A1), magnesium superoxide dismutase (MnSOD), gamma-glutamyl cystein synthetase (g-gcs), phosphoenolpyruvate carboxykinase (PEPCK)

Taqman probes contain a fluorescent reporter dye attached to the 5’ end (FAM; 6-carboxy-fluorescein), and a quencher attached to the 3’ end (TAMRA; 6-carboxy-tetramethyl-rhodamine). Sequences for probes were chosen without a guanine (G) base on the 5’ end, since the presence of a G next to the reporter dye has been shown to cause quenching of the probe. Using Primer Express software, the Tm of the probe was designed to be at least 10°C higher than the primers. Primer and probe concentrations were optimized following the manufacturers recommendations. Probes were run using a concentration of 200nM.

Sample Preparation

Real Time RT-PCR for each carp liver mRNA sample was performed using Taqman® One-Step RT-PCR Master Mix Reagent Kit (12.5 ul/well) containing 2x Master Mix without UNG (uracil N-glycosylase) to provide all necessary reagents for PCR, including dNTPs with dUTP, buffer components, AmpliTaq Gold DNA polymerase, and a passive reference dye (ROX) to normalize for well to well variations in fluorescent signal. Multiscribe™ Reverse Transcriptase enzyme from a recombinant Moloney Murine Leukemia Virus (0.625 ul/well) for the reverse transcription step is also included separately in the kit. For each gene an identical amount (4 ng/μl) of mRNA template was added. Taqman probe (0.5 ul/well), optimized forward primer and optimized reverse primer were also added for each
gene. DepC water was additionally added to bring the final well volume to 25 μl. Reagents were purchased from Applied Biosystems.

For each individual liver mRNA sample for each gene tested, a no reverse transcriptase enzyme control was included on the PCR plate. Including no mRNA amplification controls for each gene allows for a quantitative measure of unwanted amplified DNA, as opposed to amplified mRNA. For all PCR runs, no amplification control values for all genes were a minimum of 10 PCR cycles behind unknown values at the threshold values examined, indicating that DNA amplification accounted for no greater than 0.1% of nucleic acid quantified for all samples.

Additionally, for each gene a four point standard curve and a no template control was also included on each 96 well PCR plate. Standard curves were run using 1, 2, 4, and 8 ng mRNA for all genes for all laboratory fish and filed fish examined (except for mnsod after 90 and 120 days of exposure 2, 4, 8, and 16 ng were used). Standard curve template mRNA values were chosen that generated correlation coefficients near $r^2=0.99$ and had a slope of $-3.3 \pm 0.3$, indicating high PCR reaction efficiencies (PCR product doubles with each cycle).

96-well plates were centrifuged at 2254 x g at RT for 1 min. to remove any air bubbles, then loaded into an ABI Prism® 7700 Sequence Detection System. Thermal cycling parameters for all RT-PCR runs were set to 48°C, 30 min, 10 min at 95°C, then 40 cycles of 95°C for 15 sec alternating with 60°C for 1 min.
Data Analysis

After each PCR run, a threshold value ($C_T$) was manually set in the exponential growth phase of the PCR reaction to relate the normalized reporter fluorescent signal (fluorescence signal of the reporter dye divided by the reference dye, minus the baseline signal in the first few PCR rounds) to PCR cycle number. Data were then quantified using the relative standard curve method (Applied Biosystems). Using this method, four-point standard curves for each gene were used to relate the threshold value ($C_T$) to the log of mRNA concentrations. Using the standard curve for each gene, the relative amount of mRNA from each unknown sample was then determined from $C_T$ values using linear regression analysis. Samples for standard curves and unknowns were run in duplicate.

To correct for extraction efficiency, 50 ng of yeast mRNA was spiked into each carp liver prior to total RNA extraction to serve as an exogenous internal control. Utilizing an exogenous control circumvents normalizing by 'housekeeping' genes, which has been shown via real time RT-PCR to vary (Kim and Kim, 2003). In each real-time RT-PCR run, primers and probe for yeast actin were additionally used to quantify relative yeast actin values for each individual. A four-point standard curve for yeast actin was also generated using identical mRNA template concentrations. Final PCR values from individual carp liver mRNA were normalized by dividing target gene mRNA PCR values by yeast actin mRNA PCR values.

PCR GC/MS Analysis
For PCB analysis in laboratory exposed carp, tissue was obtained (approximately 0.5 g) by using a spatula to scrape the muscle between the pectoral and anal fin (n=40). For PCB analysis in carp caught from the Kalamazoo River, liver tissue (approximately 0.5 g) in addition to muscle tissue (muscle between the pectoral and anal fin) was analyzed. The reagents, extraction technique, GC/MS analysis of PCB congeners and determination of method of detection limits was identical to that previously used for PCB GC/MS analysis of *Xenopus laevis* tadpoles (Chapter 2).

Results

**Laboratory Raised Carp**

PCB fed carp showed no differences in size compared to vehicle fed carp

Juvenile carp exposed to the PCB mixture A1242 through the diet did not show a difference in weight compared to vehicle-only fed carp over the duration of the experiment (30, 60, 90, and 120 days; ANOVA, p=0.3100, n=40; Table 5). Weight in both A1242 fed and vehicle fed carp increased during the acute exposure period by 38% and 37%, respectively (ANOVA, p=.0112).

Table 5. Average weight for laboratory raised carp fed vehicle only (control, n=10) or food containing A1242 (PCB, n=10) for 30, 60, 90, or 120 days.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>30 days</th>
<th>60 days</th>
<th>90 days</th>
<th>120 days</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>9.6 ± 0.72</td>
<td>9.74 ± 0.89</td>
<td>10.45 ± 0.50</td>
<td>13.28 ± 1.86</td>
</tr>
<tr>
<td>PCB</td>
<td>8.11 ± 0.36</td>
<td>11.00 ± 0.19</td>
<td>10.07 ± 0.81</td>
<td>11.22 ± 1.10</td>
</tr>
</tbody>
</table>

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**Muscle PCB values**

Levels of PCBs measured in muscle tissue increased significantly in carp fed PCBs versus vehicle fed controls over the duration of the experiment (ANOVA, p<0.0001, Figure 8). Muscle tissue PCB concentrations in carp fed PCBs ranged from 8.5-55.1 μg/g (ppm) over the duration of the experiment, with average values at 1, 2, 3, 4 months of feeding of 10.0±0.5, 13.0±0.6, 21.3±1.1, and 50.0±1.6 μg/g, respectively. PCB muscle values among all time points (except between months 1 and 2) were significantly different.

**Figure 8.** Muscle PCB levels are significantly different between carp fed PCBs (Aroclor 1242) and carp fed vehicle only (ANOVA; p<0.0001). Carp fed for 1, 2, 3, and 4 months were all significantly different among one another.

Bioaccumulation factors (BAF) were calculated by dividing the muscle level PCBs (μg/g) by the food PCB exposure (μg/g) (Table 6). A linear regression between average muscle PCB values and total PCB food consumed yielded a correlation of...
$r^2=0.89$. The load per time point (muscle PCBs/fish weight) increased over the course of the experiment for PCB fed fish, from 1.22 at 1 month to 6.83 at 4 months.

There was no difference in muscle PCB values in carp fed food with background levels of PCBs only (0.7 ug/g dry weight) among the different time points, denoting that the PCB concentrations were in equilibrium with the muscle tissue compartment.

Table 6. Average muscle PCBs (ppm), PCBs consumed (ppm), and bioaccumulation factor (BAF) after 1, 2, 3, and 4 months of feeding food with PCBs added or vehicle treated only.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Average Muscle PCBs (ppm)</th>
<th>PCBs consumed (ppm)</th>
<th>BAF</th>
</tr>
</thead>
<tbody>
<tr>
<td>Month 1</td>
<td>control 4.478</td>
<td>0.472</td>
<td>8.86</td>
</tr>
<tr>
<td></td>
<td>PCB 9.968</td>
<td>41.7</td>
<td>0.24</td>
</tr>
<tr>
<td>Month 2</td>
<td>control 4.896</td>
<td>1.247</td>
<td>3.17</td>
</tr>
<tr>
<td></td>
<td>PCB 12.97</td>
<td>86.6</td>
<td>0.14</td>
</tr>
<tr>
<td>Month 3</td>
<td>control 3.174</td>
<td>6.4844</td>
<td>0.27</td>
</tr>
<tr>
<td></td>
<td>PCB 21.286</td>
<td>138.05</td>
<td>0.23</td>
</tr>
<tr>
<td>Month 4</td>
<td>control 3.178</td>
<td>9.0338</td>
<td>0.27</td>
</tr>
<tr>
<td></td>
<td>PCB 50.042</td>
<td>204.2</td>
<td>0.38</td>
</tr>
</tbody>
</table>

by 1 month. Muscle PCB values in these vehicle fed control carp averaged 3.9 ug/g. The BAF for these fish after 1 month of feeding was 8.86, and decreased to a steady-state value of 0.27 after 3 months of feeding. The load per time point for vehicle fed control fish was lower than for PCB fed fish, and decreased from 0.41 after 2 months of feeding to 0.19 after 4 months of feeding.
CYP1A1 mRNA expression levels from carp hepatopancreas were significantly elevated in carp fed A1242 in laboratory experiments compared to vehicle fed controls (Figure 9; ANOVA, p=0.0012). In carp fed up to 4 months, CYP1A1 mRNA expression levels were elevated from 1.4 fold at 1 month to 12.6 fold at 4 months compared to control carp. CYP1A1 mRNA expression levels for control carp did not change over the course of the experiment. In all laboratory carp molecular analysis, n=10 carp per time point (N=5 control and n=5 PCB fed), except after 4 months of exposure, n=4 for PCB fed, and after 1 month of feeding, n=4 for control and n=4 for PCB fed.

Figure 9. CYP1A1 mRNA expression is significantly elevated in laboratory carp fed PCBs (Aroclor 1242) compared to vehicle fed controls (p=0.0012).
Real time RT-PCR mRNA values involved in oxidative stress did not change with PCB ingestion

In addition to using primers and probes specific for CYP1A1, I performed real-time RT-PCR for carp hepatopancreas mRNA extracts using primers and probes specific for two oxidative stress genes involved in responses to toxic oxidation products, mnsod (magnesium superoxide dismutase) and gamma-gcs (gamma-glutamyl cysteine synthetase). Mnsod is an anti-oxidant enzyme that catalyzes the reduction of the superoxide anion radical, and gamma-gcs is the rate-limiting enzyme in glutathione synthesis. Neither mnsod nor gamma-gcs mRNA levels significantly differed in laboratory PCB exposed carp compared to vehicle fed controls (Figure 10).

Figure 10. Relative mRNA expression levels of mnsod and g-gcs in carp either fed PCBs (black bars) or vehicle fed controls (stippled bars) after 1, 2, 3, and 4 months of feeding. Differences were not statistically different.
10. There was a slight trend, however, for increased expression for both genes in PCB exposed carp.

**Real time RT-PCR mRNA values involved in metabolism did not change with PCB ingestion**

To examine potential metabolic or growth differences I also tested pepck (phosphoenolpyruvate carboxykinase) and nucleolin mRNA expression levels in PCB exposed and vehicle fed control carp for potential bioindicators of health effects (Figure 11). To test if PCBs alter metabolism, I chose to examine expression levels

Figure 11. Relative mRNA expression levels of pepck and nucleolin in carp either fed PCBs (black bars) or vehicle fed controls (stippled bars) after 1, 2, 3, and 4 months of feeding. Differences were not statistically different.

```
<table>
<thead>
<tr>
<th>Months</th>
<th>pepck mRNA (relative units)</th>
<th>nucleolin mRNA (relative units)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>2</td>
<td>2</td>
<td>2</td>
</tr>
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<td>3</td>
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<td>3</td>
</tr>
<tr>
<td>4</td>
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</tbody>
</table>
```
of pepck, an important regulatory gene involved in the conversion of glycogen to glucose during gluconeogenesis. Gluconeogenic enzyme levels have been shown to be altered by 3,3',4,4',5'-pentachlorobiphenyl and TCDD administration (Weber et al., 1991; Ishii et al., 2001), and in response to 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD), pepck mRNA and protein levels are decreased (Stahl et al., 1993). Therefore, it is possible that exposure to high environmental PCB levels alters the ability to mobilize stored glycogen for energy production. In addition, carp are specialized for metabolic depression. Winter acclimatized carp show profound changes in nuclear cytoarchitecture and in nucleolar rRNA associated genes, which regulates ribosome synthesis (Alvarez et al., 2003). To test if high levels of PCBs in carp alter protein metabolism and potentially affect winter acclimatization, I investigated mRNA levels of nucleolin, a nucleolar repressor of protein synthesis. There was no significant difference in mRNA expression levels of these genes in the laboratory carp examined. There was a trend for increased pepck values, which would indicate slightly elevated mobilization from stored glycogen for metabolic energy conversion. After 1 month of feeding, both carp fed vehicle-only food and carp fed food with A1242 added showed reduced pepck mRNA expression values compared to later time points.

Kalamazoo River carp

Size

Carp were sampled from 2 PCB contaminated sites within the Superfund site and 2 upstream sites (Figure 7) in the Kalamazoo River during the summer, 2001.
Carp from the reference sites were longer (62.6± 4.0 cm; n=6) than the carp from the PCB contaminated superfund sites (41.3± 1.9 cm; n=11) (p<0.0001).

PCB values

PCB values were significantly higher in carp hepatopancreas sampled from PCB sites (15.42± 1.95 ug/g) than from carp hepatopancreas sampled from reference sites (1.19 ±1.6 ug/g) (t-test, t=0.0001, n=18, Figure 12). Hepatopancreas PCB values were also significantly different among sites (ANOVA, p=0.0014). In a post-hoc test for significant comparisons (Fisher’s PLSD), there was no significant difference in PCB levels between the two PCB sites examined (p=0.2038) and no significant difference in PCB levels between the 2 reference sites examined (p=0.9711).

Figure 12. PCB values for carp (muscle, solid bars; hepatopancreas [hp] stippled bars) sampled from Kalamazoo River reference and PCB contaminated sites, 2001.
PCB values in muscle were not higher in carp sampled from PCB sites (2.01 ± 0.29 ug/g) than from carp sampled from reference sites (1.18 ± 0.24 ug/g, t-test = 0.0813, n=18). Additionally, there was no significant difference between hepatopancreas and muscle PCB values in carp from reference sites (paired t-test, t=0.9648). However, there was a significant difference between hepatopancreas and muscle PCBs from carp from PCB contaminated sites (paired t-test, t<0.0001, Figure 12).

CYP1A1 real time RT-PCR mRNA values increased in PCB contaminated carp.

CYP1A1 mRNA expression levels from carp hepatopancreas were significantly elevated in carp from PCB contaminated Superfund sites (3.4 fold) compared to upstream reference sites (t-test = 0.0302; Figure 13).

Figure 13. CYP1A1 mRNA expression is significantly different between carp sampled at reference versus contaminated Kalamazoo River sites (t-test; p=0.0302, n=18).
Real time RT-PCR mRNA values involved in oxidative stress did not change in PCB contaminated carp

Neither mnsod nor gamma-gcs mRNA levels significantly differed between PCB exposed field carp compared to upstream reference carp (Figure 14). There was a slight trend, however, for increased expression in PCB exposed carp. Two carp from a PCB contaminated superfund site (Lake Allegan) displayed high mnsod mRNA expression compared to other fish (reflected by a high standard error). One of these carp also displayed a high pepck mRNA level, also reflected in the relatively

Figure 14. Relative mRNA expression levels of mnsod and g-gcs in carp caught from PCB contaminated (black bars) and reference sites (stippled bars) from the Kalamazoo River. Differences were not statistically different.
large standard error for that gene.

Real time RT-PCR mRNA values involved in metabolism did not change in PCB contaminated carp

There was no significant difference between carp caught from reference and carp caught from contaminated sites in mRNA expression levels for pepck and nucleolin hepatopancreas mRNA (Figure 15).

Figure 15. Relative mRNA expression levels of pepck and nucleolin in carp caught from PCB contaminated sites (black bars) and reference sites (stippled bars) from the Kalamazoo River. Differences were not statistically different.
Discussion

Upregulation of CYP1A1: Carp are biologically responding to PCB exposure

In this report I utilize real time RT-PCR to test for bioindicators of exposure and health effects in PCB exposed carp, *Cyprinus carpio*, in both an acute laboratory study in carp exposed to PCBs through the diet and in adult carp collected from PCB contaminated sites in the Kalamazoo River Superfund Area. I demonstrate that CYP1A1 mRNA is induced in carp in the laboratory and in the field. CYP1A1 induction increased from 1.4 fold after 1 month of feeding to 12.6 fold after 4 months of feeding PCBs in the laboratory, and was induced 3.4 fold in carp from PCB contaminated sites compared to upstream reference sites. Significantly elevated CYP1A1 mRNA levels in the laboratory and in the field suggest that carp are biologically responding to PCB contamination at exposure levels currently present in the Kalamazoo River.

The significant induction of CYP1A1 mRNA in carp hepatopancreas chronically exposed to PCB contamination is contrary to recent reports of CYP1A suppression in liver of fish chronically exposed to PCBs in other PCB-contaminated Superfund Sites. For example, the *Fundulus heteroclitus* (Atlantic killfish or mummichog) population inhabiting New Bedford Harbor, MA (Nacci et al., 1999; Bello et al., 2001), and the Atlantic tomcod population (*Microgadus tomcod*) population inhabiting the Hudson River (Courteney et al., 1999) show reduced inducibility of CYP1A. Reduced CYP1A inducibility in these highly contaminated fish populations is advantageous in conferring reduced impacts of adverse health
effects mediated through the induction of CYP1A (Prince and Cooper, 1995; Nacci et al., 2002). Despite progress into elucidating the mechanisms of reduced inducibility, CYP1A expression cannot be reliably used a biomarker of exposure in these fish. In contrast, CYP1A1 mRNA expression in carp sampled from the Kalamazoo River Superfund site appears to be a reliable biomarker of PCB exposure.

Select health bioindicator genes are not significantly different in PCB exposed carp

In this research I also utilized real time RT-PCR to define bioindicators of health effects in laboratory raised and field carp exposed to PCBs. Bioindicators are commonly used as tools to predict contaminant-induced health effects on organisms, and are helpful in understanding integrated biological responses to environmental contamination. Further, applying a single tool to monitor multiple endpoints for use in environmentally based risk assessment studies could be an expeditious and efficient tool for biologically based decision making regarding clean-up of contaminated sites. Molecular tools such as RT-PCR to measure mRNA expression have shown to be highly effective and repeatable, and have many advantages over conventional bioindicator assays. Here I use Taqman® real time RT-PCR to quantify expression of multiple genes by measuring fluorescence levels of gene-specific probes during PCR cycling.

However, based on several select bioindicator endpoints selected for this study, I did not see evidence of PCB induced health effects in carp. Expression levels for specific genes involved in oxidative stress (g-gcs, mnsod) and in metabolism
(pepck, nucleolin) were not significantly different in PCB exposed laboratory carp hepatoancreas as well as in carp hepatopancreas caught from PCB contaminated versus reference sites in the Kalamazoo River.

Why CYP1A1 mRNA activity is increased whereas g-gcs and mnsod oxidative stress genes are not appears puzzling. CYP1A1 mRNA is an inducible ligand-activated gene, mediated through activation of the aryl hydrocarbon (Ah) receptor. CYP1A protein can metabolize Ah-ligands, but also is a source of reactive oxygen species (ROS) and activated metabolic breakdown products. To metabolize ROS produced in association with CYP1A expression, oxidative stress protein products that are either part of the inducible Ah gene-battery or expressed in the cell are increased (e.g., g-gcs and mnsod).

It is also possible that oxidative stress genes and proteins were inactivated by a post-transcriptional effect on CYP1A protein. It has been shown that high doses of planar PCBs competitively inhibit CYP1A protein expression (Schlezinger and Stegeman, 2001; Besselink et al., 1998; Gooch et al., 1989), and CYP1A protein activity is a source of reactive oxygen species (Schlezinger et al., 1999). It could therefore be hypothesized that induced CYP1A1 mRNA expression in carp can be uncoupled from reactive oxygen species production and health effects through PCB effects on CYP1A after translation. However, if CYP1A1 protein levels were elevated enough to cause oxidative stress, and subsequently a reduction in oxidative stress proteins concomitant with a decrease in CYP1A1 protein expression, a reduction in the oxidative stress mRNA levels with increasing exposure time should have been seen in the laboratory dose-response experiment over time.
It is additionally possible that PCB levels present in carp were not sufficient to significantly induce an oxidative stress response. In the laboratory dosing experiment, the most contaminated carp had an average of 50.0± 1.6 ug/g. After an extensive literature review, Niimi (1996) concludes that in PCB studies in aquatic organisms, tissue concentrations around 50-100 ug/g may be needed to adversely affect growth and reproduction.

RT-PCR can be utilized to test multiple health effect endpoints

In the environment, PCBs are present as mixtures that can elicit multiple biological effects. Possible health endpoints mediated by PCB congeners that can bind to the Ah receptor also include differences regarding vitamin A (Zile, 1992), glucose (Ishii et al., 2001) and lipid (Borlakoglu et al., 1991) metabolism. Health effects can additionally be mediated in an Ah-independent manner. For example, ortho-substituted congeners that cannot bind to the Ah receptor have been shown to alter calcium signaling in neurons (Pessiah and Wong, 2001), and alter cognitive function such as learning and memory (Schantz, 2001).

Recently, the advent of molecular tools has made it possible to test for additional health effect endpoints in select tissues. For example, McClain et al. (2003) used real time RT-PCR to examine the effects of 3 genes postulated to be biomarkers of exposure in liver and gill tissue of rainbow trout (Oncorhynchus mykiss) downstream of a source of creosote contamination: CYP1A1 (planar aromatic hydrocarbons), vitellogenin (estrogenic compounds), and metallothionein (metals). In order to define health effects of PCBs in carp from the Kalamazoo River superfund
site, I used real time RT-PCR to examine gene expression changes in hepatopancreas (liver plus pancreas) tissue. Using a PCR method to test for gene expression levels of single or multiple genes in signaling pathways in field organisms could prove to be a great advance in understanding gene function and characterizing health effects endpoints correlated with environmental chemical exposure.

Summary

This study uses real time RT-PCR to examine mRNA expression levels of select genes in an environmental risk assessment context as bioindicators for health effects. Significant changes in levels of mRNA expression were found in CYP1A1 in both a laboratory PCB feeding experiment and field studies using carp caught from PCB contaminated and reference sites from the Kalamazoo River. The real time RT-PCR technique presented in this chapter demonstrates that it is possible to relate changes at the physiological level in organisms in a population to a xenobiotic environmental stressor (PCBs). In addition, real-time RT-PCR can be used to detect health effects affecting a population without extensive and prior characterization of the population, which is often the case involving studies concerning xenobiotics that are already present in the environment. Additional studies that monitor suites of gene expression changes in multiple species would be useful in assessing ecosystem-wide contaminant profiles of health effects.
CHAPTER 4

HISTOPATHOLOGICAL ANALYSIS OF MACROPHAGE AGGREGATES IN CARP (CYPRINUS CARPIO) FROM THE KALAMAZOO RIVER

Abstract

The Kalamazoo River MI, USA is a superfund site due to contamination with polychlorinated biphenyls (PCBs). To test if chronic exposure to PCBs in the environment impacts the health of resident fish species, analyses were performed in carp (Cyprinus carpio) to examine hepatopancreas macrophage aggregates. Macrophage aggregates function in the immune response and in degradation, sequestration, and detoxification of effete cellular materials, and increases in density have been proposed to be indicators of environmental stress. In this study carp were caught from PCB contaminated sites and upstream reference sites from the Kalamazoo River, and hepatopancreas macrophage aggregate densities, PCB levels, and pigment components were investigated. Percent area (ANOVA, p=0.0263) and number per area (ANOVA, p=0.0453) of macrophage aggregates were greater in carp from PCB contaminated sites compared to carp from reference sites, although carp from PCB contaminated sites were smaller and younger than carp from reference sites. Additionally, macrophage aggregate number per area in carp caught from Lake Allegan, a PCB contaminated site, was significantly correlated with PCBs (r=0.9068, p=0.0337). Pigmented macrophage aggregates in hepatopancreas shows an
abundance of lipofuscin in carp from all sites. Despite the significant relationship between macrophage aggregate number and PCBs at Lake Allegan, further investigations are needed until a causal link can be established.

Introduction

The Kalamazoo River, MI, USA is listed by the U.S. Environmental Protection Agency as a Superfund Site in the Great Lakes watershed due to the high presence of polychlorinated biphenyls (PCBs). PCBs were heavily discharged into the Kalamazoo River as part of the de-inking process of carbonless copying paper by several paper companies located along the river until production was banned in 1977. Levels of polycyclic halogenated aromatic hydrocarbons (PHAHs) other than PCBs and polynuclear aromatic hydrocarbons (PAHs) are relatively low (Blasland, Bouck & Lee, 2000). Since PCBs are hydrophobic and lipophilic, PCBs accumulate in river sediments and bioaccumulate in aquatic organisms. In field studies on fish, PCBs in fish tissue have been associated with adverse health effects, such as decreased fish size, increased mortality, and increased hepatic lesion frequencies (reviewed in Niimi, 1995).

To test if PCBs pose a health threat to resident fish species in the Kalamazoo River Superfund Site, we examined hepatopancreas tissue for lesions in carp (Cyprinus carpio) from PCB contaminated Kalamazoo River sites and relatively uncontaminated upstream reference sites. Previous molecular research (Chapter 3) showed significant induction of cytochrome P4501A1 (CYP1A1) mRNA in carp hepatopancreas from PCB contaminated Kalamazoo River sites compared to carp

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from reference sites, suggesting that carp from the Kalamazoo River are responding to PCB exposure and therefore may show hepatic and pancreatic lesions associated with exposure.

Macrophage aggregates (MAs), or melano-macrophage centers (MMC) have been proposed to be biomarkers of environmental health and degradation (Fournie et al., 2001; Blazer et al., 1987). Macrophage aggregates are pigmented focal lesions that function in immune processes, iron recycling, and in storage, destruction, and detoxification of endogenous and exogenous materials (Wolke 1992). In response to environmental stress including toxicant exposure, macrophage aggregates have been observed to change in density, frequency, and size (Angius and Roberts, 2003; Wolke et al., 1985).

In the present study, carp were collected from the same PCB contaminated and reference sites from the Kalamazoo River that were previously sampled for CYP1A1 and gene expression analysis. Carp hepatopancreas were examined histologically to determine if the density, number, and size of macrophage aggregates differed among sites and between reference and PCB sites, and hepatopancreas PCB levels were also measured and correlated with macrophage aggregate densities. Macrophage aggregates were also stained with Gamori's Prussian Blue to reveal the proportion of macrophage aggregates staining for hemosiderin, lipofuscin/ceroid, and melanin. Hepatopancreas from an additional carp from a PCB site was prepared for ultrastructural analysis to confirm the nature of the macrophage aggregate lesions. Since macrophage aggregates have been previously shown to increase with age, the age of carp were determined from scales removed near the lateral line.
Hepatosomatic index, gonadal somatic index, and condition factor of carp were additionally evaluated as bioindicators of PCB exposure.

Materials and Methods

Carp collection

Mature carp were collected in August-September of 2003 from PCB contaminated (Trowbridge, Lake Allegan) and reference Kalamazoo River sites (Morrow Lake, upstream of Morrow Lake, Ceresco) for histological examination of health effects. Carp were sampled by electroshocking (Trowbridge, n=5 and Ceresco, n=9) or by fishing with doughballs (upstream of Morrow Lake, n=3 and Morrow Lake, n=5) and by fishing with corn (Lake Allegan, n=5). Carp were sampled at Morrow Lake in addition to upstream of Morrow Lake, since sediment PCB data and fish data from other studies are available for this site. Due to the short distance between these sites (approximately 3 km) and since carp can move freely between these sites, for analysis these sites are grouped together as Morrow. Ceresco was sampled at a second date in order to try to catch fish comparable in size (i.e., smaller) to the PCB contaminated sites, Lake Allegan and Trowbridge (Figure 16). Carp caught from Ceresco, Morrow River and Morrow Lake, and Lake Allegan were immediately brought back to the laboratory live in holding pens, euthanized with MS-222 (1:2000) for approximately 1 min., measured, and dissected. Carp caught from Trowbridge were euthanized with MS-222 (1:2000) for approximately 1 min and processed on site similar to laboratory processed carp except that a spring-loaded balance was used for fish weight determination.
An additional carp was sampled from Lake Allegan with a fishing rod and prepared for ultrastructural analysis of hepatopancreas. Fresh hepatopancreas tissue was processed on site immediately following harvest. Tissue was minced with a razor blade to less than 1 mm³ pieces and placed in 20 ml scintillation vials containing cold 3% EM grade glutaraldehyde (Polysciences, Inc., Warrington, PA) in a 0.1M phosphate buffer (pH 7.3). Samples were kept on ice for transport back to the laboratory for processing, and after 2 hours samples were changed into 0.1M phosphate buffer and stored for 48 hours at 4C.

Figure 16. Map of the Kalamazoo River in MI, USA. Carp were sampled from reference sites (Ceresco, upstream of Morrow Pond, and in Morrow Pond (black arrows) located upstream of the superfund site and PCB contaminated sites within the superfund sites (Lake Allegan, Trowbridge; light arrows). Date of capture and number of carp caught at each site are shown.

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Carp processing

For each carp, the weight, length, hepatopancreas wet weight, gonad wet weight, and sex were recorded. In carp, liver plus pancreas tissues are combined into a single organ, referred to as hepatopancreas. For histological analysis, samples no greater than 1 cm in one direction were cut with scissors or a razor blade from hepatopancreas, spleen, gonad, head kidney, and intestine. Organs from each carp were placed in pre-labeled cassettes which were immediately placed in neutral buffered formalin (NBF) for fixation. Samples of liver and muscle (anterior to the pectoral fin) were stored at -20C for PCB analysis. For future analysis, hepatopancreas and brain were also frozen in liquid nitrogen, and stored at -80C. Several scales from each fish, taken near the lateral line, were also saved in -20C for age determination analysis. For age determination, a subset of these scales were placed on a Nikon SMZ-U dissecting microscope (Tokyo, Japan) and high contrast images were captured using Metamorph software, with a Javelin Chromachip II (Torrance, CA, USA) high resolution camera system. Annuli were manually counted, and false annuli were identified by counting annuli at several locations on the same scale, and by counting annuli on different scales from the same fish to try to estimate variance.

Histology sampling and macrophage aggregate analysis

Samples for histological analysis were dehydrated and infiltrated with paraffin using an automated histological processing unit. Organs were then oriented and
embedded in paraffin using a paraffin embedding station. Six μm sections were cut using a microtome and floated onto slides in a 45°C water bath containing 10 ml of Tissue Bond (Fisher Scientific). Slides were stained for hematoxylin and eosin (H&E) in batch and/or stained using periodic acid Schiff's reagent (PAS; Sigma Chemical Co, St. Louis, MO) using an aqueous solution of fast green as a counterstain. Intestine samples on each slide served as a positive control indicator for PAS stained slides.

Images for macrophage aggregate densities were taken from 23.3 ± 1.8 mm² of each carp hepatopancreas using Metamorph software, and imported into NIH Image J for image analysis. One slide and one section on each slide for each carp were chosen randomly for analysis. Total area of each hepatopancreas examined was taken with a 4x lens. Macrophage aggregates in each hepatopancreas were individually traced in hepatocytes and in exocrine pancreas using a 10x lens, allowing quantification of total macrophage aggregate area, macrophage aggregate number/mm², and average macrophage aggregate size. Macrophage aggregates for each carp hepatopancreas were added from approximately 7 images.

To examine the relative pigment composition of macrophage aggregates, slides were stained using Gamori's Prussian Blue (Mallory, 1942). Slides were not counterstained in order to distinguish orange/yellow (lipofuscin) and brown/black granules (melanin). Using a Prussian Blue stain, hemosiderin stains bright blue. Positive control slides for hemosiderin and melanin were included.
Ultrastructure sampling

After 48 hours in 0.1M phosphate buffer, carp tissue samples were washed (2×) in buffer and post-fixed in buffered 1% osmium tetroxide (Polysciences, Inc., Warrington, PA) for one hour at RT. The samples were then dehydrated with a graded ethanol series and infiltrated with propylene oxide and PolyBed 812® (Polysciences, Inc., Warrington, PA) epoxy resin. Samples were embedded in PolyBed 812-filled Beem capsules, allowed to polymerize (70°C, 48 hours), and thin sectioned (60-90 nm) with a diamond knife using a LKB (Bromma, Sweden) microtome. Thick sections (750 nm) were also stained with toluidine blue for preliminary examination. Samples were then placed on 200 mesh copper grids (Ted Pella, Inc., Redding, CA) and stained with 5% methanolic uranyl acetate and Reynolds lead citrate. Ultrastructural images were examined with a JOEL 1230 TEM at 80 kV.

PCB Analysis

Samples of hepatopancreas and muscle tissue were collected from each carp for PCB determination, and stored at -20°C. The sampling techniques, reagents, extraction technique, GC/MS analysis of PCB congeners and determination of method of detection limits identical to that previously used for PCB GC/MS analysis of carp caught from the Kalamazoo River (Chapter 2) was used to analyze the samples.
Data Analysis

Data were analyzed using SAS® software (SAS, Cary, NC, USA). Nested analysis of variance (ANOVA) was used to compare effects between reference sites and PCB sites and among the reference sampling locations (Ceresco, Morrow) and the PCB sampling locations (Trowbridge, Lake Allegan) in the Kalamazoo River. Brown and Forsythe’s test for homogeneity of variance for all ANOVAs were >0.05. Pearson correlation coefficients were used to compare the correlation (r) and probability of significant effect (p) between variables, including carp length (cm), age, PCB levels, sex, and hepatopancreas macrophage aggregate densities.

Condition factor, liver weight, HSI, gonad weight and GSI were also compared between sex, between treatments (PCB sites versus reference sites), and among sites (ANOVAs or nested ANOVAs). Condition factor, hepatosomatic index (HSI) and gonadosomatic index (GSI) were calculated for each fish as follows:

Condition factor = \( \frac{\text{weight(g)}}{\text{length(cm)}}^2 \)

HSI = 100 \times \left( \frac{\text{liver weight (g)}}{\text{carp weight(g)}} \right)

GSI = 100 \times \left( \frac{\text{gonad weight (g)}}{\text{carp weight(g)}} \right)

Results

Physiological condition indices

A total of 27 carp were examined for liver histopathology from Kalamazoo River reference and PCB contaminated sites (Table 7). In the table, values with different letters indicate significance at \( \alpha=0.05 \), whereas values with the same letters...
denotes no significant difference. Carp weight and length ranged from 0.68 kg to 3.43 kg, and from 39.6 cm to 6.32 cm at Lake Allegan and Ceresco, respectively. Carp caught from Ceresco were longer than carp caught from the other sites (Morrow, Trowbridge, and Lake Allegan). The relatively larger carp caught from Ceresco had a significantly higher condition factor than the smaller carp caught from the other sites (ANOVA, p=0.0460).

Table 7. Physiological condition indices for carp caught from Kalamazoo River PCB contaminated and reference sites.

<table>
<thead>
<tr>
<th>Area</th>
<th>Site</th>
<th>Weight (Kg)</th>
<th>Length (cm)</th>
<th>Condition Factor</th>
<th>HSI</th>
<th>GSI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reference</td>
<td>Ceresco n=9</td>
<td>3.43±0.64 a</td>
<td>63.2±2.8 a</td>
<td>0.013±0.001 a</td>
<td>2.42±0.17 a</td>
<td>11.49±0.63 a</td>
</tr>
<tr>
<td>Morrow n=8</td>
<td>1.09±0.06 b</td>
<td>45.2±0.9 bc</td>
<td>0.012±0.001 ab</td>
<td>1.91±0.22 a</td>
<td>4.26±0.42 b</td>
<td></td>
</tr>
<tr>
<td>PCB Trowbridge</td>
<td>n=5</td>
<td>nd</td>
<td>49.5±2.2b</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
</tr>
<tr>
<td>Lake Allegan</td>
<td>n=5</td>
<td>0.68±0.12 b</td>
<td>39.6±1.6 c</td>
<td>0.010±0.001 b</td>
<td>2.24±0.18 a</td>
<td>6.45±2.0 b</td>
</tr>
</tbody>
</table>

different letters = significantly different
nd = not determined. Field processed fish used a spring balance (see materials and methods)

Hepatosomatic index, an indicator of relative liver size, did not differ among fish caught from reference and PCB sites. There was, however, a significant correlation between HSI and fish sex at reference sites (r=0.6290, p=0.0068), with males having a higher HSI. At Morrow, a reference site, macrophage aggregate area and number was significantly negatively correlated with HSI (r=-0.6965, p=0.0548; r=-0.7433, p=0.0346, respectively) and macrophage aggregate size was significantly positively
correlated with HSI ($r=0.8394$, $p=0.0092$). The relatively larger carp caught from Ceresco had a higher gonadal somatic index than carp caught from Morrow Lake and Lake Allegan (ANOVA, $p<0.0001$).

In carp hepatopancreas, both hepatocytes (h) and exocrine pancreas islets (p) contained macrophage aggregates. To test for differences in the relative proportion of macrophage aggregates in each tissue among sites, analyses were performed (nested ANOVA) comparing both the differences (h-p) and the ratios (h/p) of % of macrophage aggregate area occupied in each tissue. Since there was no difference utilizing both parameters between reference and PCB sites and among sites, hepatocyte and exocrine pancreas numbers were combined for the hepatopancreas macrophage aggregate profile of each carp.

**Carp hepatopancreas PCBs are significantly different among sites**

PCBs measured in carp hepatopancreas were significantly different between upstream reference sites (1.207± 0.357 ug/g) and downstream PCB sites (7.560± 1.144 ug/g) ($p<0.0001$) and among sites (Ceresco, 0.121± 0.026 ug/g; Morrow 2.428± 0.465 ug/g; Trowbridge, 10.246± 1.791 ug/g; Lake Allegan, 4.873± 1.144 ug/g) ($p=0.0005$; Figure 17).

**Significant differences in macrophage aggregate percent area and number**

Percent macrophage aggregate area ($p=0.0263$) and macrophage aggregate number ($p=0.0453$) in hepatopancreas was significantly different between reference...
Figure 17. Carp hepatopancreas PCBs sampled from reference sites (bars with diagonal lines) and from PCB contaminated sites (black bars). PCB values are significantly different among sites (p=0.0005).

and PCB sites (Figure 18). Additionally, macrophage aggregate number per area was different among sites (p=0.0079), with Lake Allegan (a PCB contaminated site) carp macrophage aggregate number per area significantly higher than that from Ceresco, Morrow (reference sites) and Trowbridge (a PCB contaminated site) (Table 8). There was no significant difference in macrophage aggregate size between reference and PCB sites. Macrophage aggregate size was significantly correlated with HSI at Morrow Lake (r=0.8394, p=0.0092), suggesting that macrophage aggregate size increases with relative liver size at this site.

Macrophage aggregate parameters not positively correlated with fish length or fish age.

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Macrophage aggregate area/mm$^2$, number per mm$^2$, and size were examined for correlation with carp length (cm) to test if these parameters increase with fish size. Carp lengths from reference sites, with an average length ranging from 45.2±

Figure 18. Percent macrophage aggregate (MA) area (mm$^2$) and macrophage aggregate number (#) per area (mm$^2$) in carp hepatopancreas are greater in PCB sites compared to reference sites.

0.9 cm (Morrow) to 63.1± 2.8 cm (Ceresco) were not significantly correlated with any macrophage aggregate parameters, although there was a positive association. Carp lengths from PCB contaminated sites also did not show a significant correlation,
displaying a negative association with macrophage aggregate parameters. There was a significant negative correlation between macrophage aggregate number and length \((r=-0.6424, p=0.0452)\), which can be explained by the significantly larger macrophage aggregate numbers per area present in smaller, Lake Allegan carp.

Table 8. Percent macrophage aggregate (MA) area, number per area, and size in hepatopancreas for adult carp sampled from Kalamazoo River PCB contaminated and reference sites.

<table>
<thead>
<tr>
<th>Area</th>
<th>Site</th>
<th>%MA Area* (\text{mm}^2)</th>
<th>MA#/Area* (\text{mm}^2)</th>
<th>MA Size/Section (\text{mm}^2)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reference</td>
<td>Ceresco ((n=9))</td>
<td>1.47±0.54</td>
<td>3.15±0.92 a</td>
<td>0.0042±0.001</td>
</tr>
<tr>
<td></td>
<td>Morrow ((n=6))</td>
<td>0.30±0.05</td>
<td>0.91±0.19 a</td>
<td>0.0037±0.0003</td>
</tr>
<tr>
<td>PCB</td>
<td>Trowbridge ((n=5))</td>
<td>2.67±1.67</td>
<td>1.87±0.21 a</td>
<td>0.012±0.006</td>
</tr>
<tr>
<td></td>
<td>Lake Allegan ((n=5))</td>
<td>3.28±1.33</td>
<td>6.20±1.99 b</td>
<td>0.0052±0.001</td>
</tr>
</tbody>
</table>

different letters = significantly different
*Reference sites significantly different than PCB sites

Age for each carp was also determined using scales collected near the lateral line. Using the age data collected, carp from reference sites (Ceresco 11.9±0.9; Morrow, 9.5±0.5) were significantly older than carp from PCB sites (Trowbridge 8.25±0.8; Lake Allegan 8.8±1.0) \(p=0.0180\). Age was not correlated with length in carp from PCB sites, but was correlated with length in carp from reference sites \((r=0.7237, p=0.0510)\). Carp age was not significantly correlated with macrophage aggregate densities in PCB sites (slight negative correlations) and carp age was not significantly correlated with macrophage aggregate densities in reference sites taken together (slight negative correlations). Carp age from Morrow was significantly
negatively correlated with macrophage aggregate area and number ($r=-0.8034$, $p=0.0163$; $r=-0.7057$, $p=0.0505$). Although macrophage aggregate parameters have been shown to increase with age (Wolke et al., 1985; Couillard and Hodson, 1996), hesitation is warranted in generating conclusions in this study due to uncertainty in inferring age from scales and small sample size.

Macrophage aggregate number/area is significantly correlated with Lake Allegan carp PCBs

Correlation analysis was performed to examine the relationship between hepatopancreas macrophage aggregate densities and hepatopancreas PCB values (Table 9). At reference sites, $r$ is relatively small, and $p$ is relatively high, suggesting a poor relationship between macrophage aggregate densities. Since the reference sites contain low, background PCB levels, a poor relationship between macrophage aggregate densities and PCB levels was predicted. At PCB sites, $r$ is relatively high

<table>
<thead>
<tr>
<th>Reference Sites</th>
<th>Correlation Coefficient</th>
<th>% MA Area (mm²)</th>
<th>MA #/ Area (mm²)</th>
<th>MA size (mm²)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ceresco</td>
<td>$r$</td>
<td>-0.19</td>
<td>-0.16</td>
<td>-0.38</td>
</tr>
<tr>
<td></td>
<td>$p$</td>
<td>0.61</td>
<td>0.67</td>
<td>0.31</td>
</tr>
<tr>
<td>Morrow</td>
<td>$r$</td>
<td>0.27</td>
<td>0.06</td>
<td>0.15</td>
</tr>
<tr>
<td></td>
<td>$p$</td>
<td>0.51</td>
<td>0.88</td>
<td>0.72</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>PCB Sites</th>
<th>Correlation Coefficient</th>
<th>% MA Area (mm²)</th>
<th>MA #/ Area (mm²)</th>
<th>MA size (mm²)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Trowbridge</td>
<td>$r$</td>
<td>0.60</td>
<td>0.60</td>
<td>0.58</td>
</tr>
<tr>
<td></td>
<td>$p$</td>
<td>0.29</td>
<td>0.28</td>
<td>0.31</td>
</tr>
<tr>
<td>Lake Allegan</td>
<td>$r$</td>
<td>0.81</td>
<td>0.91</td>
<td>-0.03</td>
</tr>
<tr>
<td></td>
<td>$p$</td>
<td>0.09</td>
<td>0.03*</td>
<td>0.96</td>
</tr>
</tbody>
</table>
and p is relatively low, suggesting a stronger relationship between macrophage aggregate parameters and PCB levels. At Lake Allegan, macrophage aggregate number per area was significantly correlated with PCBs at alpha = 0.05 (r = 0.91, p = 0.03). A strong relationship also exists between percent macrophage aggregate area and PCBs (r = 0.81, p = 0.09). Conversely, macrophage aggregate size shows a lack of relationship. Although sample sizes in this study were relatively small, the significant correlation suggests a relationship between macrophage aggregate number and PCB levels at this site.

**Hepatopancreas Macrophage Aggregate Pigment Composition**

Since the area and number of macrophage aggregates were increased in carp hepatopancreas from PCB sites compared to carp from reference sites, the macrophage aggregate pigment composition in hepatopancreas was examined to determine if the relative proportion of lipofuscin/ceroid, iron, and/or melanin increased in carp from PCB sites. The macrophage aggregates in hepatopancreas in carp from PCB and reference sites were dominantly composed of lipofuscin, with small amounts of hemosiderin and melanin (Figure 19). This is consistent with reports that hemosiderin is primarily observed in the spleen (Angius and Agbede, 1984), and all 3 pigment components have been observed in fish liver (Blazer et al., 1987). One fish from Trowbridge, however, did not appear to have any lipofuscin/ceroid, and stained positive for hemosiderin and melanin. This carp had an exceptionally large macrophage aggregate size, and abnormal, atretic oocytes adjacent to hepatopancreas upon histological observation.
Figure 19. Carp hepatopancreas macrophage aggregate (*) consisting primarily of lipofuscin/ ceroid, also containing hemosiderin (white arrow) and melanin (black arrow). Gamori’s Prussian Blue; no counterstain used.

Ultrastructural images were also examined of carp hepatopancreas caught from Lake Allegan to verify the nature of the macrophage aggregate lesions. Images indicate activated phagolysosomes processing lipids, resulting in phospholipid whorl remnants (Figure 20).

Discussion

**Significant changes in macrophage aggregates from PCB contaminated sites**

Hepatic lesions in aquatic species have served as early warning indicators for the presence of high levels of environmental contaminants, and are considered indicators of ecosystem health. Macrophage aggregates are focal accumulations of pigmented aggregates commonly found in liver, spleen, and kidney that accrue with age in Osteichthyes (Wolke, 1992) and are indicators of environmental stress (Blazer...
et al., 1987, Angius and Roberts, 2003). Macrophage aggregates accrue slower in liver compared to spleen and kidney, and liver macrophage aggregate densities have been proposed to be sensitive indicators of stress in piscine species chronically exposed to environmental stress (Ziegenfuss and Wolke, 1991). Previously, it was
shown that small mouth bass (*Micropterus dolomieu*) examined from a PCB contaminated site in the Kalamazoo river showed several altered biochemical and toxicopathic indices, including hepatic pathologies such as increased macrophage aggregates and glycogen depletion (Anderson et al., 2003).

Carp from PCB contaminated sites compared to carp from reference sites sampled from the Kalamazoo River showed increased percent macrophage aggregates per area (ANOVA, $p=0.0263$) and macrophage aggregate number per area (ANOVA, $p=0.0453$), despite that carp from reference sites were significantly longer (ANOVA, $p=0.0149$) and older ($p=0.0180$) than carp from PCB contaminated sites. In addition, macrophage aggregate number per area was significantly different among sites (ANOVA, $p=0.0079$). Carp from Lake Allegan had increased macrophage numbers per area than carp from the other reference and PCB contaminated sites. Compared to Lake Whitefish exposed to contaminants in the St. Lawrence River (Quebec, Canada) of similar length range, carp from Trowbridge were relatively comparable, whereas carp from Lake Allegan showed a higher relative percentage of macrophage aggregate numbers in liver (Mikaelian et al., 2002). Additionally, hepatic macrophage aggregate numbers in carp from Lake Allegan were highly and significantly correlated with hepatopancreas PCB levels ($r=0.9068$, $p=0.0337$). These data suggest that Lake Allegan carp may be biologically responding to chronic PCB exposure, and macrophage aggregate number may be a biomarker of stress in this population.

Interestingly, hepatopancreas PCB levels in Lake Allegan carp (4.9±1.1 ug/g) were lower than hepatopancreas PCB levels at Trowbridge (10.2±1.8 ug/g). It has
been demonstrated that fish populations inhabiting different sites can respond differentially to chemical contaminants, attributed to impacts on site-specific population life-history parameters (Munkittrick and Dixon, 1989). In the Kalamazoo River a series of dams prevent fish from downstream PCB contaminated sites from swimming upstream, although it is possible that fish from upstream reference sites could migrate downstream. Thus although carp are not a territorial species, distinct populations are predicted to exist in the Kalamazoo River. In Lake Allegan there is a high density of carp compared to carp inhabiting Trowbridge and other Kalamazoo River sites, which is thought to cause density dependant stunted growth and explain empirical observations of smaller carp routinely caught at this site. In this study, the carp that were caught were, in general, shorter and also older than carp caught from Trowbridge. However, uncertainty exists if the observed growth decrease in this carp population is also associated with chronic PCB exposure. Although macrophage aggregate densities have previously been shown to increase with starvation (Angius and Roberts, 1981), there was a high correlation between macrophage aggregate numbers and PCB levels despite the small sample size examined in this study, suggesting that carp from Lake Allegan may be impacted by PCBs.

**Correlation of macrophage aggregates with fish length and age**

Since macrophage aggregate densities have been previously shown to increase with age, correlation analysis was performed to see if length and age was increased in carp from PCB sites. Carp length was not significantly correlated with macrophage aggregate densities. In fact, correlation coefficients were negatively associated
between percent macrophage aggregate area/mm², aggregate number per mm², aggregate size and length of carp from PCB sites. Similarly, carp age was not significantly correlated with macrophage aggregate densities in reference and PCB sites, and showed negative associations. On the other hand, previous studies using white suckers (*Catostomus commersoni*) exposed to bleached-kraft mill effluent (Couillard and Hodson, 1996) showed positive and significant correlations between liver macrophage aggregate number and age at both reference and contaminated sites.

In healthy and stressed largemouth bass from Par Pond, a cooling reservoir, macrophage aggregate parameters were also positively correlated with age, although healthy fish had more significance than stressed fish (Blazer et al., 1987). Also, in a large whitefish study from the St. Lawrence River the percent number of liver macrophage aggregates increased with both fish age and fish length, and was also significantly higher in fish with cholangiocarcinoma (Mikaelian et al., 2002). The lack of correlation between macrophage aggregate densities and age in this study could reflect small sample sizes. In addition, inferring age from scales can be quite variable. Nevertheless, in this study macrophage aggregate densities were increased in carp from PCB sites, which were smaller and younger than carp from reference sites.

**Increased lipofuscin/ceroid in hepatopancreas macrophage aggregates in PCB exposed carp: links to mechanistic models**

Examination of macrophage aggregate pigment composition suggests that in carp hepatopancreas, lipofuscin/ceroid is the dominant component. Thus, the
increased area and number of macrophage aggregates in PCB exposed carp may reflect increased lipofuscin/ceroid. Also, ultrastructural analysis of macrophage aggregate lesions from a carp caught from a PCB contaminated site (Lake Allegan) showed abundant activated lysosomes and phospholipid inclusions, indicating that the increase in macrophage aggregates in PCB exposed carp may result from an increased need for lipid clearance and storage in macrophage aggregates. Although correlations among PCBs, lipid metabolism, and increased macrophage aggregates have not been directly demonstrated, it can be hypothesized that the Ah stereospecific properties of PCBs and/or CYP1A1 activated polar PCB metabolites interfere with lipid metabolism. A mechanistic model has previously been proposed that links elevated CYP1A1 to enhanced oxidative stress and lipid peroxidation, and consequently proliferation of macrophage aggregates in fish exposed to bleached-kraft mill effluent (Couillard and Hodson, 1996). Since carp caught from PCB contaminated sites in the Kalamazoo River show increased CYP1A1 mRNA expression compared to carp caught from reference sites (Chapter 3), it is possible that oxidative damage resulting from CYP1A1 activation causes increased lipid peroxidation and hence increased macrophage aggregate proliferation to remove and store the lipofuscin in PCB exposed carp. Although it has been proposed that melanin in macrophage aggregates may function in detoxification to reduce oxidative damage (Angius and Agbede, 1984), melanin was not commonly observed in carp hepatopancreas macrophage aggregates.

Alternately, the accumulation of lipofuscin/ceroid in macrophage aggregates may result from a reduction in hepatocellular lipid uptake. The ultrastructural images
of carp macrophage aggregates closely resemble the accumulation of polar lipids in lysosomes observed with the administration of cationic amphiphilic drugs (CAD) in mammalian models. CADs are a group of compounds characterized by a hydrophilic portion containing a charged primary or secondary nitrogen group and a hydrophobic portion containing an aromatic or aliphatic ring structure often substituted with halogen atoms (Ruddman et al., 2004). Thus, it could be hypothesized that, like CADs, metabolically activated PCBs could function in a similar manner to induce phospholipidosis. Although no mechanisms of PCB induced phospholipidosis have been proposed, multiple mechanisms of mechanisms of action for CADs have been proposed, including inhibition of lysosomal phospholipid activities by the unavailability of the phospholipid substrate due to drug binding, and direct binding of CADs to phospholipid enzymes (Reasor, 1989; Horn et al., 1996). Although it is presumed that PCBs with long half lives are stored in body fat, it could be hypothesized that these PCBs act similar to CADs to alter lipid metabolism.

Direct binding of PCBs or other Ah ligands to the Ah receptor could also effect lipid metabolism. For example, fat depletion (Van der Weiden et al., 1994) and lipidosis accompanied by hypertrophy of liver hepatocytes (Zodrow et al., 2003) are seen in liver of fish exposed to low doses of the synthetic polycyclic aromatic hydrocarbon 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD). TCDD is a more potent ligand than PCBs of the aryl hydrocarbon receptor (AhR), which, like co-planar PCBs, mediates health effects through induction of the [Ah] gene battery and CYP1A expression (Nebert et al., 2000). Although the endogenous ligands for the Ah receptor are not known, it has been postulated that endogenous roles of the Ah
receptor involves adipocyte differentiation, constitutive inhibition of triglyceride synthesis, and interference with cell cycle arrest and differentiation (Hanlon et al., 2003). Thus, it is also possible that xenobiotics such as PCBs bind to the Ah receptor and alter lipid metabolism directly.

**Other indicators of stress**

Hepatosomatic index (HSI) was not elevated in carp from Lake Allegan, a PCB contaminated site, compared to carp from both reference sites. Excluding carp caught from Ceresco, which were significantly larger than carp caught from other sites, there were no differences in GSI or condition factor among PCB and reference sites. Hepatic neoplasms were not observed in carp from reference or PCB contaminated sites, although carp sampled from PCB contaminated sites showed increased basophilic profiles of hepatocytes compared to carp sampled from reference sites. Interestingly, 2 female carp out of the 8 females sampled at PCB contaminated sites showed altered gonadal profiles. One carp from Trowbridge displayed no gonads upon morphological examination for sex determination, but had oocytes adjacent to hepatopancreas tissue upon histological examination. Conversely, a female carp sampled from Lake Allegan had gonads with increased weight, displaying round white opaque droplets mixed into the gonadal tissue.

**Summary**

Increases in macrophage aggregate percent area and number were seen in hepatopancreas of carp caught from PCB contaminated sites in the Kalamazoo River.
compared to carp caught from upstream reference sites. Additionally, macrophage aggregate number was increased in carp from Lake Allegan, and this increase was significantly correlated with PCB exposure. Histological and ultrastructural observations indicate that the macrophage aggregates are primarily composed of lipofuscin/ ceroid. Although the role of macrophage aggregate aggregation is poorly understood, it has been hypothesized that macrophage aggregates function in innate immunity to sequester endogenous and exogenous substances for storage, destruction, or detoxification (Bols et al., 2001). It can be hypothesized that the increase in lipofuscin is related to an increased need for lipid clearance due to chronic PCB exposure. However, the interrelationships among PCBs, lipid damage, macrophage aggregate densities, and fitness are not clear.
Appendix A

ADDITIONAL REAL TIME RT-PCR DATA
Introduction

In addition to the 5 sets of primers and probes used in Chapter 3 to examine mRNA expression of 5 genes in carp hepatopancreas, 3 additional sets of primers and probes were used to test for significant effects in the expression of 3 additional genes. There were different problems associated with quantifying expression in each of these genes, and because there appeared to be no difference with treatment or expense involved, these genes were not further examined for statistically significant differences.

The 3 genes tested were heat shock protein 90 (hsp 90), heat shock protein 70 (hsp 70), and vitellogenin. Heat shock proteins are widely conserved multigene families that are considered part of the cellular stress response system. In response to chemical or physical stress, these proteins are thought to be rapidly transcribed above constitutive levels to help mitigate the stressor. Hsp90 dimerizes with the aryl hydrocarbon receptor and ligand in the cytoplasm as part of the first step in activation of the aryl hydrocarbon (Ah) gene battery, which includes the transcription of CYP1A1 (Whitlock, 1999). Since I found that CYP1A1 expression is upregulated in PCB exposed carp, I therefore wanted to test if an additional gene related to the transcriptional pathway was also upregulated.

Hsp70 assists the folding of nascent polypeptide chains, mediates the repair and degradation of altered or denatured proteins, and acts as a molecular chaperone (Basu et al., 2002). Hsp 70 has also been proposed to be an excellent candidate for a
biomarker for general stress (Sanders, 1990). I therefore tested PCB fed and vehicle fed control laboratory carp for increased mRNA expression.

Vitellogenin is an egg-yolk precursor protein that is synthesized in the liver in response to estrogen secreted by the ovarian follicles. It is then transported through the bloodstream and taken up by developing oocytes to form the yolk (Kime, 1998). Although the vitellogenin gene is present in males, it is not normally expressed. In response to estrogen (Lattier et al., 2001) and environmental contaminants such as that present in sewage effluent, vitellogenin has been shown to be upregulated in male fish including carp (Purdom et al., 1994; Folmar et al., 1996), suggesting an endocrine disruption effect that may affect fitness. Vitellogenin mRNA expression was therefore examined in hepatopancreas in carp caught from the Kalamazoo River via real time RT-PCR to test for potential endocrine disrupting effects of PCBs.

Materials and Methods

The primer and probe sequences for hsp70, hsp90, and vitellogenin and concentration used for PCR reactions are listed in Table 10. Sequences were designed using Primer Express software, the primers were optimized, and the experiments were run in PCR reactions using identical procedures to that for the 5 genes quantified in Chapter 3 and described the materials and methods therein.

Results and Discussion
The mRNA expression of hsp90 was tested in PCB fed and vehicle fed control laboratory carp fed for 4 months. After 4 months, carp fed PCBs accumulated an average of 50.042± 1.562 ppm PCBs in muscle tissue and showed a 4.5 fold increase in CYP1A1 mRNA expression compared to controls. Therefore, it was hypothesized that if expression levels of this gene were to change with PCB exposure it would be evident after 4 months of feeding PCBs in the laboratory.

Table 10. Primer and probe sequences and concentrations used for heat shock protein 90 (HSP90), heat shock protein 70 (HSP70), and vitellogenin.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Sequence</th>
<th>Amount Used</th>
</tr>
</thead>
<tbody>
<tr>
<td>HSP90</td>
<td></td>
<td></td>
</tr>
<tr>
<td>forward primer</td>
<td>AGGCCGGAGCGGGACA</td>
<td>300 nM</td>
</tr>
<tr>
<td>reverse primer</td>
<td>CCACTAGGTAGGGCGGAATAGAAAC</td>
<td>300 nM</td>
</tr>
<tr>
<td>probe</td>
<td>FAM-CCACTCCGAAACTGACGGATCATGGA-TAMRA</td>
<td>200 nM</td>
</tr>
<tr>
<td>HSP70</td>
<td></td>
<td></td>
</tr>
<tr>
<td>forward primer</td>
<td>TCGCTTGGATTGAATGTCCTT</td>
<td>900 nM</td>
</tr>
<tr>
<td>reverse primer</td>
<td>CAGGACGTTCGCTCTGA</td>
<td>300 nM</td>
</tr>
<tr>
<td>probe</td>
<td>FAM-TGCAGCCATTGCCTACGCCCTT-TAMRA</td>
<td>200 nM</td>
</tr>
<tr>
<td>vitellogenin</td>
<td></td>
<td></td>
</tr>
<tr>
<td>forward primer</td>
<td>ACCCCTGGTAAAGACCTATGTGTACA</td>
<td>300 nM</td>
</tr>
<tr>
<td>reverse primer</td>
<td>TACCTGGCTCTAGCCAGACCTCA</td>
<td>900 nM</td>
</tr>
<tr>
<td>probe</td>
<td>FAM-CTATGAGGCTCTACTCTGCGGCGGCTCTCC-TAMRA</td>
<td>200 nM</td>
</tr>
</tbody>
</table>

There was, however, a problem concerning high copy number of this gene in hepatopancreas tissue, such that the real time RT-PCR results cannot be completely conclusive. In order to confirm changes in mRNA expression levels, as a rule of thumb the PCR reaction must have a 10 cycle difference between mRNA and DNA available (i.e., $2^{10} = 1000$ fold mRNA compared to DNA). Performing a total RNA...
extraction followed by a message RNA extraction succeeded in amplifying this
difference to at least 14 cycles in the 5 genes first tested (Chapter 3) in carp
hepatopancreas; treatment with the DNAses available at that time or attempts to dilute
out the DNA were unsuccessful.

Examination of hsp90 mRNA expression levels after 4 months of feeding in
the laboratory revealed the presence of unacceptably high levels of DNA, as shown in
the cycle difference. PCR reactions without the reverse-transcriptase enzyme (No
Enzyme Control or NEC) minus the PCR reactions containing the reverse-
transcriptase enzyme did not exceed the necessary 10 cycle difference (Ct) (Table 11).
The standard curve used was \( y=-3.3734x+28.905 \).

<table>
<thead>
<tr>
<th>carp number</th>
<th>NEC Ct</th>
<th>Average Ct</th>
<th>Difference (NEC Ct-Ct)</th>
</tr>
</thead>
<tbody>
<tr>
<td>p71</td>
<td>33.73</td>
<td>27.135</td>
<td>6.60</td>
</tr>
<tr>
<td>c72</td>
<td>35.03</td>
<td>26.115</td>
<td>8.82</td>
</tr>
<tr>
<td>p74</td>
<td>34.41</td>
<td>26.17</td>
<td>8.24</td>
</tr>
<tr>
<td>p75</td>
<td>32.85</td>
<td>28.87</td>
<td>3.98</td>
</tr>
<tr>
<td>c76</td>
<td>34.17</td>
<td>25.88</td>
<td>8.31</td>
</tr>
<tr>
<td>c77</td>
<td>34.33</td>
<td>23.215</td>
<td>11.12</td>
</tr>
<tr>
<td>p78</td>
<td>33.56</td>
<td>25.605</td>
<td>7.96</td>
</tr>
<tr>
<td>p79</td>
<td>33.33</td>
<td>25.455</td>
<td>7.88</td>
</tr>
<tr>
<td>c80</td>
<td>31.53</td>
<td>28.66</td>
<td>2.87</td>
</tr>
</tbody>
</table>

In all gene expression data for this time point including hsp90, carp c77 was excluded
since there was a problem regarding the yeast spike value compared to the extraction
value.
However, despite the unacceptable differences in cycle numbers, it is still possible to examine the results for a significant trend. For example, since PCR is an exponential reaction, a cycle difference of 5 would still yield a 32 fold difference in mRNA abundance compared to DNA. Analyzing the results, it was evident that there was no significant difference in hsp90 gene expression levels between PCB fed and vehicle fed control carp after 4 months of feeding (p-value=0.3087; control =1.131±0.337, n=4; PCB=2.577±1.333, n=5).

Since hsp90 and CYP1A1 genes are part of the same induction pathway, it would be expected that both genes might be upregulated in concert. However, due to lag time, transport, and differences in muscle PCB congener composition versus liver congener composition, the relationship is not clear.

Hsp70

Primer concentration optimization for hsp70 was repeated due to deviations from the typically observed PCR curve. Both times, however, forward and reverse concentrations of 900 uM and 300 uM, respectively, were observed at a reasonable Ct values (27.67, 26.14). Expression levels of this gene were also tested in laboratory carp fed for 4 months, since these carp had the highest muscle PCB contaminant loads and were responding to PCB exposure via induction of CYP1A1. However, threshold cycles (Ct) observed in this gene for both the standard curve and unknown carp hepatopancreas samples were all 40 or above. Due to the expense and complex nature of troubleshooting poor PCR curves, and since expression values of this gene could
have been additionally altered by transport, handling, and other non-PCB treatment factors, expression levels of this gene were not further pursued.

**Vitellogenin**

Vitellogenin mRNA expression levels were tested for Kalamazoo River carp to see if 1) males from PCB contaminated sites had higher expression levels than males from uncontaminated sites, and 2) females from PCB sites had sites had higher expression levels than females from uncontaminated sites. Ceresco-4, a female carp, was used for standard curves on each of 2 PCR plates needed to accommodate the 18 field carp. Four male carp were caught from PCB contaminated sites (from May 15 and June 26, 2001), and 3 male carp were caught from reference sites (July 30 and October 7, 2001). In all male carp, real time RT-PCR detected very low levels of mRNA, indicated by a high PCR cycle number at the threshold level (Ct) used for comparison. Using yeast normalized PCR values, there was no significant difference between males from reference and PCB sites. The mRNA levels detected in reference as well as PCB exposed carp were not much greater than the levels of DNA present, with an average cycle difference of 1.89±0.65, or a 3.7 fold increase in mRNA abundance over DNA. Additionally, there was no difference in Ct values between males from reference and PCB sites (t-test, t=0.2401).

Eight female carp were caught from PCB contaminated sites from June 20 to August 28, 2001, and 2 female carp were caught from a reference site (Ceresco) on October 2. There was no significant difference in vitellogenin mRNA expression (t-
test, p-value = 0.1413; reference = 3.821±2.956; PCB = 1.391±0.441). A comparison of vitellogenin mRNA Ct values from RT-PCR raw data did not show a difference between females from reference and PCB sites (t-test, t = 0.5059). Additionally, vitellogenin mRNA expression from 5 PCB contaminated female fish caught from Lake Allegan on one date, June 26, varied 30.2 fold, and vitellogenin mRNA expression from 2 female reference carp caught on October 7 from Ceresco varied 7.9 fold.

Although sample size of each sex was small and the sampling was confounded with date (all of the reference carp sampled after all of the PCB carp), preliminary evidence relating PCB exposure to increased vitellogenin mRNA expression suggests that there is no significant effect in Kalamazoo River male and female carp, although in female carp plasma vitellogenin appeared elevated. In a recently published field study sampling small mouth bass (*Micropterus dolomieu*) from a reference (Ceresco) and a contaminated PCB site (downstream of Kalamazoo) in the Kalamazoo River, males had no detectable plasma vitellogenin protein. There was, however, a significant increase in plasma vitellogenin in PCB exposed females, which was correlated with liver PCB concentrations (Anderson et. al., 2003).
Appendix B

LABORATORY EXPOSED CARP TRANSMISSION ELECTRON MICROSCOPE (TEM) ULTRASTRUCTURAL IMAGES
Introduction

In order to select genes whose expression changes may be indicative of ongoing health effects, I examined hepatopancreas ultrastructure in addition to histopathology to gain insights into the nature of pathologic changes that underlie health effects. For example, histopathology of laboratory raised carp hepatopancreas (liver plus pancreas) showed basophilic cytoplasmic profiles of hepatocytes in carp fed PCBs compared to vehicle fed references, suggesting decrease protein synthesis, storage, and/or disposition. However, there were no other histological lesions that could be clearly interpreted in terms of health effects. Carp liver sections from PCB as well as reference fed carp examined displayed high levels of heterogeneity, with multi-focal areas of increased hepatocyte cell size. Often, no nuclei were visible in these multi-focal areas. Additionally, there were no neoplasms in the carp sections observed.

Therefore, a survey of transmission electron microscope (TEM) images of hepatopancreas were made of laboratory fed carp in order to further elucidate health effects of PCBs. Hepatopancreas samples were collected for fixation and processing for ultrastructural investigation from a second laboratory carp exposure experiment, where juvenile carp were fed PCBs in the diet up to 4 months, as in the first feeding experiment.
Materials and Methods

Carp hepatopancreas samples were collected from a second feeding experiment for TEM and histological processing from the posterior right liver. Additional samples from these carp were collected for future analysis; 1/2 of the brain and 1/4 of the muscle were sampled for histological analysis, 1/2 of the brain, approximately 1/4 of the muscle, and the left liver was collected for mRNA extraction and PCR, and anterior of the right liver and 1/2 of the muscle was collected for PCB analysis. After 2 months and 4 months of feeding, 6 carp fed reference food (0.6 μg/g dry weight PCBs, Chapter 3) and 6 carp fed food with the PCB mixture Aroclor 1242 (18.1 μg/g dry weight PCBs, Chapter 3) were sampled as above, for a total of 12 carp sampled at each time point. There was no significant difference in weight or condition factor between reference and PCB carp after 2 months and after 4 months of feeding, although the carp gained weight from the start of the experiment (6.029±0.255 g) to the end of the experiment after 4 months (8.298±0.748 g; ANOVA, p=0.0369).

Carp samples for ultrastructural analysis were prepared identically to that mentioned in Chapter 4. Due to problems making thin sections, likely from incomplete sample dehydration, the above process was repeated with hepatopancreas samples taken cut with a razor blade from neutral buffered formalin fixed histology samples.

Four carp hepatopancreas samples from carp fed reference food for 2 months and 4 carp hepatopancreas samples from carp fed PCBs for 4 months were chosen for thin sectioning, staining, and TEM viewing.
Results and Discussion

An ultrastructural survey of the laboratory raised carp showed a high degree of heterogeneity of organelle disposition in hepatocyte cells, between reference and PCB carp, within each treatment, and within individual carp hepatocyte regions (Figure 21). For example, organelle disposition was either clustered or spread out in

Figure 21. Ultrastructural images displaying heterogeneity in hepatocyte disposition. Two different carp that are fed PCBs show hepatocyte cellular organelles that are A) spread out as well as B) clustered. Similar heterogeneity is also seen in the same animal, demonstrated by organelles that are C) clustered or D) spread out in a reference carp. Scale bar = 0.5μm.
the cell. Proliferation of endoplasmic reticulum, often observed in association with increased cyp 1A activity and exposure to PCBs, was not observed. Additionally, huge inclusions, indicative of a storage problem, were not observed.

Reasons for no significant trends include the possibility that the carp were not dosed high enough to elicit observable health impacts. Also, since PCBs are present in all specimens, including references, it is possible that reference specimens are already impacted by PCBs. Given the lack of a true reference, comparative differences might therefore not be evident.
Appendix C

LABORATORY EXPOSED CARP: IMMUNOCYTOCHEMISTRY
Introduction

Immunocytochemistry (ICC) is a widely used technique that utilizes the specificity of antibody binding to detect and visualize proteins of interest with high sensitivity and discrimination. Immunocytochemistry has been successfully used in PCB toxicity studies in fish to detect CYP1A abundance in multiple organs (Stegeman et al., 1991; Grinwis et al., 2001). In addition to affecting CYP1A, PCBs have been shown to display endocrine disruption and immunomodulatory properties. PCBs have been shown to mimic or inhibit the production or action of a wide variety of steroids (reviewed in Cooke et al., 2001). For example, synthesis of the yolk protein vitellogenin, synthesized in the liver in response to activation of the estrogen receptor, has been shown to be increased in association with PCBs. The immune system has also been proposed as a sensitive target for PCB-induced toxicity (reviewed in Tryphonas and Feeley, 2001). However, limited data is available on the effects of cellular mediated immunity.

In this experiment, slides containing transverse sections through carp viscera (20 um) were examined using ICC for possible endocrine disruption (i.e., vitellogenin) and immune effects (humoral mucosal Ig antibody and cellular mucosal T cell marker) in carp exposed to PCBs for 4 months through the diet or references in a laboratory carp feeding experiment (Chapter 3).

Materials and Methods

Laboratory raised carp that were fed either reference or PCB food for 4 months was tested for ICC. Carp viscera were cut into 3 equal sections, and the
middle section processed for histology. Carp sections were not perfused. Carp sections were dehydrated in an ethanol series, infiltrated with methyl salicylate, then placed in a vacuum oven (60C) for 1 hour with a 50:50 mixture of methyl salicylate:paraffin. Sections were then transferred to paraffin and kept in a vacuum oven (60C) overnight. Twenty micron sections were cut using a Micron HM 325 microtome and floated on to 2x gel-subbed slides in a 45C water bath, sprinkled with gelatin (Sigma Chemical Co, St. Louis, MO).

Vitellogenin monoclonal mouse and polyclonal rabbit antibodies were donated from Biosense Laboratories AS (Bergen, Norway) for potential development for use in fish immunocytochemistry. Typically, vitellogenin assays are performed either by ELISA (enzyme-linked immunosorbent assay) or by radioimmunoassay, although new PCR-based methods are currently being developed (i.e., Appendix 1). WCI12 (monoclonal mouse), which reacts with the H chain of mucus Ig in carp, and WCL38 (monoclonal mouse), a putative carp mucosal T cell marker, was purchased from Dr. Jan Rombout, Wageningen Agricultural University (The Netherlands) for preliminary trials in PCB exposed carp.

For ICC, slides were first dehydrated to buffer, using either TBS buffer for alkaline phosphatse (AP) visualization, or PBS buffer for diaminobenzidine (DAB) visualization. Non-specific staining was next blocked for 2 hours at room temperature using a solution containing either normal goat serum (if goat anti-rabbit antibody), or normal horse serum (if horse anti-mouse antibody). If slides were visualized with DAB, endogenous peroxidase were quenched with a 3% H$_2$O$_2$ solution and rinsed with PBS. Slides were then incubated in 100 ul of primary
antibody with a plastic coverslip overnight in a humid chamber (4C). For vitellogenin antibodies, primary antibody concentrations ranging from 1:50 to 1:1000 were tested. For WCL38 and WCI12 the recommended dilution of 1:100 was used. After washing with buffer, either 100 ul of goat anti-rabbit or horse anti-mouse secondary biotinylated antibody (1:200) was placed on a slide with a plastic coverslip for 1 hour at room temperature. After washing with buffer, slides were treated with avidin-biotin complex (ABC) using either alkaline phosphatase (ABC-AP; Vecastain Alkaline Phosphatase Kit #AK-5000) or DAB (ABC, Vectastain Elite Kit PK-6100) for visualization. DAB solutions were tested in used ABC complex to verify staining quality. Color development for both visualization techniques was monitored under a microscope. Antibody-labeled slides were dehydrated into xylene, and mounted with Permount for further examination and storage. In all ICC runs, negative references using one slide with no primary antibody (blocking solution only) and a second slide with no primary and no secondary antibody (blocking solution only) were used.

Results and Discussion

Staining using the polyclonal vitellogenin antibody using reference female carp sections did not work using primary antibody concentrations of 1:50, 1:100, 1:500, or 1:1000 with AP visualization. Additionally, using reference female carp sections primary polyclonal antibody concentrations of 1:25, 1:50, 1:100, and 1:250 staining did not work using DAB visualization. Using the monoclonal vitellogenin antibody there was staining at a dilution of 1:50 for the primary antibody and
visualized with DAB in multiple organs of both reference male and female carp (2 males and 2 females) and PCB fed male and female carp (2 males and 2 females). In reference males and females, staining was observed in gonads, gut, liver, spleen, and kidney. Staining in PCB fed males and females was the same as above, with the addition of staining observed in the hepatopancreas. The relative staining intensities were similar in males (reference and PCB) and in females (reference and PCB).

No staining at low concentrations of antibody and a high degree of nonspecific staining at a high (1:50) primary antibody concentration indicates that these vitellogenin antibodies are not working in the carp slides tested. Additionally, reference male fish should contain relatively little vitellogenin compared to reference female fish. In these trials, however, males that stained at high primary antibody concentration showed the same degree of staining as females, in both reference and PCB carp. Although this antibody is purported to work in carp to measure serum levels, it is possible that using ICC to localize vitellogenin is not practical.

Vitellogenin is present in plasma, which might account for the high levels of nonspecific staining in multiple organs at relatively high antibody dilutions. Fish also have a poorly defined lymph system, which might also hamper the use of a vitellogenin antibody in ICC. Another possible alternative explaining negative ICC results could be that the carp tested were too young for strong positive results. The laboratory carp were juveniles, approximately 11 months old at the start of the 4 month feeding experiment. Although sexually dimorphic gonadal morphology was evident in the carp sampled after 4 months and used for ICC, these carp could still be pre-vitellogenic, and not react with a vitellogenin antibody. Staining results using the
carp immune markers, WCL38 and WCI12, were inconclusive after a few trials and were not considered further.
Appendix D

PCB CONGENER DATA
Table 12. PCB congeners measured in laboratory raised carp muscle fed the PCB mixture Aroclor 1242 or fed vehicle only (control) for 1 and 2 months.

<table>
<thead>
<tr>
<th>PCB congener</th>
<th>1 month exposure (ng/g wet weight)</th>
<th>2 month exposure (ng/g wet weight)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>control (n=5)</td>
<td>PCB fed (n=5)</td>
</tr>
<tr>
<td>1</td>
<td>352.2±149</td>
<td>521.8±20.5</td>
</tr>
<tr>
<td>5</td>
<td>0</td>
<td>529.8±20.5</td>
</tr>
<tr>
<td>29</td>
<td>0</td>
<td>539.8±20.9</td>
</tr>
<tr>
<td>50</td>
<td>0</td>
<td>1065.6±41.1</td>
</tr>
<tr>
<td>77</td>
<td>0</td>
<td>588.8±22.7</td>
</tr>
<tr>
<td>89</td>
<td>478.6±119.9</td>
<td>615.0±23.7</td>
</tr>
<tr>
<td>101</td>
<td>479.8±120.2</td>
<td>617.2±24.5</td>
</tr>
<tr>
<td>87</td>
<td>1374±344.3</td>
<td>1750.8±67.9</td>
</tr>
<tr>
<td>105</td>
<td>213.2±53.6</td>
<td>279.6±10.7</td>
</tr>
<tr>
<td>118</td>
<td>210.6±129.1</td>
<td>0</td>
</tr>
<tr>
<td>126</td>
<td>0</td>
<td>566.2±22.7</td>
</tr>
<tr>
<td>154</td>
<td>639.2±261.0</td>
<td>1075.8±41.5</td>
</tr>
<tr>
<td>169</td>
<td>644.2±51.7</td>
<td>609.2±23.2</td>
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<tr>
<td>153</td>
<td>660.4±53.4</td>
<td>624.2±24.1</td>
</tr>
<tr>
<td>128</td>
<td>261.4±19.7</td>
<td>243.4±8.4</td>
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<tr>
<td>138</td>
<td>222±80.2</td>
<td>255.6±10.3</td>
</tr>
<tr>
<td>156</td>
<td>281.6±74.7</td>
<td>322.4±12.5</td>
</tr>
<tr>
<td>188</td>
<td>973.6±397.6</td>
<td>1638.4±63.2</td>
</tr>
<tr>
<td>180</td>
<td>487.4±122.1</td>
<td>624.8±24.1</td>
</tr>
<tr>
<td>183</td>
<td>368.2±150.6</td>
<td>370.2±152.7</td>
</tr>
<tr>
<td>170</td>
<td>234.8±59.0</td>
<td>305.8±11.7</td>
</tr>
<tr>
<td>205</td>
<td>0</td>
<td>1677.8±64.8</td>
</tr>
<tr>
<td>194</td>
<td>0</td>
<td>624.8±24.1</td>
</tr>
<tr>
<td>209</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>
Table 13. PCB congeners measured in laboratory raised carp muscle fed the PCB mixture Aroclor 1242 or fed vehicle only (control) for 3 and 4 months.

<table>
<thead>
<tr>
<th>PCB congener</th>
<th>3 month exposure (ng/g wet weight)</th>
<th>4 month exposure (ng/g wet weight)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>control (n=5)</td>
<td>PCB fed (n=5)</td>
</tr>
<tr>
<td>1</td>
<td>11.4±11.4</td>
<td>6.0±4.8</td>
</tr>
<tr>
<td>5</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>29</td>
<td>0</td>
<td>15.2±9.4</td>
</tr>
<tr>
<td>50</td>
<td>0</td>
<td>0.8±0.8</td>
</tr>
<tr>
<td>77</td>
<td>6.4±6.4</td>
<td>23.4±11.1</td>
</tr>
<tr>
<td>69</td>
<td>0</td>
<td>36.4±12.5</td>
</tr>
<tr>
<td>101</td>
<td>27.4±27.4</td>
<td>49.0±10.2</td>
</tr>
<tr>
<td>87</td>
<td>5.6±5.8</td>
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<tr>
<td>105</td>
<td>0</td>
<td>88.8±15.4</td>
</tr>
<tr>
<td>118</td>
<td>67.6±19.3</td>
<td>53.0±10.8</td>
</tr>
<tr>
<td>128</td>
<td>34.0±15.5</td>
<td>21.8±9.9</td>
</tr>
<tr>
<td>154</td>
<td>0</td>
<td>0.4±0.4</td>
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<td>169</td>
<td>38.4±15.7</td>
<td>37.2±6.5</td>
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<td>153</td>
<td>3.2±3.0</td>
<td>17.0±8.0</td>
</tr>
<tr>
<td>128</td>
<td>4.8±4.8</td>
<td>24.8±8.9</td>
</tr>
<tr>
<td>138</td>
<td>272.4±147.8</td>
<td>23±7.3</td>
</tr>
<tr>
<td>156</td>
<td>0</td>
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<td>188</td>
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<tr>
<td>183</td>
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<td>5.6±5.1</td>
</tr>
<tr>
<td>170</td>
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<td>3.4±3.4</td>
</tr>
<tr>
<td>205</td>
<td>0</td>
<td>0</td>
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<tr>
<td>194</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>209</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>
Table 14. Muscle and hepatopancreas PCB congener data from carp collected from reference sites (Ceresco and Morrow) and from PCB sites (Trowbridge and Lake Allegan), 2001. Measurements are in ng/g wet weight.

<table>
<thead>
<tr>
<th>PCB congener</th>
<th>Muscle (ng/g wet weight)</th>
<th>Hepatopancreas (ng/g wet weight)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>reference sites (n=6)</td>
<td>PCB Sites (n=11)</td>
</tr>
<tr>
<td>1</td>
<td>0</td>
<td>0.9±0.3</td>
</tr>
<tr>
<td>5</td>
<td>0</td>
<td>0.2±1.6</td>
</tr>
<tr>
<td>29</td>
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<td>0.9±2.3</td>
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<tr>
<td>50</td>
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<td>0.2±2.3</td>
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<td>77</td>
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<td>13.0±2.3</td>
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<td>87</td>
<td>0</td>
<td>0.9±2.3</td>
</tr>
<tr>
<td>116</td>
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<tr>
<td>126</td>
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<td>0.9±2.3</td>
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<tr>
<td>154</td>
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<td>0.9±2.3</td>
</tr>
<tr>
<td>169</td>
<td>0</td>
<td>0.9±2.3</td>
</tr>
<tr>
<td>153</td>
<td>0</td>
<td>0.9±2.3</td>
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<tr>
<td>128</td>
<td>0</td>
<td>0.9±2.3</td>
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<tr>
<td>133</td>
<td>0</td>
<td>0.9±2.3</td>
</tr>
<tr>
<td>156</td>
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<td>0.9±2.3</td>
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<tr>
<td>188</td>
<td>0</td>
<td>0.9±2.3</td>
</tr>
<tr>
<td>180</td>
<td>0</td>
<td>0.9±2.3</td>
</tr>
<tr>
<td>183</td>
<td>0</td>
<td>0.9±2.3</td>
</tr>
<tr>
<td>170</td>
<td>0</td>
<td>0.9±2.3</td>
</tr>
<tr>
<td>205</td>
<td>0</td>
<td>0.9±2.3</td>
</tr>
<tr>
<td>194</td>
<td>0</td>
<td>0.9±2.3</td>
</tr>
<tr>
<td>209</td>
<td>0</td>
<td>0.9±2.3</td>
</tr>
</tbody>
</table>
Appendix E

IACUC APPROVAL LETTERS
PROJECT OR COURSE TITLE: Neurochemical and molecular affects of Aroclor 1242 on carp, Cyprinus carpio

IACUC Protocol Number: 01-05-01
Date of Review Request: 06/30/03
Date of Last Approval: 5/2001

Purpose of project (select one): ☑ Teaching ☑ Research ☑ Other (specify):

PRINCIPAL INVESTIGATOR OR ADVISOR
Name: Charles Hie Title: Director/Assoc. Director
Department: EL Electronic Mail Address: charles.hie@wmich.edu

CO-PRINCIPAL OR STUDENT INVESTIGATOR
Name: Marla Fisher Title: Student
Department: BIO Electronic Mail Address: Marla.Fisher@wmich.edu

1. The research, as approved by the IACUC, is completed:
☐ Yes (Continue with items 4-5 below.) ☒ No (Continue with items 2-5 below.)

   If the answer to any of the following questions (items 2-4) is “Yes,” please provide a detailed explanation on an attached sheet of paper. Include details of any modifications made to the protocol based on new findings or publications, adverse events or mortalities.

2. Have there been any changes in Principal or Co-Principal Investigators? ☐ Yes ☐ No

3. Have there been any new findings or publications relative to this research? ☐ Yes ☐ No
   Describe the sources used to determine the availability of new findings or publications:
   ☐ No search conducted (Please provide a justification on an attached sheet.)
   ☑ Animal Welfare Information Center (AWIC)
   ☑ Search of literature databases (select all applicable)
   ☒ AGRICOLA ☐ Biological Abstracts ☐ Current Research Information Service (CRIS)
   ☐ Other (please specify): PubMed
   Date of search: 06/03/03
   Years covered by the search: 1965-present
   Key words:
   ☐ Additional search strategy narrative:

4. Are there any adverse events, in terms of animal well being, or mortalities to report as a result of this research? ☐ Yes ☐ No
   Cumulative number of mortalities: 4

5. Animal usage: Number of animals used during this quarter (3 months): 0
   Cumulative number of animals used to date: 100

Principal Investigator/Faculty Advisor Signature: 1/30/03
Co-Principal or Student Investigator Signature: 6/30/03

IACUC REVIEW AND APPROVAL
Upon review of the relevant information regarding this protocol, the IACUC approval for this project has been extended for one year from the date of this signature.

IACUC Chair Signature: 7/11/03

Revised 10/01 WMU IACUC
All other copies obsolete.
PROJECT OR COURSE TITLE: Neurochemical And Molecular Affects Of Aroclor 1242 On Carp, Cyprinus Carpio
IACUC Protocol Number: 01-05-01
Date of Review Request: 07/31/01
Date of Last Approval: 08/08/01
Purpose of project (select one): ☐Teaching ☒Research ☐Other (specify):

PRINCIPAL INVESTIGATOR OR ADVISOR
Name: Charles Ide
Title: Director/Assoc. Director
Department: El
Electronic Mail Address: charles.idealwmich.edu

CO-PRINCIPAL OR STUDENT INVESTIGATOR
Name: Title: Select one
Department: Electronic Mail Address:

1. The research, as approved by the IACUC, is completed:
☐Yes (Continue with items 4-5 below.) ☒No (Continue with items 2-5 below.)

If the answer to any of the following questions (items 2-4) is "Yes," please provide a detailed explanation on an attached sheet of paper. Include details of any modifications made to the protocol based on new findings or publications, adverse events or mortalities.

2. Have there been any changes in Principal or Co-Principal Investigators? ☒Yes ☐No
3. Have there been any new findings or publications relative to this research?
☐Yes ☒No

Describe the sources used to determine the availability of new findings or publications:

☐ No search conducted (Please provide a justification on an attached sheet.)
☐ Animal Welfare Information Center (AWIC)
☐ Search of literature databases (select all applicable)
☐ AGRICOLA ☒ Current Research Information Service (CRIS)
☐ Biological Abstracts ☒ Medline
☐ Other (please specify):
Date of search: 07/31/02 Years covered by the search: 1990-present
Key words:
☐ Additional search strategy narrative:

4. Are there any adverse events, in terms of animal well being, or mortalities to report as a result of this research?
☐ Yes ☒No

Cumulative number of mortalities: 107

5. Animal usage:

Number of animals used during this quarter (3 months): 24
Cumulative number of animals used to date: 104

Principal Investigator/Faculty Advisor Signature Date

Co-Principal or Student Investigator Signature Date

IACUC REVIEW AND APPROVAL

Upon review of the relevant information regarding this protocol, the IACUC approval for this project has been extended for one year from the date of this signature:

IACUC Chair Signature Date

IACUC Protocol Number: 01-05-01
Date of Review Request: 07/31/01
Date of Last Approval: 08/08/01
Purpose of project (select one): ☐Teaching ☒Research ☐Other (specify):

PRINCIPAL INVESTIGATOR OR ADVISOR
Name: Charles Ide
Title: Director/Assoc. Director
Department: El
Electronic Mail Address: charles.idealwmich.edu

CO-PRINCIPAL OR STUDENT INVESTIGATOR
Name: Title: Select one
Department: Electronic Mail Address:

1. The research, as approved by the IACUC, is completed:

☐Yes (Continue with items 4-5 below.) ☒No (Continue with items 2-5 below.)

If the answer to any of the following questions (items 2-4) is "Yes," please provide a detailed explanation on an attached sheet of paper. Include details of any modifications made to the protocol based on new findings or publications, adverse events or mortalities.

2. Have there been any changes in Principal or Co-Principal Investigators? ☒Yes ☐No
3. Have there been any new findings or publications relative to this research?

Describe the sources used to determine the availability of new findings or publications:

☐ No search conducted (Please provide a justification on an attached sheet.)
☐ Animal Welfare Information Center (AWIC)
☐ Search of literature databases (select all applicable)
☐ AGRICOLA ☒ Current Research Information Service (CRIS)
☐ Biological Abstracts ☒ Medline
☐ Other (please specify):
Date of search: 07/31/02 Years covered by the search: 1990-present
Key words:

☐ Additional search strategy narrative:

4. Are there any adverse events, in terms of animal well being, or mortalities to report as a result of this research?
☐ Yes ☒No

Cumulative number of mortalities: 107

5. Animal usage:

Number of animals used during this quarter (3 months): 24
Cumulative number of animals used to date: 104

Principal Investigator/Faculty Advisor Signature Date

Co-Principal or Student Investigator Signature Date

IACUC REVIEW AND APPROVAL

Upon review of the relevant information regarding this protocol, the IACUC approval for this project has been extended for one year from the date of this signature:

IACUC Chair Signature Date

IACUC Protocol Number: 01-05-01
Date of Review Request: 07/31/01
Date of Last Approval: 08/08/01
Purpose of project (select one): ☐Teaching ☒Research ☐Other (specify):

PRINCIPAL INVESTIGATOR OR ADVISOR
Name: Charles Ide
Title: Director/Assoc. Director
Department: El
Electronic Mail Address: charles.idealwmich.edu

CO-PRINCIPAL OR STUDENT INVESTIGATOR
Name: Title: Select one
Department: Electronic Mail Address:

1. The research, as approved by the IACUC, is completed:

☐Yes (Continue with items 4-5 below.) ☒No (Continue with items 2-5 below.)

If the answer to any of the following questions (items 2-4) is "Yes," please provide a detailed explanation on an attached sheet of paper. Include details of any modifications made to the protocol based on new findings or publications, adverse events or mortalities.

2. Have there been any changes in Principal or Co-Principal Investigators? ☒Yes ☐No
3. Have there been any new findings or publications relative to this research?

Describe the sources used to determine the availability of new findings or publications:

☐ No search conducted (Please provide a justification on an attached sheet.)
☐ Animal Welfare Information Center (AWIC)
☐ Search of literature databases (select all applicable)
☐ AGRICOLA ☒ Current Research Information Service (CRIS)
☐ Biological Abstracts ☒ Medline
☐ Other (please specify):
Date of search: 07/31/02 Years covered by the search: 1990-present
Key words:

☐ Additional search strategy narrative:

4. Are there any adverse events, in terms of animal well being, or mortalities to report as a result of this research?

Cumulative number of mortalities: 107

5. Animal usage:

Number of animals used during this quarter (3 months): 24
Cumulative number of animals used to date: 104

Principal Investigator/Faculty Advisor Signature Date

Co-Principal or Student Investigator Signature Date

IACUC REVIEW AND APPROVAL

Upon review of the relevant information regarding this protocol, the IACUC approval for this project has been extended for one year from the date of this signature:

IACUC Chair Signature Date

Revised 10/01  WMU IACUC
All other copies obsolete.
July 16, 2001
Re: Animal Use Protocol # 01-05-01

Dear Dr. Bejcek,

Please add the following revisions to my protocol (01-05-01) entitled, Neurochemical and molecular effects of Aroclor 1242 on carp, *Cyprinus carpio:*

1) The number of carp that will be used in the proposed experiments was determined from previous quantitative RT-PCR and immunohistochemical studies with frogs exposed to Aroclor 1254. A minimum of five replicates per treatment group was required to attain statistical significance. One way ANOVA followed by Fisher’s post-hoc test was used to calculate statistical significance between the treatment groups. Similar statistical methods will be used for the carp studies.

2) All animal care personnel have been advised regarding procedures for working with the PCBs and all tanks containing PCBs treated fish have been clearly marked.

3) The PubMed database was searched for relevant articles from the mid 1960’s to the present.

4) The field caught animals will be euthanized immediately. Brain and liver tissues will be extracted and the carcass will be stored at -20°C for analysis of tissue contaminants.

5) A Standard Operating Procedure will be filed with the Animal Facilities manager for the carp that will be housed in the facility.

Please feel free to contact me if you need any additional information.

Sincerely,

Charles F. Ide
Director, Environmental Institute
Title of Project: Interleukin-1 System Molecules as Molecular Biomarkers of Exposure

The information included in this IACUC application is accurate to the best of my knowledge. All personnel listed recognize their responsibility in complying with university policies governing the care and use of animals.

I declare that all experiments involving live animals will be performed under my supervision or that of another qualified scientist. Technicians or students involved have been trained in proper procedures in animal handling, administration of anesthetics, analgesics, and euthanasia to be used in this project.

If this project is funded by an extramural source, I certify that this application accurately reflects all procedures involving laboratory animal subjects described in the proposal to the funding agency noted above.

Any proposed revisions to or variations from the animal care and use data will be promptly forwarded to the IACUC for approval.

Disapproved  Approved  Approved with provisions listed below

Provisions or Explanations: [signature]

IACUC Chairperson  7/21/99  Date

Acceptance of Provisions  9/1/99  Date

Signature: Principal Investigator/Instructor  Date

IACUC Chairperson Final Approval  9/24/99  Date

Approved IACUC Number  99-07-01

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BIBLIOGRAPHY


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Coletti D, Palleschi S, Silvestroni L, Cannavo A, Vivarelli E. 2001. Poly- 
chlorobiphenyls inhibit skeletal muscle differentiation in culture. Toxicol 

Connell D, Hawker D, Warne M, Vowles P. 1997. Basic Concepts of 
Environmental Chemistry, p. 125-151. CRC Press LLC, Boca Raton, FL.

Cooke PS, Tomomi S, Buchana, DL. 2001. Disruption of steroid hormone signaling 
by PCBs, p. 257-263. In PCBs: recent advances in environmental toxicology and health effects. Ed Robertson LW, Hansen LG. The University Press of 
Kentucky.

Couillard CM, Hodson PV. 1996. Pigmented macrophage aggregates: A toxic 
response in fish exposed to bleached-kraft mill effluent? Environ Toxicol 

Courtenay SC, Grunwald CM, Kreamer G, Fairchild WL, Arsenault JT, Ikonomou M, 
Wirgin II. 1999. A comparison of the dose and time response of CYP1A1 
mRNA induction in chemically treated Atlantic tomcod from two populations. Aquatic Toxicol 47: 43-69.

Biochemical responses in aquatic animals: A review of determinants of 

PCBs: recent advances in environmental toxicology and health effects. Ed 
Robertson LW, Hansen LG. The University Press of Kentucky.

Fernie K, Bortolotti G, Smits J. 2003. Reproductive abnormalities, teratogenicity, 
and developmental problems in American kestrels (Falco sparverius) exposed 

Folmar LC, Denslow ND, Rao V, Chow M, Crain DA, Enblom J, Marcino J, Guillette 
LJ Jr. 1996. Vitellogenin induction and reduced serum testosterone 
concentrations in feral male carp (Cyprinus carpio) captured near a major 


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