

2017

Endocrine Disruption in Largemouth Bass (*Micropterus Salmoides*) from a Pcb- Contaminated Reservoir

Stephanie B. LaPlaca
University of South Carolina

Follow this and additional works at: <https://scholarcommons.sc.edu/etd>

 Part of the [Environmental Public Health Commons](#)

Recommended Citation

LaPlaca, S. B. (2017). *Endocrine Disruption in Largemouth Bass (Micropterus Salmoides) from a Pcb-Contaminated Reservoir*. (Master's thesis). Retrieved from <https://scholarcommons.sc.edu/etd/4185>

This Open Access Thesis is brought to you by Scholar Commons. It has been accepted for inclusion in Theses and Dissertations by an authorized administrator of Scholar Commons. For more information, please contact dillarda@mailbox.sc.edu.

ENDOCRINE DISRUPTION IN LARGEMOUTH BASS (*MICROPTERUS SALMOIDES*)
FROM A PCB-CONTAMINATED RESERVOIR

by

Stephanie B. LaPlaca

Bachelor of Science
University of South Carolina, 2014

Submitted in Partial Fulfillment of the Requirements

For the Degree of Master of Science in

Environmental Health Sciences

The Norman J. Arnold School of Public Health

University of South Carolina

2017

Accepted by:

Geoffrey I. Scott, Director of Thesis

Dwayne E. Porter, Reader

James B. Glover, Reader

Cheryl L. Addy, Vice Provost and Dean of the Graduate School

© Copyright by Stephanie B. LaPlaca, 2017
All Rights Reserved.

DEDICATION

To the one person who has continuously been on my mind throughout the completion of this thesis: my Papa. I miss you and I hope I made you proud. Thank you for encouraging my love for fish from such a young age. I can't wait to reel in the big ones with you when I see you again.

ACKNOWLEDGEMENTS

I have many people to thank for their support, advice, guidance, and time spent in the completion of this thesis. Firstly, thank you to Chad Altman, Scott Castleberry, and James Glover of the South Carolina Department of Health and Environmental Control (SC DHEC) for their help with fish collections, work up, and technical support. Without you, this project would have never happened. I am very thankful for the SC DHEC being open and flexible in supporting my pursuits of this research project. I am extremely grateful for my visits to USGS Leetown Science Center and the assistance I received with running my samples from Vicki Blazer, Heather Walsh, Kathy Spring, and Darlene Bowling. I am thankful for Peter van den Hurk (Clemson University) for reaching out and offering additional valuable input on this project. Thank you to David Wyker (Clemson University) for guiding me through laboratory procedures for the YES assay and taking the time to run all my samples with me. Thank you to my advisors Geoffrey Scott and Dwayne Porter for their continued advice and support.

Most importantly, thank you to my parents, Peter and Stacy LaPlaca, for your continued love and unwavering support. I am so thankful for the opportunities you have given me.

ABSTRACT

There are numerous cases of intersex condition in fish, specifically immature oocytes in testicular tissue, documented in recent literature. Typically, these cases identify a point source input of endocrine disrupting compounds impacting the fish, such as wastewater treatment effluent. Legacy contaminants such as polychlorinated biphenyls (PCBs) have been suggested as endocrine disruptors in fish species. The objective of this study was to assess endocrine disruption in wild largemouth bass (*Micropterus salmoides*) from exposure to PCBs at a Superfund site in South Carolina on Lake Hartwell (SV-107), where high levels of PCBs in sediment and fish tissue samples have been detected for many years. A less severely PCB-contaminated site on Lake Hartwell (SV-535) and a reference site upstream on Lake Keowee (SV-311) were also sampled. Endocrine disruption in fish was measured by analyzing plasma vitellogenin (VTG), intersex severity, and intersex prevalence. Measurable levels of plasma VTG, an egg yolk precursor protein, were detected in male fish from all sites. There were no cases of intersex observed in females collected for this study. Intersex in males manifested as testicular oocytes (TO). Histological analysis indicated that testicular oocytes were present in 23 of 33 (69%) of male largemouth bass sampled and was found in fish from all three sites. These data suggest there may be a significant association between PCB concentration in fish tissue and endocrine disruption in the form of VTG production, intersex severity, and intersex prevalence in male largemouth bass.

TABLE OF CONTENTS

DEDICATION	iii
ACKNOWLEDGEMENTS.....	iv
ABSTRACT	v
LIST OF TABLES	viii
LIST OF FIGURES	ix
LIST OF ABBREVIATIONS.....	xi
CHAPTER 1: BACKGROUND	1
1.1 POLYCHLORINATED BIPHENYLS	1
1.2 LARGEMOUTH BASS (<i>MICROPTERUS SALMOIDES</i>).....	7
1.3 ENDOCRINE DISRUPTION	13
CHAPTER 2: OBJECTIVES AND METHODS	19
2.1 HYPOTHESES	19
2.2 STUDY AREA	20
2.3 FIELD COLLECTION AND PROCESSING	24
2.4 LABORATORY ANALYSES	26
2.5 STATISTICAL ANALYSES.....	29
CHAPTER 3: RESULTS.....	36
3.1 MORPHOMETRIC PARAMETERS.....	36
3.2 PCB CONCENTRATIONS IN FISH	37
3.3 REPRODUCTIVE PARAMETERS	37

CHAPTER 4: DISCUSSION.....	46
4.1 OVERALL HEALTH.....	46
4.2 VITELLOGENIN	47
4.3 INTERSEX.....	50
4.4 PCBs AS ENDOCRINE DISRUPTORS.....	53
CHAPTER 5: CONCLUSIONS	68
REFERENCES	70
APPENDIX A – SITE DESCRIPTIONS AND LOCATIONS	91

LIST OF TABLES

Table 3.1 Morphometric and biological parameters for largemouth bass (LMB) collected in spring 2016	41
Table 3.2 Reproductive parameters for LMB collected in spring 2016	42
Table 4.1 Spearman rank correlation results for vitellogenin, intersex severity score, and intersex prevalence.....	59
Table A.1 Station names, descriptions, and locations for Lake Hartwell and Lake Keowee stations sampled and sites from Hinck et al. (2009)	91

LIST OF FIGURES

Figure 1.1 General chemical structure of a chlorinated biphenyl.....	18
Figure 1.2 Chemical structure of estrogenic compounds.....	18
Figure 2.1 Lake Hartwell and Lake Keowee in South Carolina and Georgia, USA.	31
Figure 2.2 Polychlorinated Biphenyls (PCBs) in largemouth bass (LMB) Fillets from 1990 – 2014 in Lake Hartwell sites	32
Figure 2.3 Early stages of oocyte development in a cross section of LMB female ovary	33
Figure 2.4 Late stages of oocyte development in a cross section of LMB female ovary	33
Figure 2.5 Stage 3 testis cross section from male LMB	34
Figure 2.6 Stage 4 testis cross section from male LMB	34
Figure 2.7 Intersex severity scores from male LMB	35
Figure 3.1 Mean weight and standard deviation in normal and intersex LMB	43
Figure 3.2 Mean length and standard deviation in normal and intersex LMB	43
Figure 3.3 Mean condition factor and standard deviation in normal and intersex LMB.....	44
Figure 3.4 Distribution of intersex severity scores, 0-4, from all cross sections of LMB testicular tissue examined for each site.....	44
Figure 3.5 Percentage of intersex severity scores 0-4 from each site in all cross sections examined of male LMB	45
Figure 4.1 Mean log-VTG and standard deviation in normal and intersex LMB.....	60
Figure 4.2 Box and whisker plot of LMB plasma log VTG concentrations.....	60

Figure 4.3 Average PCBs in LMB fillets and intersex prevalence at three sampling sites..	61
Figure 4.4 Land use in surrounding areas near Lake Hartwell and Lake Keowee in South Carolina and Georgia, USA.	62
Figure 4.5 Quantile regression analysis of PCBs and Log-VTG in largemouth bass from Lake Hartwell and Lake Keowee sites.	63
Figure 4.6 Sampling locations from USGS widespread intersex in black bass study (Hinck et al., 2009) and current study.	64
Figure 4.7 Percentage of intersex male LMB collected from sites with PCBs in fish tissue and sites with no PCBs.	65
Figure 4.8 Percentage of land use types comprising the average male VTG ($\mu\text{g/ml}$), average intersex severity score, and intersex prevalence at each site	66
Figure 4.9 USDA 2012 Census of Agriculture map of farms with broiler inventory for United States	67

LIST OF ABBREVIATIONS

11-KT	11-ketotestosterone
ANOVA	Analysis of variance
APEs	Alkylphenol ethoxylates
BPA.....	Bisphenol A
CERCLA.....	Comprehensive Environmental Response, Compensation, and Liability Act
CF.....	Condition factor
DDT	<i>dichloro-diphenyl-trichloroethane</i>
E2	17- β estradiol
EDCs.....	Endocrine disrupting compounds
ELISA	Enzyme-linked immunosorbent assay
EPA.....	Environmental Protection Agency
FDA.....	Food and Drug Administration
FSH	Follicle-stimulating hormone
GSI	Gonadosomatic index
GtH.....	Gonadotropin
GtHR.....	Gonadotropin receptor
H&E	Hematoxylin and eosin
HAAs	Hormonally active agents
IARC.....	International Agency for Research on Cancer
LH	Luteinizing hormone
LMB.....	Largemouth bass

PCB..... Polychlorinated biphenyl
SC DHEC.....South Carolina Department of Health and Environmental Control
SMB Smallmouth bass
T Testosterone
TBST..... Tris-buffered saline and Tween 20
TO Testicular oocytes
USGS United States Geological Survey
VTG Vitellogenin
WWTPWastewater treatment plant

CHAPTER 1

BACKGROUND

1.1 POLYCHLORINATED BIPHENYLS

Polychlorinated biphenyls (PCBs) are synthetic organic compounds composed of two benzene rings and varying numbers of chlorine atoms (Agency for Toxic Substances and Disease Registry [ATSDR], 2000). These chemicals were commercially manufactured from the 1920's until their ban in the United States in the 1970's. PCBs were produced by a variety of companies and sold worldwide under different trade names including: Aroclor, Clophen, Phenoclor, and Santotherm. The Monsanto Chemical Company was the primary manufacturer of PCBs for the United States (ASTDR, 2000). Aroclor compounds were typically named according to their percentage by weight chlorine content; for example, Aroclor 1254 contains 54% by weight chlorine as indicated by the last two numbers in its four-digit name (Safe, 1992). There are 209 individual chlorinated biphenyls, or congeners. Homologs include all PCBs with the same number of chlorines (e.g., trichloro-biphenyls) and isomers are homologs with different patterns of chlorine substitution (ATSDR, 2000).

The chemical structure of PCBs consists of two benzene rings connected by a carbon-carbon bond (Figure 1.1). The biphenyl structure can rotate around its central bond which causes planarity of the congener to be determined by the chlorine atoms in the *ortho*-positions (2, 2', 6, 6'). PCB congeners with two or more chlorine atoms in the *ortho*-position twist the biphenyl ring around the bond, thereby making the structure non-

planar or non-coplanar, while non-*ortho* and mono-*ortho* substituted PCB congeners are planar or coplanar (ATSDR, 2000). The toxicity of PCB congeners to different organisms varies (Alhborg et al., 1994) depending on a number of factors including age, sex, and species of organism, route of exposure, duration of exposure, and chlorine content of the PCB mixture (Safe, 1992).

PCBs were used in many industrial and chemical applications because they are non-flammable, chemically stable, have a high boiling point, and have good electrical insulating properties. These characteristics also make them environmentally persistent pollutants and hinder their degradation. PCBs have been used as hydraulic lubricants, dielectric fluids for transformers and capacitors, plasticizers, pesticide extenders, carbonless copy paper, and flame retardants. It is estimated that the total production of PCBs in the United States from 1930 to 1975 was more than 635 million kg (ATSDR, 2000). Improper disposal practices of PCBs and their wastes resulted in their introduction to the environment. They are found globally and are distributed throughout the air, water, sediment, and wildlife due to their chemical stability and lipophilicity.

The first evidence suggesting PCBs were estrogenic was reported in the early 1970's (Bitman & Cecil, 1970). Since then, numerous studies on the endocrine effects of PCBs have been conducted (National Research Council, 1999; Geyer et al., 2000; Baldigo et al., 2006) with insight into different modes of action for endocrine disruption. The varying structure of PCBs allows them to mimic natural hormones such as estrogens which have similar ring structures (Figure 1.2). The non-coplanar congeners with lower chlorination are typically estrogenic, although some congeners with higher degrees of chlorination are also estrogenic (Cooke et al., 2001). PCBs have the potential to

recognize estrogen receptor binding sites, react directly or indirectly with hormone structure to alter it, and the ability to alter the pattern of hormone synthesis or modulate hormone receptor numbers and affinities (McKinney & Waller, 1994). One study (Kester et al., 2000) found that some hydroxylated PCBs inhibit the enzyme estrogen sulfotransferase, which is critical for the eventual inactivation of estradiol (E2). This inhibitory action results in decreased inactivation and increased E2 bioavailability in an organism. This action may result in increased production of the egg yolk precursor protein, vitellogenin (VTG), as E2 is responsible for VTG production. It is important to note that not all PCBs are estrogenic. Anti-estrogenic effects of some PCBs (Moore et al., 1997) have also been studied in both *in vitro* and *in vivo* studies. Mixtures of compounds in the environment and interactions with other contaminants may influence the degree of estrogenicity of PCBs.

Health effects of PCB exposure in humans have been widely researched (Weisglaskuperus et al., 1995; Fierens et al., 2003; Hauser, et al., 2003; Schantz, et al., 2003). The major route of exposure for humans is through consumption of fish containing PCBs, maternal transfer during pregnancy, or transfer to infants via breast milk (Rogan et al., 1987; Jacobson et al., 1989). Due to their lipophilic nature, PCBs accumulate in fatty tissue in humans and remain there for long periods of time. Direct exposure to relatively high levels of PCBs has also occurred in people who worked in plants that manufactured or used PCBs or PCB-containing products. Occupational exposure studies have revealed abnormal liver function tests in PCB manufacturing plant workers and dermatologic effects in the form of chloracne (Longnecker et al., 1997). Some studies show an increased risk of cancer. Suggested types of cancer seen in occupationally exposed

cohorts include liver, gallbladder, biliary (Brown, 1987), and brain cancers (Sinks et al., 1992), as well as melanoma (Loomis et al., 1997). PCBs are considered probable human carcinogens according to the International Agency for Research on Cancer (IARC), based on data from epidemiological and animal studies. Most of these studies involve exposures to other chemicals in addition to PCBs, such as solvents, which could affect cancer rates (Persky, 2001) and not all studies yield the same results in terms of cases where individuals develop cancer from exposure. There are limited data on specific effects of occupational exposures and findings are inconsistent among studies due to lack of follow up.

Reproductive effects in humans have been investigated in some case-control studies but result in inconsistent conclusions. Using birthweight as a measure of reproductive success, few studies (Fein et al., 1984; Patandin et al., 1998) found that PCB levels were inversely associated with birthweight however; in few studies with high exposure levels (Rylander et al., 1998; Bjerregaard & Hansen 2000), there was no significant association between birthweight and PCB exposure. There have been multiple studies on Great Lakes fish consumption that attempt to link adverse reproductive outcomes such as spontaneous fetal death (Mendola et al., 1995), conception delay (Buck et al., 1997), and menstrual cycle length (Mendola et al., 1997) to PCBs in fish tissue, but the only significant adverse association determined was shorter menstrual cycle length in women. Some studies have observed negative effects in males, such as lower sperm count and poor sperm quality, in those who were exposed to PCBs (Carlsen et al., 1992; Sharpe & Skakkebaek, 1993) although these studies are also inconclusive in directly and specifically linking PCBs to reproductive health outcomes. There remains a limited

knowledge on the environmental effects of PCBs on human reproductive health and possible effects may vary among populations, exposures, or PCB mixtures.

Perhaps the most relevant and frequently studied harmful outcome of PCB exposure is neurological development effects in children from mothers who consumed PCB contaminated fish (Gladen & Rogan, 1991; Huisman et al., 1995; Walkowiak et al., 2001). State fish tissue monitoring programs began in the 1970's with goals to inform the public of risks of eating highly contaminated fish and reduce the contaminant load to humans. Newborns can be exposed *in utero* when they are more vulnerable and sensitive due to lack of protective barriers such as the blood-brain barrier. Newborns are exposed additionally from feeding on mother's breast milk, which may contribute to the majority of their lifetime exposure to PCBs as lactation is a major route of release of the toxic burden in females. One study on offspring from mothers that consumed Lake Michigan fish found that infants with elevated cord serum PCB levels were more likely to score lower on visual recognition memory performance tests and suggested that intrauterine exposure to PCBs may be harmful to child development (Jacobson et al., 1985). Other studies on Great Lakes fish consumers yielded similar results with exposed children showing poorer performance on neurological tests including abnormal reflexes, less mature autonomic responses, and delayed habituation responses (Stewart et al., 2000) compared to less exposed children. Additional studies on neurologic and intellectual function in infants and young children exposed to PCBs *in utero* were conducted (Jacobson & Jacobson, 1996) with major findings concluding that prenatal exposure to PCBs was associated with lower IQ scores and poorer intellectual function. In general, most evidence seems to point to a general decline in cognitive functioning of children

who have been exposed to PCBs from maternal plasma or breast milk (Patandin et al., 1999).

PCBs are found in a variety of wildlife and laboratory species. They have been analyzed in aquatic invertebrates (Lazorchak et al., 2015) and used to assess relative sediment PCB levels, in Arctic species such as whales, sea birds, and pinnipeds (Letcher et al., 2010), loggerhead sea turtles, bivalves, alligators (Guillette et al., 1999), mink (Heaton et al., 1995), and in laboratory animals such as monkeys (Allen & Barsotti, 1976) and mice.

Effects from PCB exposure vary among species and degree of exposure. Evidence of estrogenic and anti-estrogenic effects on laboratory animals exposed to PCB mixtures and congeners have been observed in some studies but is difficult to interpret their environmental significance because most PCB extracts from environmental samples do not resemble dosages of commercial PCB mixtures used in laboratory studies (Simmons et al., 2005 and Vega-Lopez et al., 2006). It is still important to consider laboratory results in assessing environment and wildlife exposure effects. Laboratory studies suggest certain PCBs have estrogenic effects on some species including Aroclor 1242 in rats (Jansen et al., 1993), Aroclor 1221 and 1248 in fish (Geyer et al., 2000). PCB-fed male rats that mated with unexposed females had significantly lower incidence of implantation and a lower number of live births (Sager, 1983). Estrogenic effects were also observed in the offspring of PCB-fed guinea pigs (Lundkvist, 1990) who yielded significantly reduced testis weight. Oskam et al. (2003) found that male polar bear offspring exposed to PCBs differed significantly from a control group with respect to plasma hormone and

testosterone levels and had a significantly higher percentage of sperm with damaged DNA.

It is unclear what mechanism is responsible for endocrine modulation caused by PCBs and there may be multiple modes of action for endocrine disruption (Seegal, 1996; Royland & Kodavanti, 2006). In fish, modulation of receptor function depends on the binding affinity and the receptor's ability to recognize hormones and may be a factor in the expression of VTG in male fish. Vitellogenesis may be stimulated in males by activating estrogen receptor mediated expression of VTG. In a study with rainbow trout (*Oncorhynchus mykiss*), nonylphenol, chlordecone, PCBs (Aroclor 1245), and lindane were all able to induce VTG and estrogen receptors in trout hepatocytes *in vitro* (Flouriot et al., 1995). In the Hudson River, male largemouth bass (*Micropterus salmoides*, LMB) plasma VTG and lipid-based PCB residues in fish tissue were significantly positively correlated (Baldigo et al., 2006), indicating a potential moderate estrogenic effect from PCB exposure. Anderson et al. (2003) found evidence of intersex in some male fish from both a PCB-contaminated site and a reference site upstream, but no measurable VTG in male fish from either site. It is unknown what the background prevalence of intersex and VTG levels are in male fish.

1.2 LARGEMOUTH BASS (*MICROPTERUS SALMOIDES*)

The largemouth bass (*Micropterus salmoides*) is a freshwater predatory gamefish endemic to the Southeastern United States but now occurring in many countries throughout the world. (Rohde et al., 2009). They are in the family *Centrarchidae* which includes sunfishes, black bass and crappie. Largemouth bass are found in a variety of freshwater habitats including rivers, lakes, ponds, reservoirs, and streams. Adults are

typically solitary fish and prefer calm, warm water. They hide under the cover of logs, rock ledges, vegetation, and artificial structures. Largemouth are top predators in most freshwater ecosystems, feeding on a variety of organisms including other fish, crayfish, frogs, and insects (Mecozzi, 2008).

Spawning is dependent on water temperature and photoperiod. Spawning begins when water temperatures range between 65 to 75 °F (18°C - 24°C), typically around April to June in South Carolina (Rohde et al., 2009). Males will build nests in shallow water into which that females deposit up to 150,000 eggs. The fertilized eggs are guarded by the male until they hatch and the fry disperse. Largemouth bass are one of the most important freshwater fish in South Carolina due to their economic impact from sport fishing as well as their ecological role as top predators.

Largemouth bass were selected as a target species for this study because they have exhibited endocrine disruption in previous studies (Larkin et al., 2002; Hinck et al., 2009) and may serve as a sentinel for human health. In general, fish have been shown to be an appropriate sentinel species to monitor exposure and effects of contaminants in the aquatic environment because they are known to have major ecological roles in their aquatic environments (Beyer, 1996). Largemouth bass are found throughout the state of South Carolina and are easily collected in the field. They serve as good indicators of water quality conditions in a given area because they inhabit a relatively small home range (Lewis & Flickinger, 1967), have a long lifespan of up to 11 years in the South (Davis & Lock, 1997), and their feeding habits create the greatest potential for biomagnification of contaminants that may have an impact on their health. Largemouth bass are used in many state's fish advisory programs as well, providing a large amount of

data on tissue concentrations of contaminants such as mercury and PCBs. There is also the potential for transfer of contaminants to humans via consumption of these fish. Previous studies have shown that largemouth and smallmouth bass (SMB, *Micropterus dolomieu*) are sensitive to endocrine modulating compounds including compounds found in wastewater treatment effluent (Iwanowicz et al., 2009) and runoff from agricultural practices (Blazer et al., 2014).

Largemouth bass are gonochoristic fishes where individuals develop only as males or females and remain the same sex throughout their life spans (Devlin & Nagahama, 2002). In black bass species (*Micropterus* spp.), primordial germ cells are present in gonads before sexual differentiation and have the ability to become either oogonia or spermatogonia. Gonadal development is dependent on endocrine communication between the brain, pituitary, and gonads, allowing developmental, physical, chemical, social, and seasonal cues to be integrated with gonad maturation (Devlin & Nagahama, 2002). The production of gonadotropins (GtH) in the pituitary gland primarily controls endocrine functioning and regulates growth, sexual development, and reproductive functioning. The two primary gonadotropins in fish are GtHI and GtHII which are analogous to mammalian follicle-stimulating hormone (FSH) and luteinizing hormone (LH), respectively (Strüssmann & Nakamura, 2002). Studies on salmonid species indicate that GtHI is important in early gonadal development, vitellogenesis, and spermatogenesis while GtHII levels are low or undetectable until just prior to final oocyte maturation in females and spermination in males (Janz, 2000). When released, gonadotropins circulate via the bloodstream and interact with receptors on target tissues. Two gonadotropin receptors (GtHR) in the fish gonad have been studied: GtHRI

which binds both GtHI and GTHII gonadotropins and GtHRII which binds only GtHII (Miwa et al., 1994). The receptors are found in various stages of sexual maturation among males and females and stimulate sex steroid production for each. Gonadotropins are important for stimulating steroid synthesis in the teleost gonad and sex steroids function as feedback to the brain to alter GtH production.

Sex steroids commonly studied in fish include 17β -estradiol (E2), testosterone (T) and 11-ketotestosterone (11-KT). E2 is found at higher levels in females than in males and is believed to be the major sex steroid responsible for maintaining ovarian development (Yamamoto, 1969), although T is also important in oogenesis. Both T and 11-KT are found in males, with 11-KT being the main sex steroid responsible for testicular development (Devlin & Nagahama, 2002).

Oocyte development & hormones

In females, oogenesis is the process by which primordial germ cells become ova that are ready to be fertilized (Lubzens et al., 2009). This process consists of the formation of primordial germ cells, sex differentiation, transformation of oogonia into oocytes, growth of oocytes, maturation, and ovulation. Growth and maturation are largely influenced by vitellogenin production. Vitellogenin (VTG) is a phospholipo-protein synthesized by the liver, transported by the blood stream to the ovary, and taken up by growing oocytes (Patiño & Sullivan, 2002) and provides yolk components to mature eggs. It is often used as a biomarker for exposure to environmental estrogens as studies have shown that environmental contaminants can mimic estrogen and induce vitellogenesis (Tyler et al., 1999). Induction of hepatic vitellogenesis is controlled by communication between the brain, pituitary, and gonads. Gonadotropin I (GtHI) in the

blood induce follicular production of the hormone E2 which stimulates hepatic vitellogenesis (Specker & Sullivan, 1994). Blood levels of gonadotropin II (GtHII) rise when vitellogenic growth is completed. GtHII binds to its receptor in the follicle and stimulates the process of oocyte maturation. Ovulation and expulsion of the mature ovum is often highly synchronized with spermination in males via pheromone behaviors (Kobayashi et al., 2002).

Ovarian hormones associated with oogenesis include 17β -estradiol and testosterone. In sexually immature females, plasma sex steroid levels are very low, typically less than 0.3 ng/ml (Janz, 2000). During vitellogenesis, an increase in plasma E2 levels is positively correlated with growth of vitellogenic oocytes, and the highest levels of E2 occur at this time. Plasma E2 levels decrease after ovulation and spawning. Androgens are precursors for estrogens. Testosterone is present in females as it is the immediate precursor for E2 biosynthesis. In general, the conversion of T to E2 is catalyzed by aromatase. Aromatase converts one of the steroids in T into an aromatic state, resulting in E2. Androgens are released into the plasma when no longer needed for aromatization of T to E2 (Campbell et al., 1976).

Spermatogonia development & hormones

In males, spermatogenesis is the process by which primordial germ cells become spermatozoa (Schulz et al., 2010). The process is similar to oogenesis with phases of sexual differentiation, transformation from spermatogonia to spermatocytes, and growth and development of spermatozoa. Gonadotropins GtHI and GtHII are important pituitary hormones in regulating testicular physiology (Schulz et al., 2010). Sertoli cells are directly associated with germ cells and provide physical and chemical support during

spermatogenesis. Leydig cells found in adjoining tissue function to synthesize sex steroid hormones responsible for spermatogenesis, expression of secondary sexual characteristics, and feedback regulation of gonadotropins (Janz, 2000). The two main sex steroids involved in spermatogenesis in males are T and 11-KT. The process of spermatogenesis begins within the Sertoli cells where development of germ cells takes place. Briefly, cyst formation begins with mitotic division of spermatogonia. Spermatogonia transform into primary spermatocytes and the first meiotic division of these primary spermatocytes produces secondary spermatocytes. During the second meiotic division, they become spermatids. Although the spermatids are haploid, they must still undergo differentiation into spermatozoa which involves reorganization of the nucleus and cytoplasm with development of a flagellum.

In sexually immature males, circulating levels of androgens T and 11-KT are low. Reproductively active male salmonids can reach peak levels of 45 ng/ml of androgens (McDonald & Milligan, 1992). An increase in androgen levels typically occurs during the spawning season, specifically with the appearance of spermatozoa and even higher levels are observed when spermination occurs (Billard, 1978; Sivarajah et al., 1979). Estrogen steroids, specifically E2, has been measured in low level and trace amounts in males for a variety of species: *Carassius auratus* (Schreck & Hopwood, 1974); *Salmo trutta* (Billard et al., 1978), and *Salmo salar* (Idler et al., 1981). There is still a lack of knowledge regarding testicular hormone regulation in fish compared to what is known concerning ovarian physiology.

1.3 ENDOCRINE DISRUPTION

Largemouth bass are gonochorists, where individuals develop only as males or females, and remain the same sex throughout their life (Devlin & Nagahama, 2002). In contrast, hermaphrodites are individuals that change gonadal sex or contain both male and female germ cells in the same gonad. Hermaphroditism and sex reversal is common in some fish species, especially marine species such as black sea bass (Lavenda, 1949) but is abnormal in LMB (Blazer, 2002).

In the examination of ovaries and testes of gonochorists, the presence of male and female characteristics simultaneously in one individual is referred to as intersex. The most common intersex condition observed is ovo-testis, the presence of ovarian tissue in the testes of male fish (Blazer, 2002). Ovo-testis in male gonads as the presence of immature oocytes within male testicular tissue is referred to testicular oocytes (TO). Many feral fish species throughout the world have been observed with TO including: white sucker (*Catostomus commersonii*) in Colorado (Woodling et al., 2006), common carp (*Cyprinus carpio*) in Spain (Lavado et al., 2004), and with greater frequency, largemouth and smallmouth bass in Michigan, New York, Virginia, and West Virginia (Anderson et al., 2003; Baldigo et al., 2006; Blazer et al., 2007). The Southeastern United States, including water bodies from parts of North and South Carolina, has been reported to have some of the highest intersex prevalence rates in largemouth bass (Hinck et al., 2008).

Intersex has been linked to exposure to EDCs from various sources such as wastewater treatment discharges (Jobling et al., 1998; Bjerregaard et al., 2006), pulp and paper mill effluent (Durhan et al., 2002), and PCB contaminated areas (Baldigo et al.,

2006). The natural incidence of intersex is unknown in most species of fishes and more data are needed to accurately interpret intersex rates in relation to contaminant exposure.

The primary steroid hormone in female LMB reproduction is 17- β estradiol (E2). The structure of E2 is an aromatized carbon steroid with two hydroxyl groups and it is the most potent form of mammalian estrogenic steroids. In female ovaries, the two somatic cell layers of the follicle play a role in steroid biosynthesis. The ovarian thecal layer contains enzymes necessary for the production of testosterone and precursor androgens and the granulosa layer is where conversion of testosterone to estrogen via aromatase takes place (Devlin & Nagahama, 2002). Aromatase is not usually detectable in testicular tissue, however; evidence exists for estradiol production during some stages of testicular development (Pasmanik & Callard, 1988). Synthesis of the egg yolk precursor protein VTG is estrogen dependent and therefore measuring plasma VTG levels is commonly used as a biomarker for estrogenic exposure in fish (Jobling et al., 1998; Blazer et al., 2014). Vitellogenin is not produced under normal physiological conditions in males and detection of VTG in male blood plasma is indicative of recent or continued exposure to estrogenic compounds.

Naturally occurring and synthetic hormonally active agents (HAAs) are found in the environment. The most common natural HAAs are either from plants (phytoestrogens) or fungi that infect the plants (mycotoxins). Legumes, nuts, grains, and vegetables may contain phytoestrogen compounds like isoflavonoids (National Research Council, 1999). A large number of synthetic HAAs exist in the environment and many are now banned from production and use; PCBs, DDT (dichloro-diphenyl-trichloroethane), Bisphenol A (BPA), chlordecane, and 4-Alkyphenol have all been

linked to endocrine disruption (Vethaak et al., 2005; Monteiro et al., 2015). Many studies on endocrine modification in fish from waters that receive wastewater treatment plant (WWTP) effluent have been conducted (Larsson et al. 1999; Orlando et al., 2004; Vajda et al., 2008; Jobling et al., 2009). It is believed that estrogenic compounds from humans, specifically the synthetic estrogen found in birth control pills, ethinyl-estradiol, are the active chemicals in WWTP effluent that are causing potential endocrine effects in fish (Purdom et al., 1994). Alkylphenol ethoxylates (APEs) and their degradation products have also been suggested as estrogenic compounds found in domestic and industrial sewage effluent. Studies have shown that alkylphenols caused a significant increase in VTG concentration and a significant decrease in testicular growth in male rainbow trout (Jobling et al., 1996). In the Great Lakes, DDT and fish fry mortality has been studied and monitored for some time. Based on laboratory studies of different fish species, it is estimated that DDT is lethal to salmonid eggs at 1.0 – 10 mg/kg wet weight (Macek, 1968). DDT levels were measured up to 10 mg/kg in fish eggs from the Great Lakes (Burdick et al., 1964), so it is possible DDT has caused reproductive impairment in salmon from the Great Lakes. Another major source of HAAs to the environment is from bleached paper mill effluent. There are a variety of synthetic and natural chemicals released in the effluent mixtures from these plants. Some species have shown decreased levels of plasma sex steroids, decreased egg and gonad size, and delayed sexual maturity when exposed to bleached paper mill effluent (Van Der Kraak et al., 1992). Agricultural production is also a major source of HAAs to the environment. Intersex prevalence in SMB has been associated with percent agriculture and animal density in catchments draining to collection sites (Blazer et al., 2012), suggesting that agricultural pesticides

and natural and synthetic hormones are potential HAAs responsible for endocrine disruption in SMB.

It is important to understand the non-lethal effects that endocrine disrupting chemicals have on organisms. Sub-lethal effects from EDCs that impair reproduction may ultimately effect overall population numbers, as seen in previous studies (Kidd et al., 2007). Whether endocrine disruption observed in fish species translates to effects in humans is still unknown, but fish are important sentinel species for humans and understanding endocrine disruption in fishes utilizing the same water sources and ecosystems as humans is of value for human health implications.

The purpose of this study was to assess endocrine disruption in LMB from Lake Hartwell in South Carolina that has had historically high levels of PCBs above 2 ppm, on average, in LMB. Many studies thus far on endocrine disruption in fish have involved fish from riverine or lotic habitats (Baldigo et al., 2006; Ingram et al., 2011; Fritts et al., 2016). Lotic systems differ in how contaminants cycle through them compared to lentic waters. For flowing systems, PCBs are circulated via sediment transport mainly by adsorbing to particles. Autochthonous inputs such as benthic algae and allochthonous inputs including leaf detritus are the major vectors for adsorption and transfer of PCBs to stream consumers. The degree of heterotrophy in streams has been positively correlated to PCBs in brown trout (*Salmo trutta*) (Berglund et al., 2005) indicating that transfer of PCBs to higher order organisms is smaller in less productive systems.

In eutrophic lakes, the greatest amount of PCBs typically accumulate or are buried in the sediment but a greater fraction of the total PCB load is typically dissolved in water in oligotrophic lakes (Berglund et al., 2001). Due to the greater productivity in

lakes versus rivers and streams, it is believed that benthic invertebrates play a large role in moving contaminants from sediment to consumers (Walters et al., 2008) which facilitates bioaccumulation of contaminants to higher order organisms.

Few studies have assessed intersex rates in fishes from lakes and even less have reported rates for largemouth bass, therefore additional research on intersex rates and endocrine disruption in popular sport fish from large lakes is warranted. Intersex occurrence in fish from lakes that are relatively free of contaminants may provide insight into background levels of intersex. A survey of intersex in black basses from US rivers (Hinck et al., 2009) found some of the highest rates of intersex among LMB from South Carolina's Pee Dee River Basin as well as downstream of Lake Hartwell in the Savannah River Basin. The information gathered in the current study for LMB in Lake Hartwell will help assess potential long term impacts on fish health from PCB contamination, shed light on intersex occurrence in South Carolina fishes, and provide additional intersex data to compare to fish among Southeastern lakes.

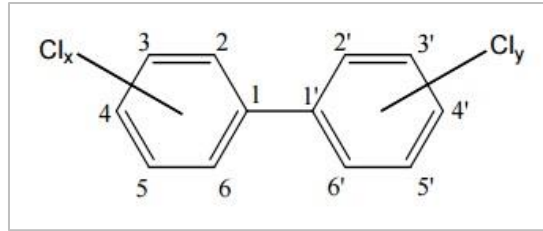


Figure 1.1: General chemical structure of a chlorinated biphenyl. Positions 2, 2', 6, 6' are *ortho* positions, positions 3, 3', 5, and 5' are *meta* positions, and positions 4, 4' are *para* positions. Benzene rings can rotate around the carbon bond that connects them. Varying numbers of chlorines are attached to the biphenyl molecule.

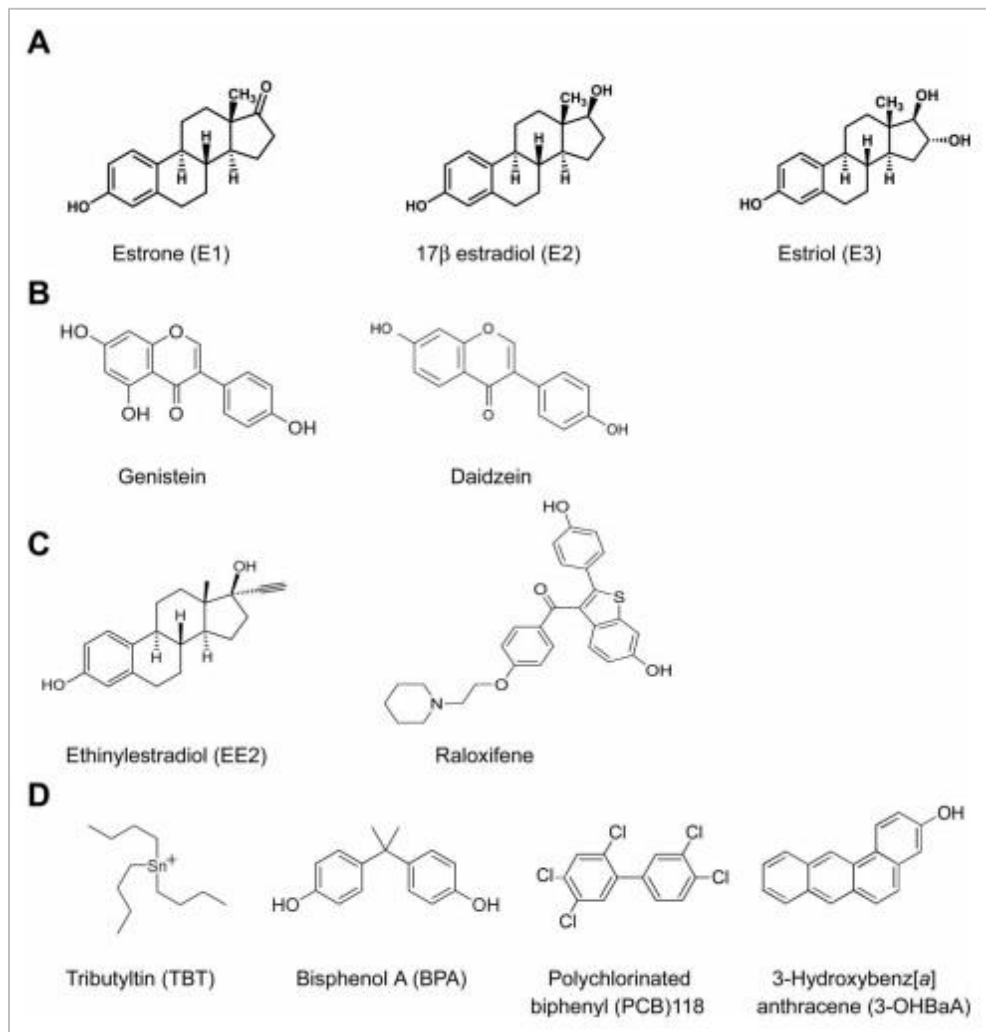


Figure 1.2: Chemical structure of estrogenic compounds: (A) natural animal estrogens; (B) natural plant estrogenic compounds (phytoestrogens); (C) synthetic pharmaceutical estrogenic compounds; (D) synthetic estrogens from other applications. From: Pinto et al., 2014

CHAPTER 2

OBJECTIVES AND METHODS

The overall goal of this study was to understand the endocrine effects in wild largemouth bass (LMB, *Micropterus salmoides*) populations exposed to polychlorinated biphenyls (PCBs). The goal was attained using the following objectives:

1. Determine if endocrine disruption is found in wild fishes from Lake Hartwell and Lake Keowee by measuring plasma VTG, intersex prevalence, and intersex severity in largemouth bass.

2. Determine whether largemouth bass collected from a contaminated site with historically high levels of PCBs found in fish tissue have significantly different VTG plasma concentration compared to largemouth bass collected from a reference site with non-detectable PCB levels in fish tissue.

3. Investigate if largemouth bass from a PCB contaminated site have a greater prevalence of intersex compared to largemouth bass collected from a reference site.

4. Investigate if largemouth bass from a PCB contaminated site have a greater severity of intersex compared to largemouth bass collected from a reference site.

5. Assess overall differences in fish health between sampling sites, if they exist.

2.1 HYPOTHESES

a) *Is there no difference in male largemouth bass plasma VTG in fish collected from a PCB-contaminated site compared to a reference site? Does VTG differ in a heavily contaminated site versus a less contaminated site?*

H0: VTG_{SV107} = VTG_{SV535} = VTG_{SV311}

HA: VTG_{SV107} ≠ VTG_{SV535} ≠ VTG_{SV311}

b) *Is there no difference in male largemouth bass intersex prevalence from fish collected at a PCB-contaminated site compared to a reference site? Does intersex prevalence in male fish differ in a heavily contaminated site versus a less contaminated site?*

H0: Intersex prevalence_{SV107} = Intersex prevalence_{SV535} = Intersex prevalence_{SV311}

HA: Intersex prevalence_{SV107} ≠ Intersex prevalence_{SV535} ≠ Intersex prevalence_{SV311}

c) *Is there no difference in male largemouth bass intersex severity from fish collected at a PCB-contaminated site compared to a reference site? Does intersex severity in male fish differ in a heavily contaminated site versus a less contaminated site?*

H0: Intersex severity_{SV107} = Intersex severity_{SV535} = Intersex severity_{SV311}

HA: Intersex severity_{SV107} ≠ Intersex severity_{SV535} ≠ Intersex severity_{SV311}

2.2 STUDY AREA

In 1955, Sangamo Weston Electric Company opened a plant in Pickens, South Carolina manufacturing electrical capacitors. PCBs were used as a dielectric fluid in the capacitors manufactured at the plant. Before their ban in 1977, PCBs and waste materials containing PCBs were landfilled in six disposal areas around the plant in Pickens County, SC. In the mid-1970's, State and Federal environmental monitoring programs detected PCBs in the sediment of Lake Hartwell and its tributaries, in the soil of Sangamo Weston's dump sites, and in fish tissue samples collected from Lake Hartwell (Gaymon, 1992). Schlumberger Technology Corporation purchased Sangamo Electric Company in 1975 and is currently responsible for investigations, cleanup activities, and treatment of

contaminated areas under the Comprehensive Environmental Response, Compensation, and Liability Act (CERCLA) (US Environmental Protection Agency [EPA], 1994).

Environmental monitoring in 1976 by the South Carolina Department of Health and Environmental Control (SC DHEC) and the United States Environmental Protection Agency (EPA) found that fish from certain areas of Lake Hartwell contained PCB levels in excess of the safe tolerance level of 5 ppm for fish consumption established by the United States Food and Drug Administration (FDA). In 1984, the FDA has reduced the safe tolerance level for PCBs in fish tissue to 2 ppm. (EPA, 1991) The action level for issuance of fish consumption advisories at present in South Carolina is 0.05 ppm for fish. SC DHEC issued fish consumption advisories for Lake Hartwell beginning in 1976 in an effort to reduce human exposure to PCBs. In 1978, Sangamo Weston closed the manufacturing plant and in 1990, the site was added to EPA's National Priorities List as a Superfund site due to contaminated debris, ground water, sludge, soil, and fish tissue from facility operations. Fish advisories for PCBs still exist throughout Lake Hartwell (SC DHEC, 2016) with the greatest restriction advising people not to consume any species of fish caught from the Twelvemile Creek (SV-107) or the Seneca River arm (SV-106) of Lake Hartwell. When the Sangamo Weston plant operated from 1955 to 1977, it is estimated that 441,000 lbs. of PCBs were released into the Twelvemile Creek system at Town Creek in Pickens and contaminated Lake Hartwell downstream. This substantial release of PCBs allowed PCBs to be taken up into the bodies of fish in water. PCBs are highly lipophilic and are rapidly accumulated by aquatic organisms then biomagnified as they move up the food chain. Concentrations of PCBs in aquatic organisms may be 2,000 to one million times higher than the concentrations found in

surrounding waters (United States Environmental Protection Agency [EPA], 1999). A total PCB value of more than 100 ppm was detected in a fish collected from the Twelve-mile Creek arm of Lake Hartwell in the 1970's (Aldridge, 1978). Generally, larger and fatty predatory fish such as largemouth bass or walleye contain higher levels of contaminants compared to smaller, leaner species like sunfish. Today, the most common route of entry of PCBs, affecting the largest number of people, is consumption of fish caught in PCB-contaminated lakes and rivers (Nadakavukaren, 2011).

Lake Hartwell is a man-made reservoir that was constructed from 1955 – 1963 by the US Army Corps of Engineers by damming the Tugaloo, Seneca, and Upper Savannah Rivers. Construction of the Hartwell Dam and Reservoir were authorized under the Flood Control Act of 1950 as part of the development of the Savannah River Basin for generating hydroelectric power and for the improvement and benefit of navigation and control of destructive flood waters (Flood Control Act of 1950). It is comprised of approximately 56,000 acres of water and 962 miles of shoreline. Lake Hartwell forms the border of South Carolina with Georgia and lies within Anderson, Pickens, and Oconee counties in the upstate of South Carolina and Stephens, Franklin, and Hart counties in Georgia. Land use in areas that drain directly into Lake Hartwell is dominated by forested land (43%), pasture and cropland (29%), and developed (e.g. urbanized) land (16%) (Homer, 2015). Fish tissue monitoring in Lake Hartwell has shown historically high PCB concentrations in fish throughout the lake due to contamination from PCB wastes from the Sangamo Weston plant. There are six fish tissue sampling sites on Lake Hartwell that were established by SC DHEC (Figure 2.1) and sampled by Schlumberger Technology Corporation annually from 1990 – 2014 and bi-annually from 2014 to present. There is

an overall decreasing trend in PCB concentrations in LMB fillet samples both spatially and temporally in Lake Hartwell. As distance from the Sangamo input site increases, PCBs in fish tissue decrease, with highest levels found at sites closest to the plant. Fish tissue monitoring data have shown that PCBs in LMB fillet samples have temporally declined overall from all sites since 1990 (Figure 2.2). Fish for this study were collected at site SV-107, Twelvemile Creek at Lake Hartwell, because it is the end of the tributary Town Creek at which PCBs entered the Lake Hartwell system. High PCB concentrations, above 2 ppm in largemouth bass, have been recorded since extensive annual monitoring began in 1990 (Gaymon 1992). Fish were also collected from site SV-535 on Lake Hartwell which is farther downstream in the lake and in the vicinity of Andersonville Island near the confluence of the Tugaloo and Seneca Rivers. This site was sampled to compare a less contaminated site to the highly-contaminated site.

Lake Keowee served as the reference site for this study. It is directly upstream from Lake Hartwell in a series of manmade reservoirs along the Savannah River Basin and is geographically separated from Lake Hartwell. It was also created under the Flood Control Act of 1950. Lake Keowee is in Oconee and Pickens counties in South Carolina and is comprised of approximately 18,500 acres of water and 300 miles of shoreline (Duke Energy, 2014). Land use in areas that drain directly to Lake Keowee is dominated by forested land (68%) followed by pasture and cropland (11%) and then developed land (10%) (Homer, 2015). Lake Keowee does not have a PCB advisory and is considered relatively less developed in comparison to Lake Hartwell. The most recent PCB fish tissue data for Lake Keowee is from 2006, with all results falling below the detection limit of 0.05 ppm. The SC DHEC does not currently monitor Lake Keowee for PCBs

because levels in fish tissue have consistently remained below the detection limits. However, in 2016, Lake Keowee was added to the fish consumption advisory list in SC due to high levels of mercury found in largemouth bass tissue samples, advising “at risk” consumers to limit their consumption of LMB to one meal per week. High levels of mercury have also been found in LMB from Lake Hartwell (SC DHEC, 2016).

2.3 FIELD COLLECTION AND PROCESSING

Largemouth bass (*Micropterus salmoides*) were the target fish species for this study. LMB are a predatory fish species found in both Lake Hartwell and Lake Keowee and generally inhabit a small home range within a water body. Largemouth have served as vertebrate sentinel species for humans because they are able to integrate natural and anthropogenic stressors in their biological response to contaminants (van der Oost et al., 2003; Iwanowicz et al., 2012). Fish were collected using protocol from the SC DHEC fish tissue monitoring and advisory program (Glover et al., 2010) using a Smith-Root electrofishing boat. Preliminary data were gathered in the fall and additional data were collected in the spring. An attempt to collect ten male and ten female LMB was made at each site during each sampling event. In the fall, only SV-107 and SV-311 were sampled due to fish seasonal movement patterns and lack of fish accessibility. A total of 21 fish comprised of 5 males and 16 females were collected between October 20 and November 17 2015. All three sites were sampled in the spring and data gathered in the spring was used in all analysis and reporting. A total of 60 LMB comprised of 33 males and 27 females were collected from three sites in the spring between April 12 and May 4 of 2016.

Fish were processed in the field using methods similar to Blazer et al. (2007). Upon collection, fish were kept in an aerated live well until processed. A few fish were placed in a holding cooler with a tricaine methanesulfonate (MS-222, Sigma-Aldrich) solution and euthanized by lethal overdose. Individual fish were weighed to the nearest 0.1 gram and measured for total length to the nearest 0.1 inch (2.54 mm). Each fish was dissected by making a ventral incision from the vent to the head of the fish. A 3 cc, 22G x 1 ½ heparinized needle was inserted into the heart of the fish to draw blood via cardiac puncture. The needle tip was removed and the blood was carefully dispensed into a heparinized vacutainer containing 75 U sodium heparin (Becton, Dickinson and Company, Franklin Lakes, NJ), gently inverted, and placed on wet ice until processed in the field. The sex of the fish was identified and the gonads were excised, weighed to the nearest 0.1 g, and placed in a bottle containing buffered zinc formalin fixative, Z-fix (Anatech Ltd., Battle Creek, MI) for histology. After all fish were dissected, blood samples were processed immediately (< 2 hours). Blood in vacutainers was centrifuged at 3500 rpm for 10 minutes to obtain plasma. Plasma was aspirated with a transfer pipette into a 2 ml cryogenic vial and transferred to dry ice immediately. Fish carcasses were placed in individual bags with a site and fish number identification tag and placed on wet ice until they could be stored in the lab freezer. Upon return to the lab, plasma samples were frozen in a -80°C freezer until ready to be analyzed for vitellogenin.

Condition factor (CF) was calculated by the formula: $((\text{body weight} - \text{gonad weight in g} / \text{length}^3 \text{ in mm}) \times 10^5)$. Gonadosomatic index (GSI) was calculated by the formula: $(\text{gonad weight (g)} / (\text{total body weight (g)} - \text{gonad weight (g)})) \times 100$

2.4 LABORATORY ANALYSES

Vitellogenin

Vitellogenin analysis was conducted with assistance from staff at US Geological Survey (USGS) Leetown Science Center. A direct enzyme-linked immunosorbent assay (ELISA) for LMB was conducted on the blood plasma samples collected. The assay used monoclonal antibodies developed for smallmouth and largemouth bass and followed methodology described in Denslow et al. (1999) and Blazer et al. (2012).

The primary antibodies used were monoclonal antibodies, including ND-3G2 for largemouth bass (Cayman Chemical Company, Ann Arbor, MI). The VTG standards used for this assay were purified from smallmouth bass and prepared at the Center for Human and Environmental Toxicology, University of Florida, Gainesville, Florida. Plasma samples were defrosted at room temperature one hour prior to beginning analysis. Once defrosted, plasma was centrifuged at 3000 rpm for 10 minutes. Two dilution plates for each plate being read in the microplate reader were prepared with dilution buffer using 96 well Round Bottom Assay Plates (Costar 3360, Corning, NY). Plasma samples were diluted 1:100 and 1:1000 with PBSZ (4.2 millimoles [mM] trisodium phosphate, 5.8 mM monosodium phosphate, 150 mM sodium chloride (NaCl), 0.02% sodium azide, pH 7.6). For the 1:100 dilution plate, 2 μ L plasma:198 μ L dilution buffer was used. For the 1:1000 dilution plate, 20 μ L of 1:100 dilution:180 μ L dilution buffer was used. Plasma samples were loaded in duplicate and VTG standards were loaded in triplicate at a volume of 50 μ L into a 96-well Easy Wash™ High Binding plate (Costar, Corning, NY) and stored overnight at 4 °C in a humidified chamber. Following the overnight incubation, the plates were washed five times with Tris-buffered saline and Tween 20

(TBST) (10 mM Sigma 7-9, 150 mM NaCl, 0.05% Tween-20, pH 7.6) and 300 μ L of blocking buffer (10 mM Sigma 7-9, 150 mM NaCl, 1% bovine serum albumin, 0.05% Tween-20, pH 7.6) was added. Plates were incubated at room temperature for one hour and washed five times with TBST. The anti-VTG antibody was diluted to 1:1000 in blocking buffer, added to all wells (50 μ L), and incubated at room temperature for 1 hour. Plates were washed again five times with TBST. The biotinylated secondary antibody (goat anti mouse IgG-biotin) was diluted 1:1000 in blocking buffer, added to all wells (50 μ L), and incubated at room temperature for one 1 hour. Plates were washed five times with TBST. Streptavidin-alkaline phosphatase (S-AP) was diluted 1:5,000 in blocking buffer, added to all wells (50 μ L), and incubated at room temperature for one hour. Plates were washed five times with TBST and color was developed by adding 50 μ L of developer solution (2.7 mM 4-nitro-phenyl phosphate, 30 mM sodium carbonate, 2 mM magnesium chloride (MgCl₂, pH 9.6)). Plates were incubated in the dark at room temperature for 30 minutes. Optical density (405 nanometers [nm]) readings were taken at the end of incubation using SpectraMax M4 microplate reader (Molecular Devices, Inc., Sunnyvale, California). Concentrations of unknowns were determined from the standard curves and using Softmax Pro TM software (Molecular Devices). The limit of detection was 1 microgram per milliliter (μ g/mL).

Histology

Histology of gonadal tissue was also conducted with assistance from staff at USGS Leetown Science Center. Tissue samples in Z-fix fixative were prepared by taking 5-10 transverse cross sections along the entire length of the gonad and placing them in up to two cassettes. Tissue pieces were dehydrated in alcohol, embedded in paraffin,

sectioned at 6 μm , and stained with hematoxylin and eosin (H&E) (Luna, 1992). Two cassettes with 5-15 cross sections in total were made for each fish. All tissue slides were examined to confirm sex, determine reproductive stage, intersex condition, and overall health. Reproductive stage was assigned based on previous work by Blazer (2002).

Largemouth are group-synchronous spawners where most of the oocytes or sperm are in the same developmental stage. Gonads were staged based on the majority of what stage was observed for each fish. Oocyte stages were scored as stage 1 – immature (nucleolar), stage 2 – early vitellogenic (cortical alveolar), stage 3 – mid-vitellogenic (yolk droplet), stage 4 – mature (yolk begins to hydrate) and stage 5 – postovulatory follicles. Figure 2.3 shows a cross section a female LMB ovary with mostly stage 1 and stage 2 oocytes.

Figure 2.4 shows a more developed oocyte in stage 3, with vitellogenic components in the egg. Male gonad stage was scored similarly, as stage 1 – exclusively immature stages (spermatogonia to spermatids); stage 2 – predominantly immature stages, some sperm may be present; stage 3 – approximately equal portions of spermatocytes and spermatids, and spermatozoa; stage 4 – primarily spermatozoa; and stage 5 – post-spawn. Figure 2.5 shows a cross section of a male LMB testis classified as stage 3. Figure 2.6 shows a more developed testis right before spawning with almost entirely spermatozoa.

For the assessment of intersex condition in the fish, only males were analyzed because there were no observations of intersex in female fish collected. Intersex was assessed in two ways – presence or absence and intersex severity score similar to previous methods outlined by Blazer et al. (2007). An individual was considered intersex if pre-vitellogenic oocytes (TO) were present in gonadal tissue (Figure 2.7). Severity score for each individual cross section was taken by examining each cross section from

the male gonad and ranking it 0-4, with 0 being no observable intersex and 4 being the most severe case with many TO present. Cross sections were first scanned at low magnification of 40X to determine the presence or absence of TO. Sections with TO were then scanned at 200X using a Zeiss Axio A1 microscope to determine severity. Severity was ranked by the distribution and number of oocytes in a field of view and each section received a final score for the field of view that ranked most severe. The ranking system used was adapted from Blazer (2007) and assigned scores based on the following criteria: 0; no oocytes in a field of view, 1; focal distribution – a single oocyte observed within a field of view (Figure 2.7A), 2; diffuse distribution – more than one oocyte in a field of view, but no physical association with neighboring oocytes (Figure 2.7B), 3; cluster distribution – more than one but less than five closely associated oocytes (Figure 2.7C), and 4; zonal distribution – more than five closely associated oocytes or numerous clusters in a field of view (Figure 2.7D). Once each fish had severity scores for all its individual cross sections, the average severity for the fish was assigned.

2.5 STATISTICAL ANALYSES

Computations and statistical analyses were performed with R Version 3.3.3 (R Core Team, Vienna, Austria) or Microsoft Excel 2013 (Microsoft Corporation, Redmond, WA). Arithmetic means and standard errors were computed for morphometric and biological parameters (total weight, total length, CF, GSI) and reproductive biomarkers (VTG, intersex severity score). Data was tested for normality using the Shapiro-Wilk test. If data was not normally distributed, it was \log_{10} transformed to approximate normality for statistical analysis. When data did not normalize, a nonparametric test was used. Welch's t-test was used to compare differences between males and females in the

parameters measured for normally distributed data (CF). For nonparametric data (TW, TL, VTG, GSI), a Mann-Whitney U test was used to compare differences between males and females. Data was tested for normality among males and females using the Shapiro-Wilk test. If data was not normally distributed, it was \log_{10} transformed to approximate normality for statistical analysis. When data did not normalize, a nonparametric test was used to compare differences between sites. An analysis of variance (ANOVA) was used to compare differences among sites for the normally distributed parameters measured (males: LogTW, LogTL, LogCF, LogVTG, GSI; females: LogCF, VTG, GSI). For nonparametric data (females: TW and TL), a Kruskal-Wallis ANOVA on ranks was used to compare differences between sites. If significant difference were detected between sites for males or females, Tukeys HSD post-hoc test was used to assess which sites differed from one another. For males, intersex severity was analyzed using a Kruskal-Wallis test to compare severity scores between sites. Nominal data (i.e. intersex prevalence) was analyzed using Fisher's exact test. Total PCBs (ppm) in LMB fish tissue from 2014 (Schlumberger Technology Corporation, 2014) were \log_{10} transformed for statistical analysis followed by Welch's t-test to compare differences between Lake Hartwell sites SV-107 and SV-535. Total PCBs from Lake Keowee (SV-311) were not used in statistical analysis as SV-311 has not been sampled for PCBs since 2006 and is presumed to still have levels below detectable limits for PCBs in fish tissue. A Spearman's rank correlation (ρ) was used to test the relation between reproductive biomarkers (i.e. VTG, intersex severity score, and intersex prevalence) and PCBs at each site. A significance level (α) of 0.05 was used for all statistical analyses.

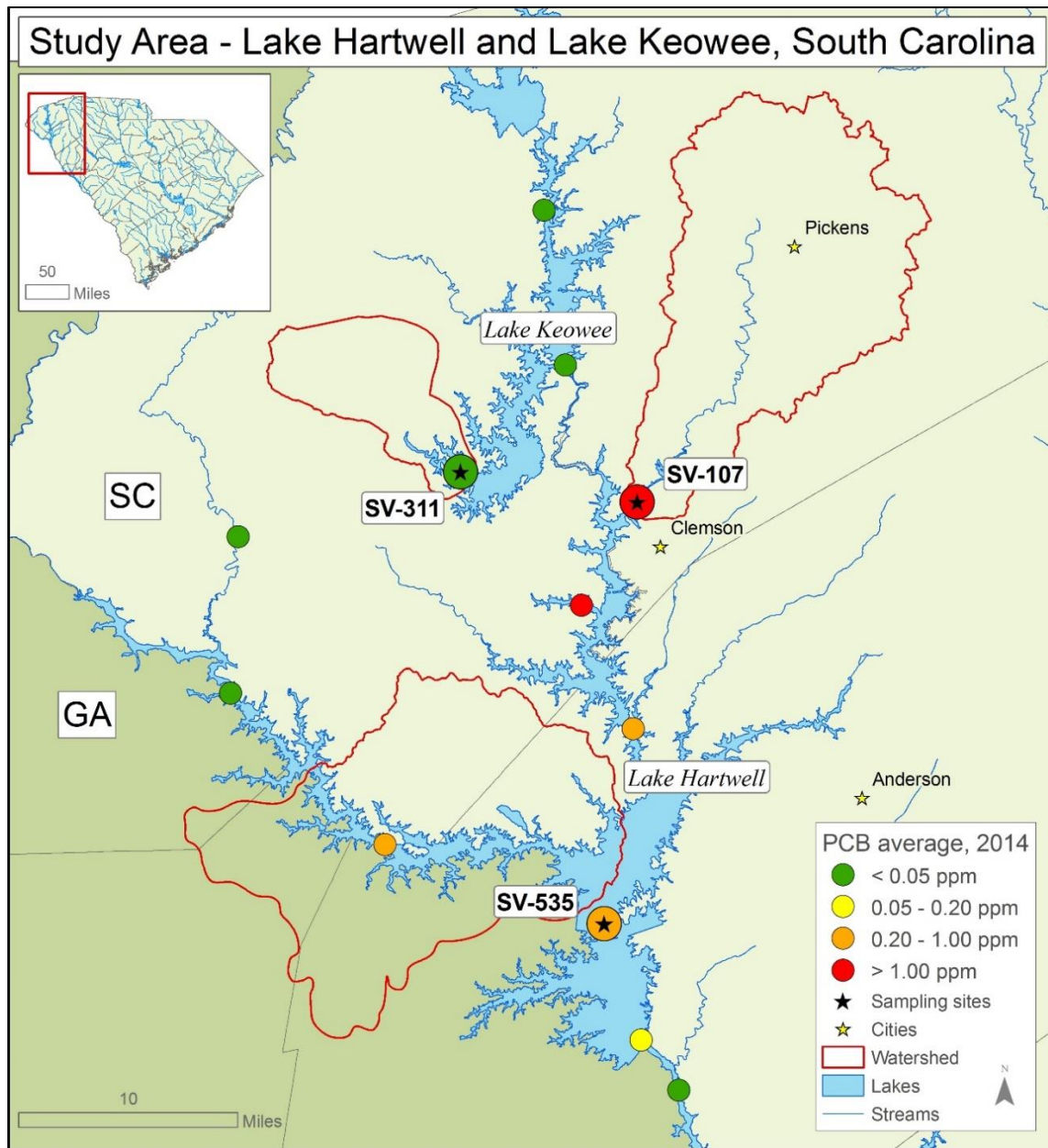


Figure 2.1: Lake Hartwell and Lake Keowee in South Carolina and Georgia, USA. Fish tissue monitoring sites are represented by the circles and average PCB values for 2014 are displayed. Sites with a star represent sites sampled for LMB for this study.

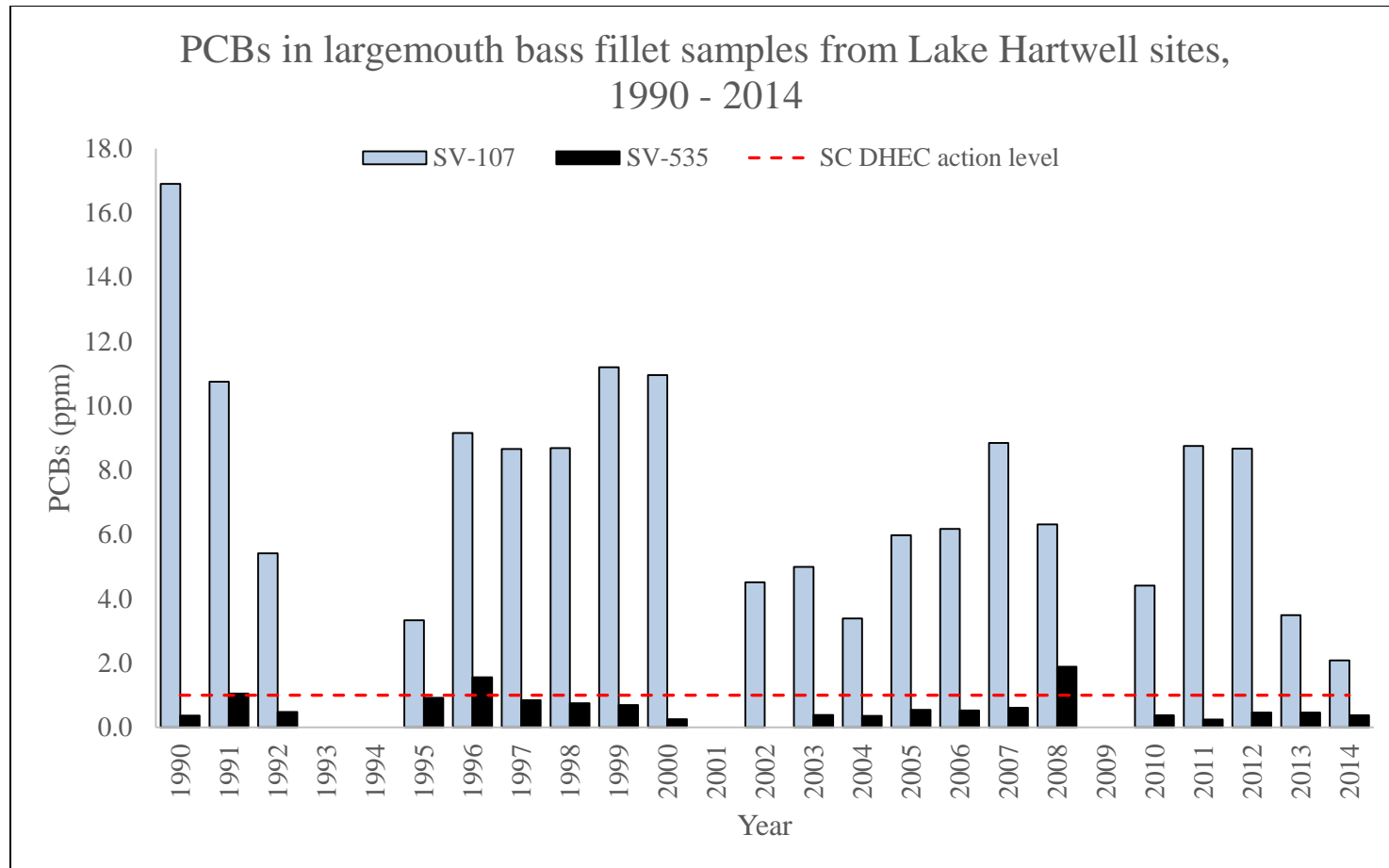


Figure 2.2: Polychlorinated Biphenyls (PCBs) in LMB Fillets from 1990 – 2014 in Lake Hartwell sites. Average PCBs (ppm) measured in both male and female LMB collected from Lake Hartwell fish tissue monitoring sites are displayed. SV-107 is the end of the tributary where PCBs entered Lake Hartwell in the 1970s. Site SV-535 is farther downstream in Lake Hartwell. Overall, there is a decline in PCB contamination from 1990 to 2014.

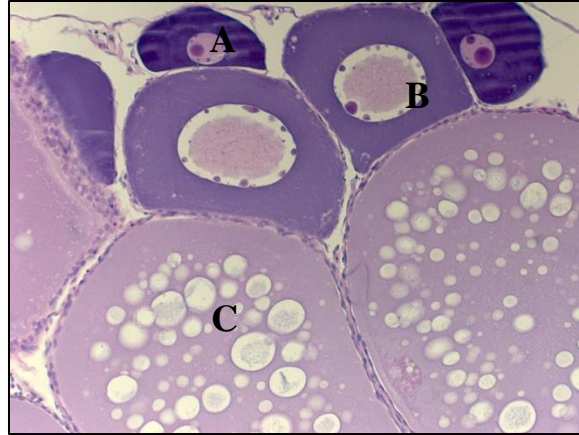


Figure 2.3: Early stages of oocyte development in a cross section of LMB female ovary. A: Stage 1 – immature; previtellogenic stage with nucleolar oocyte with little cytoplasm and centrally located nucleus containing single nucleolus. B: Stage 1 – immature; perinucleolar oocytes with increased cytoplasm, larger nuclei, and multiple nucleoli. C: Stage 2 – early vitellogenic; cortical alveoli begin moving to periphery of oocyte.

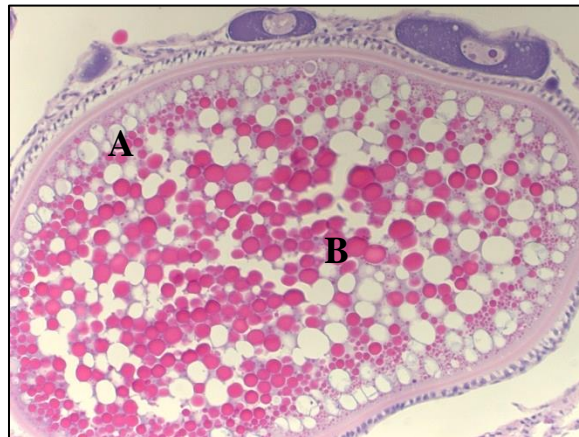


Figure 2.4: Late stages of oocyte development in a cross section of LMB female ovary. A: Stage 3 oocyte – mid-vitellogenic growth with cortical alveoli (A) pushed to periphery of oocyte and most of cytoplasm is filled with yolk globules (B).

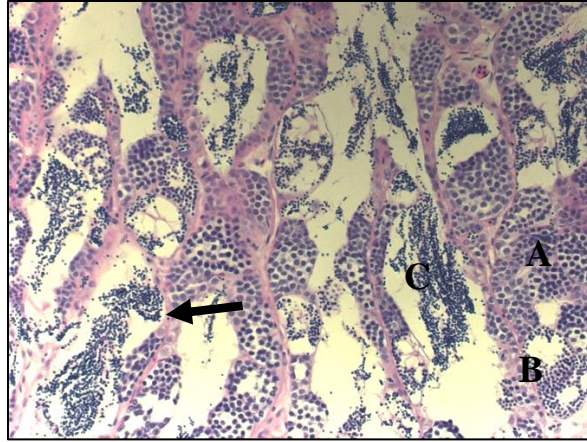


Figure 2.5: Stage 3 testis cross section from male LMB. Early to mid-spermatogenic testes. Contains predominantly spermatocytes (A), spermatids (B), and few spermatozoa (C). Arrow indicates the lobular wall.

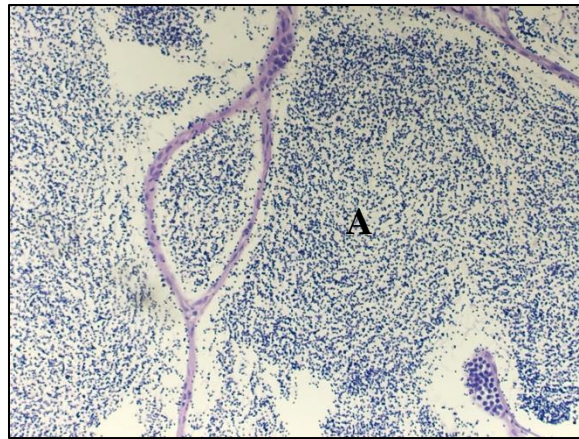


Figure 2.6: Stage 4 testis cross section from male LMB. Late spermatogenic testes containing primarily spermatozoa (A)

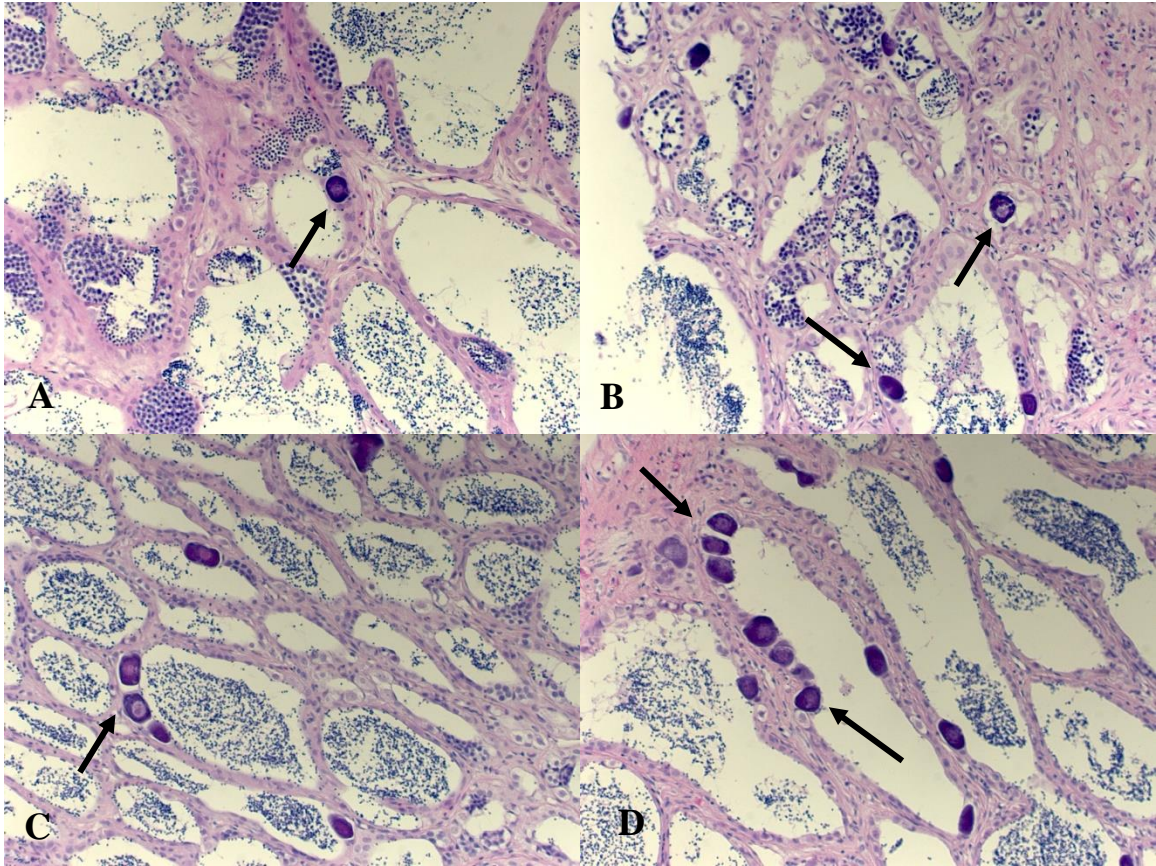


Figure 2.7: Intersex severity scores in LMB testicular tissue. A: Severity score 1 – single oocyte. An individual oocyte (arrow) in the field of view. B: Severity score 2 – multifocal. More than one oocyte per field of view. Oocytes (arrows) are not closely associated. C: Severity score 3 – cluster. Groups (arrow) of 2 – 5 oocytes that are closely associated with each other in the field of view. D: Severity score 4 – zonal. Multiple clusters or more than five closely associated oocytes in the field of view

CHAPTER 3

RESULTS

3.1 MORPHOMETRIC PARAMETERS

Arithmetic means and standard errors were computed for general morphometric parameters. Statistical analysis indicated that fish total weight was significantly different between males and females ($U = 629, p < 0.01$) with females weighing more on average across all three sites. Weight was not significantly different in comparisons of males or females between sites. Fish total length was significantly different in males and females ($U = 599, p < 0.01$), with females measuring greater in length on average. Total length did not differ significantly in comparisons of males between sites or females between sites (Table 3.1).

There was no significant difference in CF between males and females or in males between sites. The CF for males was not significantly different across sites. Female CF was significantly different between sites ($F(2, 24) = 8.21, p < 0.01$) with Lake Hartwell fish (SV-107 and SV-535) having slightly lower CF than Lake Keowee females (SV-311) on average.

A t-test was used to compare \log_{10} -transformed data for intersex and normal male LMB. When comparing intersex and normal male LMB, there was a significant difference in weight ($t(14) = 3.03, p < 0.01$, Figure 3.1) and length ($t(13) = 2.84, p < 0.01$, Figure 3.2). The CF was not significantly different ($U = 162, p = 0.06$, Figure 3.3)

among intersex and normal male fish. In general, intersex fish were smaller and weighed less compared to normal males collected in this study.

3.2 PCB CONCENTRATIONS IN FISH

Individual fish tissue PCB concentrations were not measured in fish collected for this study. PCBs for each site were estimated and calculated by taking the average PCB values in male and female LMB at each site from 2010 to 2014. Data were acquired from yearly monitoring reports completed for Schlumberger Technology Corporation as required by EPA Region IV under CERCLA. Log₁₀-transformed PCBs were significantly different between Lake Hartwell sites SV-107 and SV-535 ($t(105) = 12.02, p < 0.01$) with the greatest contamination at SV-107. Statistics were not performed on PCBs from site SV-311. PCB data for SV-311 were not available for 2010 to 2014. Historically, PCBs have remained below the detection limits in fish tissue and SV-311 has not been sampled for PCBs since 2006, at which time they were below 0.05 ppm. There was no significant difference in PCBs measured from males compared to females ($t(109) = -1.41, p = 0.16$).

3.3 REPRODUCTIVE PARAMETERS

Gonadosomatic index

There was a significant difference ($t(26) = 10.07, p < 0.01$) between male and female GSI overall with females typically having a greater GSI. There was also a significant difference ($F(2, 30) = 3.42, p = 0.04$) in GSI in males between sites. Male GSI was significantly different in SV-107 and SV-535 compared to SV-311 ($p < 0.05$). There was no significant difference in GSI among females between sites ($F(2, 24) = 1.67, p = 0.20$) (Table 3.2).

Vitellogenin levels

There was a significant difference in VTG levels when comparing all males to all females from the three sites ($t(37) = 9.03, p < 0.01$), which would be expected as females naturally produce a greater amount of VTG than males. For males, there was no significant difference in VTG levels between sites ($F(2, 30) = 1.34, p = 0.27$). For females, there was also no significant difference in VTG levels between sites ($F(2, 24) = 2.47, p = 0.10$).

All females collected had measurable levels of circulating VTG. Plasma concentrations ranged from 0.023 to 0.771 mg/ml at all sites. The female with the lowest VTG measured of 0.024 mg/ml was found at SV-535, but was likely sexually immature with a low total body weight and gonad weight of only 1.2 g. At SV-311, the greatest plasma VTG measured was 0.771 mg/ml and lowest plasma VTG measured was 0.052 mg/ml. At SV-535, the highest VTG measured was 0.755 mg/ml and lowest was 0.024 mg/ml. At SV-107, the highest VTG measured was 0.490 mg/ml and the lowest was 0.148 mg/ml.

All males collected also had measurable levels of VTG with plasma concentrations ranging from 0.015 to 0.139 mg/ml across all three sites. Males exceeding 0.10 mg/ml VTG were collected only from SV-107 ($n = 3$). The male fish with the lowest plasma VTG level of 0.015 mg/ml was found at SV-311, and may have been from a sexually immature fish due to low total body weight. From SV-311, the greatest plasma VTG measured was 0.077 mg/ml. At site SV-535, the highest plasma VTG measured was 0.087 mg/ml and the lowest was 0.036 mg/ml. At SV-107, the highest plasma VTG

measured was 0.139 mg/ml and the lowest was 0.024, accounting for the largest range of VTG measurements.

Average VTG in females collected was 0.331 mg/ml and the average VTG in males collected was 0.055 mg/ml and these differences were statistically significant. VTG was nearly six times as high in females compared to males. High levels of VTG were expected in female LMB during the spawning season, from April to June in South Carolina.

Histology results

Intersex male LMB were found at all three sites. A fish was counted as intersex if at least one oocyte was found in testicular tissue when examined microscopically. The prevalence between sites ranged from 85% at SV-107, 64% at SV-535, and 50% at SV-311. Intersex prevalence in fish was not significantly different among sites (Fisher's exact test, $p = 0.28$) There were no intersex females observed in this study.

Using a scoring method similar to Blazer et al. 2007, intersex severity was less than 1 on average at all three sites. The highest average intersex severity score was observed at site SV-535. The lowest average intersex severity score was observed at site SV-311. The most severe ranking assigned to a fish was 2.72 and was from SV-535. Only six fish total out of 33 males examined ranked above 1 for intersex severity and were only collected from Lake Hartwell sites, SV-107 and SV-535. No significant difference was found between sites for intersex severity score ($H = 1.92$, $p = 0.38$). Intersex scores ranged from 0 – 4 in individual cross sections examined in this study.

A total of 348 cross sections of testicular tissue were examined. Of these, 76% received a score of 0 and had no TO. Only 3% of cross sections examined received a

score 4 for intersex severity (Figure 3.4). The reference site, SV-311, had more than 90% of sections score a 0 for intersex severity. The high PCB-contaminated site, SV-107, had the most sections receive a score of 1 and had an even distribution of scores 2, 3, and 4 in remaining sections that scored above 0 and had the least amount (70%) of scores rank 0 (Figure 3.5). The low PCB-contaminated site, SV-535, accounted for the most sections to score 2 and 4 compared to the other sites. Individual cross section intersex prevalence at each site was also analyzed using a Fisher's exact test followed by a pairwise test for independence with Bonferroni correction. Cross section scores of fish testes from SV-107 and SV-535, the Lake Hartwell sites, were significantly different from cross sections scores of SV-311 fish ($p < 0.05$).

Table 3.1: Morphometric and biological parameters for largemouth bass collected in spring 2016.

Site	Date Sampled	Sex	No. fish	Weight (g)	Total length (mm)	Condition factor (CF)	PCB (ppm)
Lake Hartwell - SV107	4/12/2016	M	13	488.0 (258.8) ^a	339.4 (47.4) ^a	1.14 (0.10) ^a	5.56 (4.46) ^a
(Twelvemile Creek)		F	7	1068.4 (623.2) ^b	430.3 (82.0) ^b	1.16 (0.02) ^b	5.57 (4.06) ^a
Lake Hartwell - SV535	4/19/2016	M	14	654.1 (271.0) ^a	371.4 (42.3) ^a	1.20 (0.13) ^a	0.36 (0.21) ^b
(Andersonville Is.)		F	8	1022.6 (577.9) ^b	420.7 (83.9) ^b	1.20 (0.06) ^b	0.39 (0.30) ^b
Lake Keowee - SV311	5/4/2016	M	6	626.2 (410.9) ^a	364.1 (64.7) ^a	1.10 (0.10) ^a	< 0.05 ^c
		F	12	804.0 (421.6) ^b	380.6 (61.1) ^b	1.30 (0.08) ^c	< 0.05 ^c

The above table shows overall health parameters measured in LMB from three sites. Data is presented as averages with standard deviation in parentheses. Condition factor (CF) is calculated as follows: $((\text{body weight} - \text{gonad weight in g}) / \text{length}^3 \text{ in mm}) \times 10^5$. PCBs were averaged from historical data on LMB fillet samples collected for Schlumberger Technology Corporation from 2010 – 2014. Site SV-311 has not had PCB data for LMB collected since 2006 and is therefore assumed to be below laboratory detection levels at < 0.05 ppm. Parameters with the same letter superscript are not significantly different at $p = 0.05$.

Table 3.2: Reproductive parameters for largemouth bass collected in spring 2016.

Site	Sex	No. fish	GSI	VTG (mg/ml)	Intersex severity ¹	Intersex prevalence
Lake Hartwell - SV107	M	13	0.49 (0.20) ^a	0.06 (0.03) ^a	0.57 (0.59)	85%
(Twelvemile Creek)	F	7	2.87 (1.00) ^b	0.32 (0.12) ^b		
Lake Hartwell - SV535	M	14	0.44 (0.10) ^a	0.05 (0.01) ^a	0.99 (0.90)	64%
(Andersonville Is.)	F	8	2.80 (1.48) ^b	0.25 (0.23) ^b		
Lake Keowee - SV311	M	6	0.29 (0.12) ^c	0.04 (0.02) ^a	0.26 (0.07)	50%
	F	12	3.84 (1.41) ^b	0.39 (0.18) ^b		

¹ intersex severity was an average of males with TO. Males that were not intersex were excluded from the average calculation

The above chart displays average values for reproductive biomarkers measured in fish from the three sites. GSI was calculated as: (gonad weight / (total body weight-gonad weight)) × 100. Statistical comparisons were made between fish of the same sex as well as between males and females. Parameters with the same letter superscript are not significantly different at p = 0.05. If no significant differences were observed, annotation was omitted.

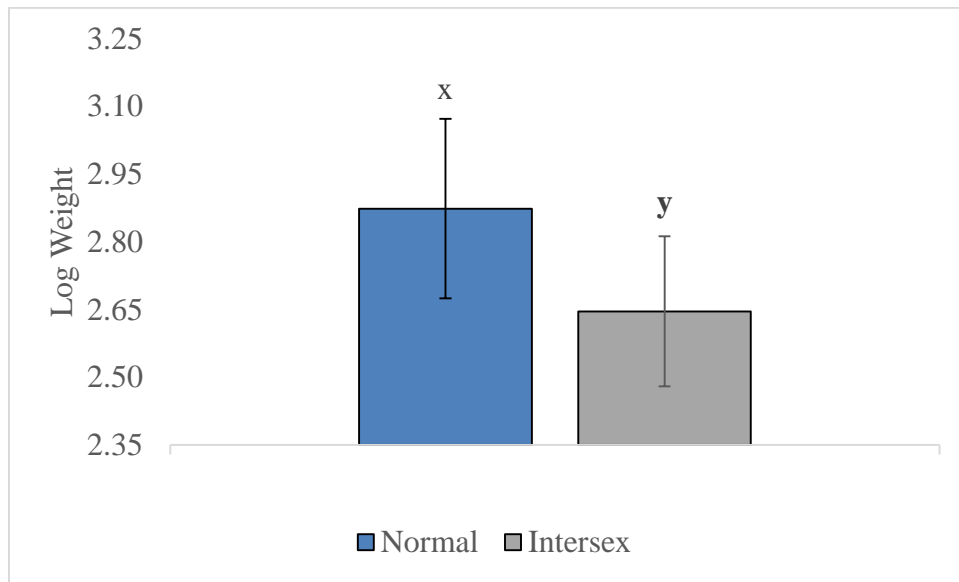


Figure 3.1: Mean weight and standard deviation in normal and intersex male largemouth bass. Weight was significantly different ($p < 0.01$) between normal and intersex bass with intersex bass weighing less on average.

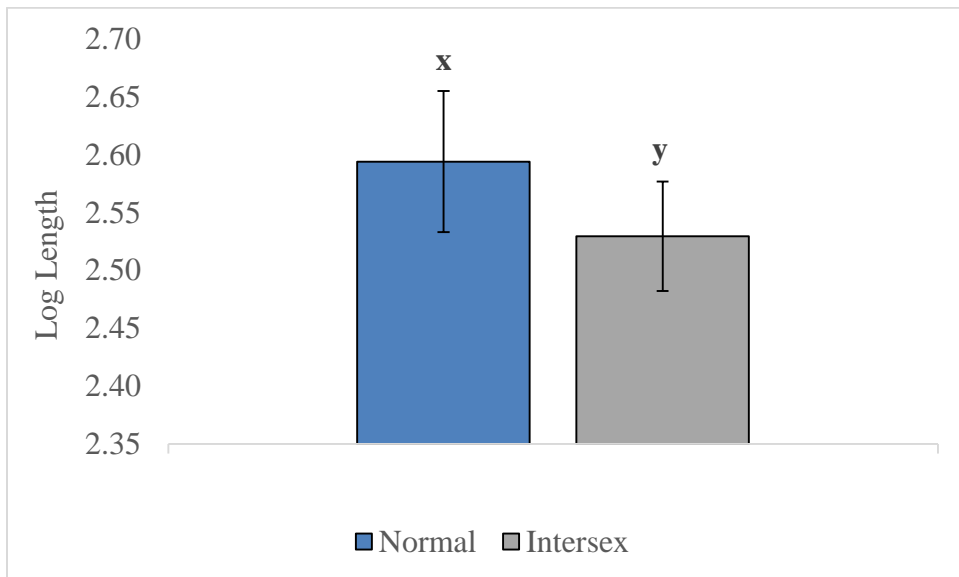


Figure 3.2: Mean length and standard deviation in normal and intersex male largemouth bass. Length was significantly different ($p < 0.01$) between normal and intersex bass with intersex bass being smaller on average.

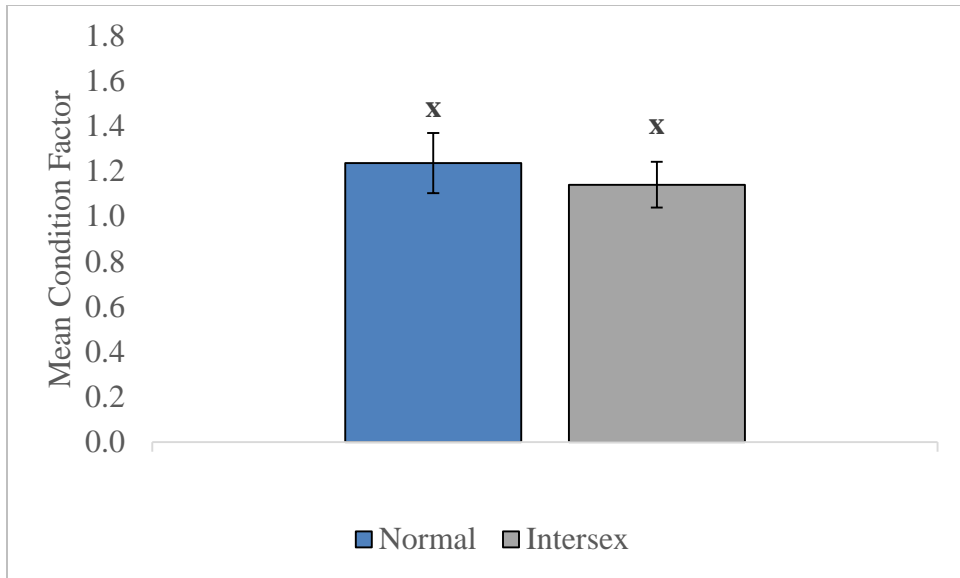


Figure 3.3: Mean condition factor and standard deviation in normal and intersex male largemouth bass. Condition factor was not significantly different between normal and intersex bass.

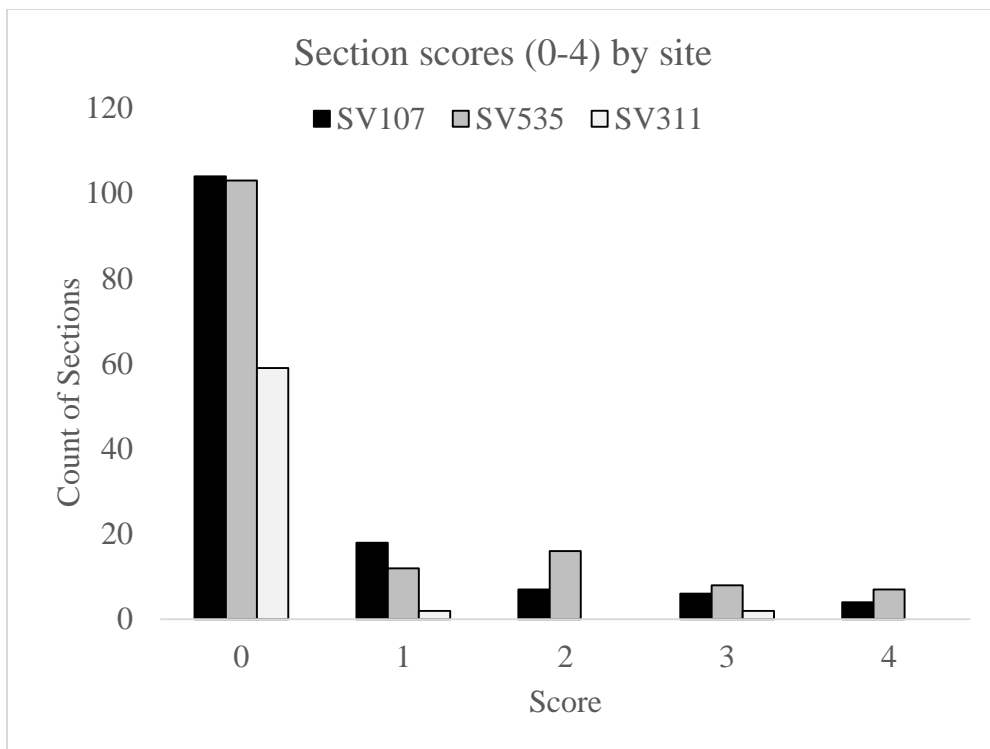


Figure 3.4: Distribution of intersex severity scores, 0 – 4, from all cross sections of LMB testicular tissue examined for each sampling site.

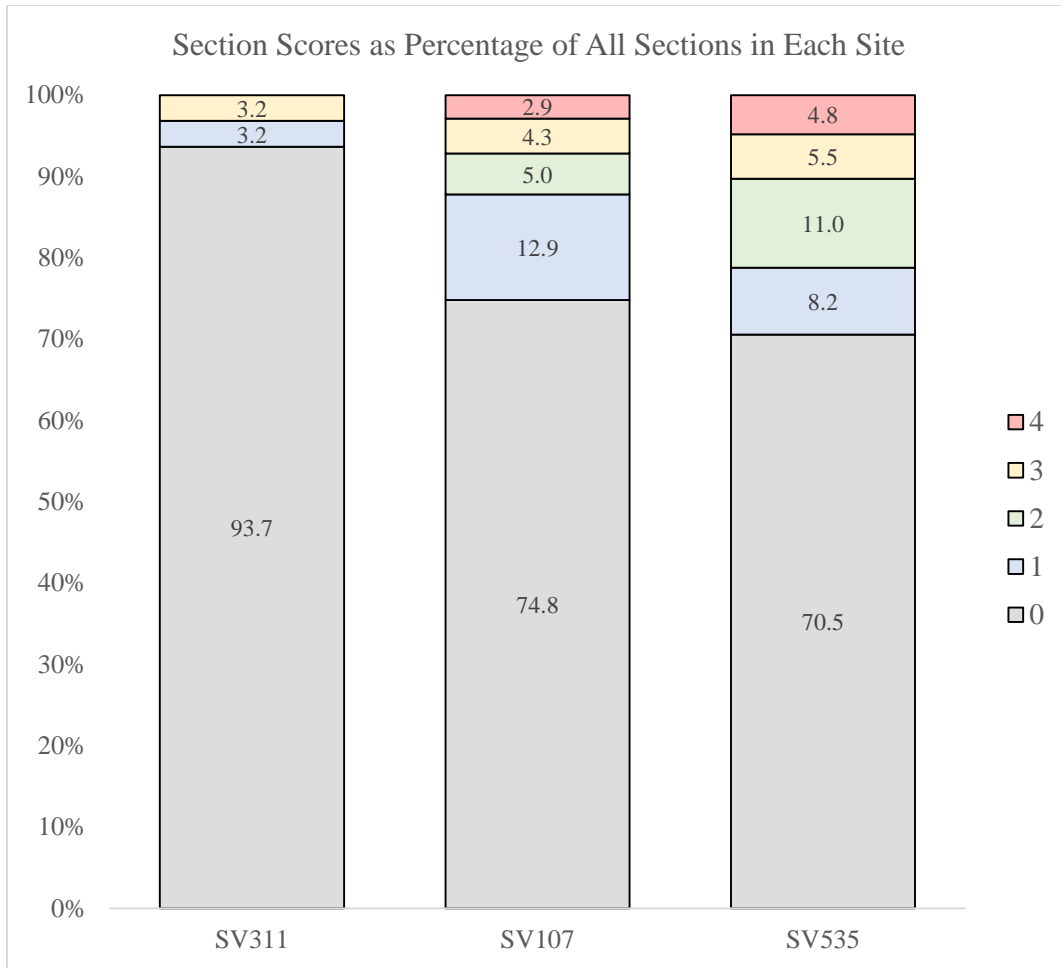


Figure 3.5: Percentage of intersex severity scores 0 – 4 from each site in all cross sections examined of male LMB testicular tissue. The similarity in patterns of severity score distribution at SV-107 and SV-535 show a distinct response to PCB exposure, whereas results from the reference site at Lake Keowee is different.

CHAPTER 4 DISCUSSION

4.1 OVERALL HEALTH

Fish collected (n = 60) appeared overall healthy in external appearance with no apparent physical abnormalities or deformities using conventional morphometric measurements. For each sex, fish total weight and total length did not vary significantly between sites. Females were typically larger than males at each site, which is common across many fish species (Parker, 1992).

Condition factor (CF) is typically used to express the degree of well-being of a fish and variations in a fish's CF reflect their state of sexual maturity and degree of nourishment. (Williams, 2000). There was no overall significant difference in CF between males and females or in males between sites. Female CF was significantly different between sites with Lake Hartwell fish (SV-107 and SV-535) having slightly lower CF than Lake Keowee females (SV-311) on average. It is possible that Lake Hartwell females had lower CF due in part to chronic exposure to PCBs or variation in spawning times between the two lakes.

Gonadosomatic index (GSI) relates the proportional size of the gonad to body mass and may reflect changes resulting from a variety of physiological factors such as reproductive stage and environmental stressors, including exposure to contaminants (Schmitt et al., 2004). Gonad weight increases during the spawning season and therefore GSI begins to increase due to gamete proliferation and maturation, peaking immediately

prior to release of eggs and sperm. Overall, there was a significant difference between male and female GSI overall with females typically having a greater GSI. The female ovary with developed eggs account for the greater GSI values typically seen in females as they contribute more to weight to total gonad weight in females used in calculating the GSI. There was no significant difference in GSI among females between sites. Male GSI was significantly different between SV-107 and SV-311 with fish from SV-311 having slightly lower GSI values on average which may be a reflection of the slight differences in spawning times between Lake Hartwell and Lake Keowee fish or the possibility of collecting more sexually immature fish at SV-311. Another possibility for the variation in male GSI may be due to the presence of intersex (testicular oocytes) which may have contributed in part to the increased the weight of gonads at SV-107 (Blazer et al., 2012).

4.2 VITELLOGENIN

Vitellogenin has been utilized as an effective biomarker in many studies on endocrine disruption in fish. In normal mature females, estradiol is synthesized primarily in the ovary and stimulates hepatic synthesis of vitellogenin. Male fish do not naturally produce VTG but possess the gene for production of VTG in their liver. The gene can be expressed, however, when male fish are exposed to exogenous or endogenous estrogens or mimics. Exposure to estrogenic compounds has been found to induce measurable levels of VTG in male fish. For PCBs, the variability in chemical structure and existence of many congeners allows for multiple potential modes of action in endocrine disruption. The various structures may be linked to receptor-mediated and non-receptor-mediated responses. While it is unclear what specific mode of action causes endocrine disruption from PCBs in fish, possible mechanisms include: acting as a mimic of sex steroid

hormones by binding to hormone receptors, blocking or preventing hormonal binding to hormone receptors, altering the production and breakdown of natural hormones, or modifying levels and functioning of hormone receptors (Matozzo et al., 2008). PCBs are likely to act as a mimic of estradiol because they have similar structure and therefore may stimulate VTG production in the liver by binding to receptors that stimulate the VTG gene.

It is believed that induction of VTG is indicative of relatively recent exposure to endocrine disrupting compounds in adult male fishes because VTG levels are regulated by synthesis and elimination rates within the body (Blazer et al., 2012). Levels of VTG vary throughout the spawning season. In females, plasma VTG decreases to baseline levels after spawning in the summer and then increase from the fall through spring spawning (Rosenblum et al., 1994). In terms of chemical exposures resulting in the induction of VTG, the duration of exposure and chemical concentration may be an important factor in the amount of VTG measured. One study found that sheepshead minnows exposed to low and high doses of estradiol (0.1 and 1.0 $\mu\text{g/l}$ respectively) induced different plasma VTG concentrations, with the higher dose yielding higher VTG production; however, both exposed groups took over 100 days to return to basal levels (Hemmer et al., 2002). Another study found that time post-exposure was also important in VTG production. After a 9-day exposure to poultry-litter derived estrogenicity, only 50% of male fathead minnows had elevated plasma VTG levels compared to 21 days later when 100% of the minnows had significantly elevated levels of VTG (Yonkos et al., 2010). High concentrations of estrogenic chemicals or prolonged exposures may result in detectable VTG measurements for months after exposure.

Overall plasma VTG levels were significantly different between males and females from all sites. This was not surprising due to normal female reproductive development typically yielding much higher VTG levels when compared to males. It is still interesting to note that all the males sampled had measurable amounts of plasma VTG (> 0.001 mg/ml) compared to other studies (Baldigo et al., 2006; Blazer et al., 2012) that found non-detectable levels of VTG in some of the male LMB collected. There was no significant difference in plasma VTG levels from intersex males and normal males (Figure 4.1). The process of forming TO and inducing intersex may have been a result of chronic exposure to EDCs or exposure at early life stages in young, immature fish. If young fish have less T and 11-KT, they could potentially have less VTG production since E2 wouldn't be converted as easily compared to older fish with more T. This hypothesis that these fish were exposed to EDCs at a young age could explain in part why there is no significant difference in plasma VTG levels in the observed intersex and normal bass from this study.

There was no significant difference in VTG levels among males or among females (Figure 4.2) from different sites. Increased VTG production in male fish from SV-107 or SV-535 compared to SV-311 would be expected if exposure to PCBs resulted in an estrogenic effect in fish. PCBs in the Twelvemile Creek system have been declining since remediation and cleanup began in the 1990's; however, declines in fish tissue PCBs have not been observed until recently (SC DHEC 2016). It is possible that the current low residual PCB contamination in Lake Hartwell does not impact current VTG production in these fish. It is also likely that the elevated VTG observed in male fish from this study may be due to contaminants other than PCBs. Potential sources may include wastewater

treatment plant effluent, pesticides from urban areas (Stone et al., 2014), or agricultural production nearby in the watershed drainage. It is currently unknown if there are potential background levels of VTG in male LMB and low levels of circulating VTG may naturally occur in male LMB.

4.3 INTERSEX

Intersex is another biomarker commonly used in endocrine disruption studies. Testicular oocytes (TO) was the type of intersex observed in LMB in this study. While testicular oocytes have been induced in experimental studies in some species of fish (Papoulias et al., 2000; Hayes et al., 2011), the presence of TO may be more indicative of early life stage exposure (Blazer et al., 2012). Larval stages are the most sensitive period of exposure for induction of TO in multiple fish species but the reproductive period of gonadal recrudescence in mature adults is also sensitive to endocrine disrupting chemicals (Ankley & Johnson, 2004). It is believed that TO prevalence may vary seasonally and differences in TO prevalence have been observed in male SMB between spring, pre-spawn sampling and summer, post-spawn sampling (Blazer et al., 2007). The higher prevalence observed in the spring versus summer in male SMB collected suggests that some TO are shed during spawning with the expulsion of sperm and may not hinder reproduction by their simple presence. However, literature suggests that individuals with higher severity of TO are more likely to suffer adverse reproductive effects than individuals with low TO severity. (Gronen et al., 1999; Blazer et al., 2012; Vandenberg et al., 2012)

Intersex male largemouth bass were found at all three sites. The prevalence of intersex in male LMB between sites ranged from 85% at SV-107, 64% at SV-535, and

50% at SV-311. There was no significant difference in intersex prevalence between sites. However, it is interesting to note that there was a similar pattern of intersex severity found in Lake Hartwell sites versus Lake Keowee and there was a general decreasing gradient of intersex prevalence as site contamination from PCBs decreases (Figure 4.3).

Individual cross sections from male LMB were analyzed and counted as “intersex” or “normal” and there was a significant difference in cross section TO prevalence between sites indicating that SV-107 and SV-535 cross section TO prevalence was significantly different from SV-311 cross section TO prevalence. Intersex prevalence may be greater at SV-107 due to historically high levels of PCBs compared to SV-311 where there are non-detectable levels of PCBs. If fish were exposed in early life stages to high PCB levels at SV-107, TO may manifest in the adult males.

While there were still TO observed in 50% of the males from SV-311, the sample size was small (n=6) and only 6% of testicular tissue cross sections examined from this site contained any TO compared to the higher prevalence of TO observed in cross sections from SV-107 (25%) and SV-535 (29%). Previous studies have shown TO prevalence correlates with percent of agricultural land use in the catchment above the collection site (Blazer et al., 2014) as well as number of animals in animal feeding operations in the catchment. It is possible fish in this study were exposed to EDCs in runoff or leachate from application of manure from animal feeding operations as there is a high amount of poultry production in this area of South Carolina and Georgia. This could explain the higher TO prevalence observed at Lake Hartwell sites (SV-107 and SV-535) as Lake Hartwell receives water from SC and GA agricultural areas compared to Lake Keowee which is entirely in SC and dominated by forested land. In addition,

urbanization may also be a significant source of contaminant exposure as Stone et al. (2014) found that > 90% of all urban sites had exceedances of aquatic life criteria compared to only 60% in agricultural areas. It is also possible the combination of poultry production and urbanization may be cumulative and additive. Additional research is needed to better clarify this.

Intersex severity was generally low (< 1), with no fish exceeding an overall score greater than 2.75 on a scale of 0 to 4. This is consistent with severity scores calculated from previous studies that utilized the Blazer ranking method (Ingram et al., 2011; Blazer et al., 2012; Fritts et al., 2016). The severity scores were not significantly different between the three sites. Although not statistically significantly different, the average severity was highest at SV-535 and lowest at SV-311. Site SV-535 encompasses a larger watershed drainage area compared to SV-311 and intersex severity may be greater in these fish due to potential exposure from a wider variety of EDCs in the watershed drainage including: wastewater effluent, septic discharge, poultry production by-products, and legacy PCBs (Figure 4.4). When looking at individual gonadal cross sections for males, a total of 348 cross sections were examined resulting in about 10 sections for each fish from each site. Out of all cross sections examined, 76% received a score of 0 and had no TO. Only 3% of cross sections examined received a score 4 for intersex severity with the majority in fish from SV-535. We found that most of the more severe scores (≥ 2) were in cross sections of fish from SV-535. The reference site (SV-311) had no cross sections with scores of 2 or scores of 4. At the Lake Hartwell sites (SV-107 and SV-535), there was a fairly even distribution of scores 1 through 4 but the majority ($> 70\%$) of sections were scored 0.

Although TO severity was measured in this study, it is difficult to report severity scores that can be used for comparison across studies. Ranking scores are interpreted by individuals who may have different opinions on the severity of each cross section. There are a variety of ranks used in assessing intersex; for example, a severity index for European flounder (*Platichthys flesus*) utilizes the distribution pattern and type of oocyte in assigning a score (Bateman et al., 2004) but a ranking system from 0 – 7 was used to score intersex in a study on roach (*Rutilus rutilus*) exposed to sewage effluent in British rivers (Jobling et al., 1998). Other studies have used testicular oocyte counts as a measure of severity (Kellock et al., 2014; Fritts et al., 2016). In Georgia, an investigation on intersex in shoal bass (*Micropterus cataractae*) suggested that intersex is a natural occurrence in this species due to overall low intersex severity observed (< 1.20 using Blazer method) from sites along the Flint River (Ingram et al., 2011). There is a significant limitation in comparing TO severity results across studies due to the issues of individual interpretation of scores, the various ways that severity scores can be calculated, and the differences in species. Future studies aimed at standardizing intersex severity ranking systems for individual species would be useful for comparing studies among geographic areas.

4.4 PCBS AS ENDOCRINE DISRUPTORS

There was a significant positive correlation between historical PCBs and VTG, intersex severity, and intersex prevalence in male LMB (Table 4.1). Urban sources are potential sources of EDCs to these sites. Assuming that an estrogenic response would manifest in fish from areas with high PCBs, SV-107 (with average PCBs in LMB fish tissue samples ranging from 16.90 ppm in 1990 to 2.08 ppm in 2014), would be expected

to yield male LMB with significantly higher plasma VTG, greater severity of TO, and/or higher prevalence of intersex compared to sites with low (< 1 ppm) and no contamination from PCBs. Although not statistically significant, vitellogenin was positively correlated with PCBs at the 80th percentile of measurements (Figure 4.5), indicating that high levels of VTG in fish may be positively correlated with PCB values. There was no significant difference in intersex prevalence or TO severity among the sites unless specifically looking at individual cross section intersex prevalence which yielded a significant difference between Lake Hartwell sites (SV-107, SV-535) and the Lake Keowee site (SV-311). Although a positive correlation between PCBs and reproductive parameters measured was observed, it is not sufficient evidence to conclude that PCBs *per se* were the single causative factor for observing elevated VTG and intersex in males as there were no significant differences among sites for the reproductive biomarkers measured in male fish, with the exception of the increased GSI in males at Lake Hartwell sites SV-107 and SV-535 which was significantly different than males from Lake Keowee.

Data was also analyzed by combining intersex prevalence documented from Hinck et al. (2009) with data from the current study. Sites from Hinck et al. (2009) sampled included three sites in the Savannah River Basin (SRB) downstream of Lake Hartwell and three sites within the Great Pee Dee River Basin (PDRB) (Figure 4.6). Intersex prevalence documented in LMB from the SRB was remarkably similar to prevalence observed in the reference site, SV-311 on Lake Keowee. Sites sampled from the PDRB showed LMB with intersex prevalence rates similar to those collected from Lake Hartwell (SV-107 and SV-535). Intersex prevalence in the three PDRB sites were ranked 1st, 2nd, and 3rd highest out of 52 sites across the US sampled for LMB.

Polychlorinated biphenyls have been found in sediment and fish tissue from the Great Pee Dee River. The SC DHEC and North Carolina Department of Health and Human Services (DHHS) have issued consumption advisories for fish due to elevated PCBs in the Great Pee Dee River and reservoirs in the PDRB. When intersex prevalence data from both Hinck et al. (2009) and the current study were combined based on PCB-contamination or lack of, there was a significant difference in intersex prevalence of fish from PCB-contaminated sites compared to intersex prevalence of fish from “reference” sites (Figure 4.7) ($\chi^2(1, N = 78) = 7.433, p < 0.05$). This indicates that background rates of intersex fish may be widespread or common but intersex has been observed at a greater frequency from sites with PCB-contamination.

Measurable levels of VTG (> 0.005 mg/ml) and some TO were found in males from all sites sampled in this study. Other xenoestrogens may be present in the Savannah River basin and may be impacting the endocrine system of these fish. Agricultural, pharmaceutical, and industrial sectors can produce estrogenic compounds in their daily operation. Vitellogenin levels were greatest at SV-107, where there was also the greatest percentage of urban land use among the sites (Figure 4.8) and high levels of agriculture.

Hinck et al. (2009) found what may be an unusually high prevalence of TO in LMB from the Pee Dee, Savannah, and Apalachicola River basins where row crops and animal agriculture dominate land use. As previously stated, the PDRB sites sampled in their study ranked 1st through 3rd highest among 52 sites sampled across the US. The SRB sites sampled ranked 5th, 6th, and 7th highest, indicating there is still potential endocrine disruption occurring in non-PCB contaminated sites.

The agricultural land use in areas draining to the sites in this study is made up of 27% agriculture at SV-107, 44% at SV-535, and 23% at SV-311. A similar study addressing TO in LMB from the Delmarva Peninsula found high TO prevalence in areas with high agricultural activity from poultry production and disposal of poultry waste via agricultural application as organic fertilizer (Yonkos et al., 2014). While poultry manure is only the animal's excreta, poultry litter encompasses a mixture of manure, bedding material, feathers, and bird carcasses. Poultry litter is typically used as fertilizer for agricultural fields. Potential contaminants of concern in litter include: feed additives (metals, antibiotics); bedding impurities (pesticides); and fecal sex steroids (estrogenic and androgenic hormones) (Yonkos et al., 2010). Lab studies have shown exposure of fathead minnows to poultry litter runoff extracts induces vitellogenesis in male fish (Yonkos et al., 2010). It is possible fish from the Savannah River Basin are being exposed to similar contaminants as Georgia is the leader in the US for broiler production and there is a high concentration of poultry farms, specifically broilers, nearby the Savannah River Basin (Figure 4.9).

While there were significant differences in weight and length between intersex and normal male LMB, PCBs may not be the single causative factor for these observations. There may be a variety of other contaminants that these fish were exposed to which could result in these findings. One hypothesis to help explain the difference in size may be that the intersex fish are younger or immature fish, and have not been through enough reproductive cycles to fully shed TO if they were present. Hinck et al. (2009) found the highest percentage of intersex LMB collected were young, between 1-3 years old from the nine river basins sampled in the US. Reproductively immature fish are

typically more sensitive in their early life stages to EDCs, and this may be the case for fish in this study as all of the fish had elevated levels of VTG indicating recent exposure to EDCs and only some of the fish had TO, which would typically take longer to manifest.

The data collected in this study show evidence of endocrine disruption in largemouth bass collected from Lake Hartwell and Lake Keowee and significant correlations between endocrine disruption biomarkers and PCBs were observed, even with limited data. Intersex prevalence rates from Lake Hartwell sites are remarkably similar to intersex prevalence rates in LMB from the Great Pee Dee River, which also contains PCBs (Hinck et al., 2009; SC DHEC, 2016). Intersex prevalence rates from Lake Keowee were similar to intersex prevalence rates in LMB from other sites downstream in the Savannah River and were generally low ($\leq 50\%$) (Hinck et al., 2009). Other possible EDCs in the area contributing to the elevated levels of VTG in males and the presence of TO are likely from agricultural sources from poultry production. Future studies that measure potential EDCs in the water and sediment of Lake Hartwell and Lake Keowee would be useful in identifying potential contaminants leading to VTG production and intersex in male LMB from these lakes. Individual fish tissue analysis for contaminants such as PCBs, DDT, and mercury would also be beneficial for more accurately correlating reproductive biomarkers to tissue contaminants. There are still many unknown factors associated with endocrine disruption in fish, especially surrounding specific modes of action which cause endocrine disruption and quantifying the background levels of VTG or intersex in males, if they exist. This study adds valuable

knowledge on the occurrence of intersex in largemouth bass in the Southeast and is one of few studies to look at intersex in large reservoirs.

Table 4.1: Spearman's ρ and p values from Spearman's rank correlation results for vitellogenin, intersex severity score, and intersex prevalence compared to historical PCB values from sampling locations.

	VTG		Severity		Intersex Prevalence	
	ρ	p	ρ	p	ρ	p
PCBs	0.71	0.03	0.72	0.02	0.79	0.01

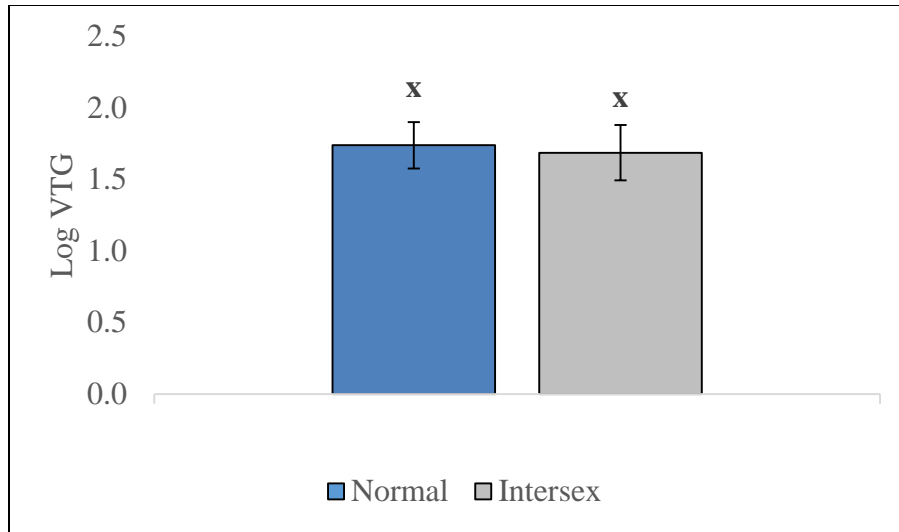


Figure 4.1: Mean log-VTG and standard deviation in normal and intersex male largemouth bass. VTG was not significantly different between normal and intersex bass collected.

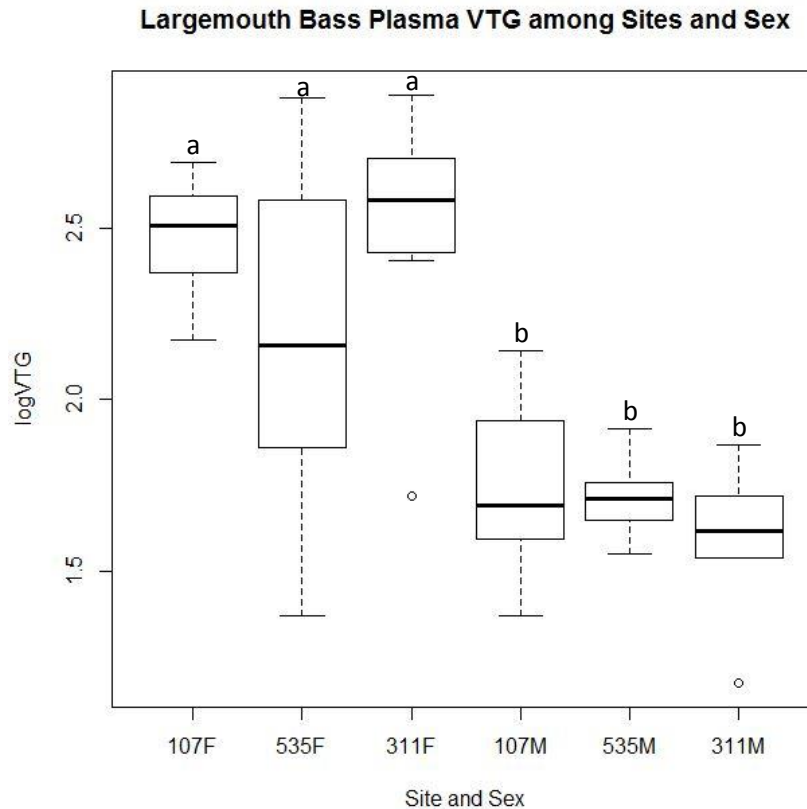


Figure 4.2: Box and whisker plot of LMB plasma log VTG concentrations. The box represents the 25th – 75th percentile, the whiskers represent the 10th and 90th percentiles, and the horizontal line within the box represents the median. Outliers are shown as points. Sites are labeled with location and sex.

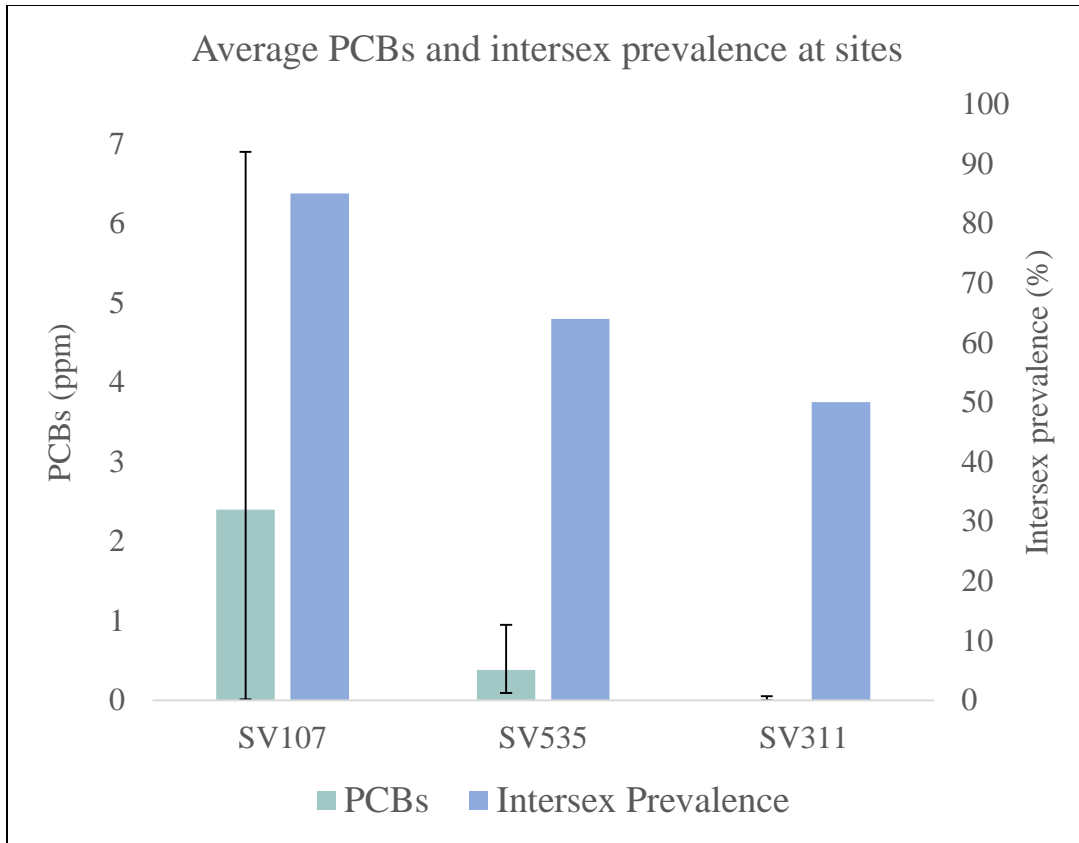


Figure 4.3: Average PCBs in LMB fillets and intersex prevalence at three sampling sites. Bars represent the average values for PCBs and the whiskers represent the maximum and minimum values. Bars for intersex prevalence are shown as percentage of fish. PCB values for SV-311 are < 0.05 ppm.

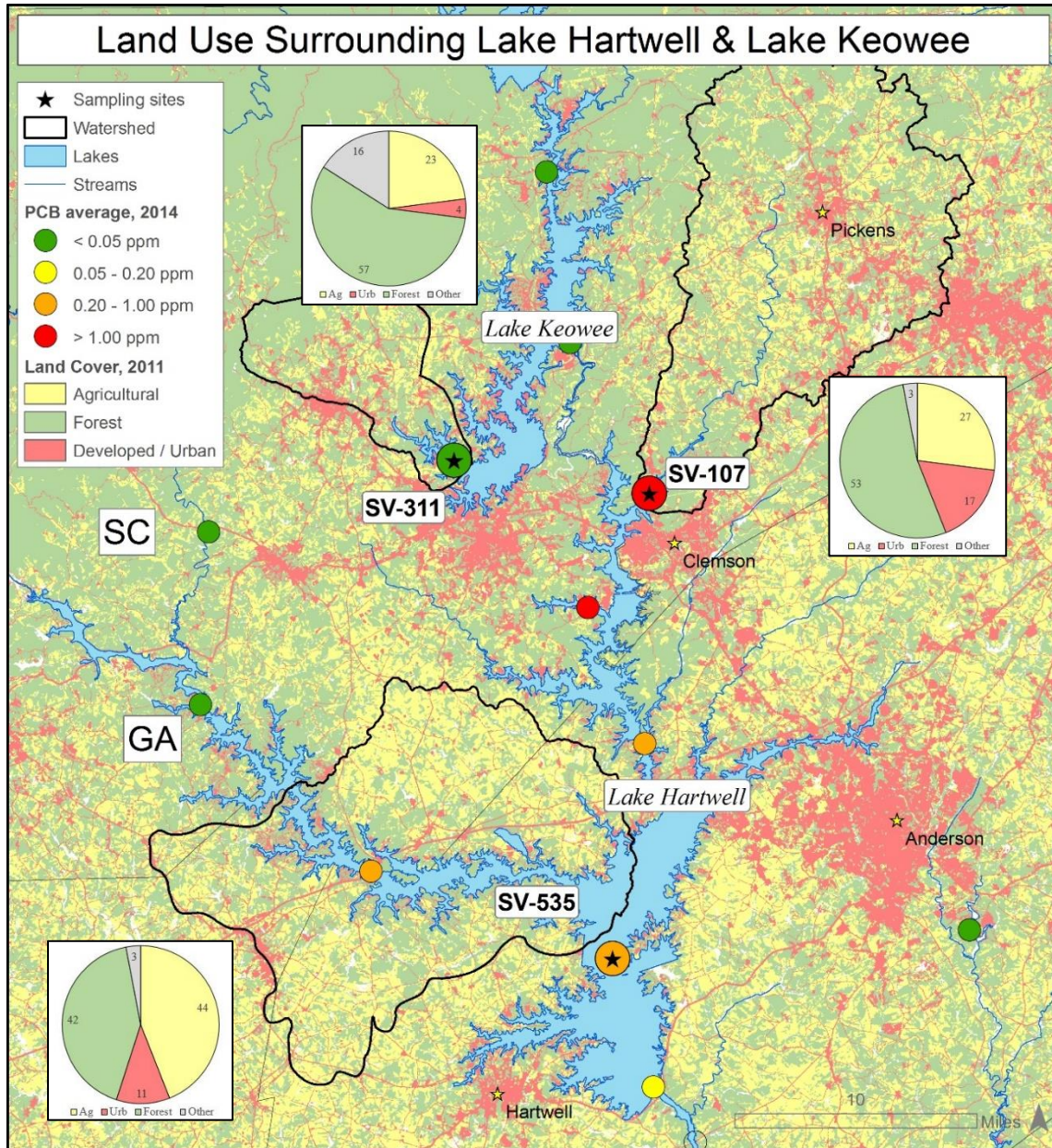


Figure 4.4: Land use in surrounding areas near Lake Hartwell and Lake Keowee in South Carolina and Georgia, USA. Fish tissue monitoring sites are represented by the circles and average PCB values for 2014 are displayed. Sites with a star represent sites sampled for LMB for this study. Land cover was retrieved from the National Land Cover Database (NLCD) 2011.

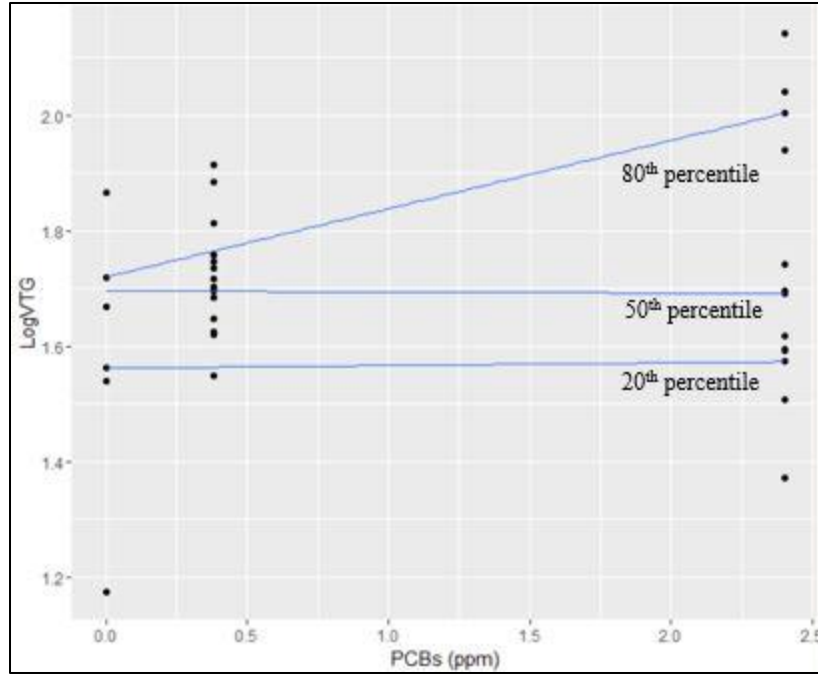


Figure 4.5: Quantile regression analysis of PCBs and Log-VTG in largemouth bass from Lake Hartwell and Lake Keowee sites. The 80th, 50th, and 20th percentiles are indicated by the lines.

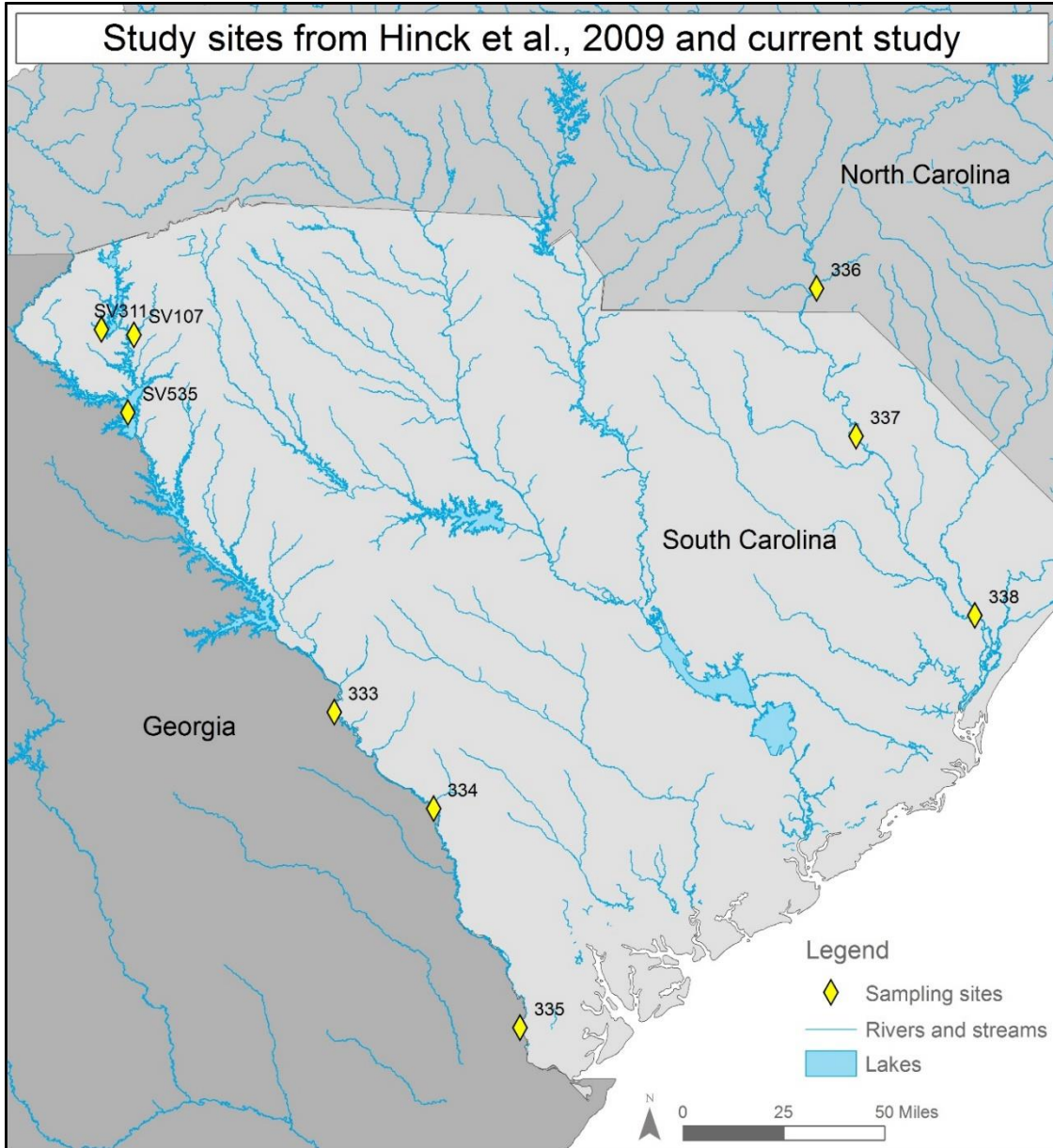


Figure 4.6: Sampling locations from USGS widespread intersex in black bass study (Hinck et al., 2009) and current study. Sampling locations in the Savannah River Basin and Great Pee Dee River Basin are displayed.

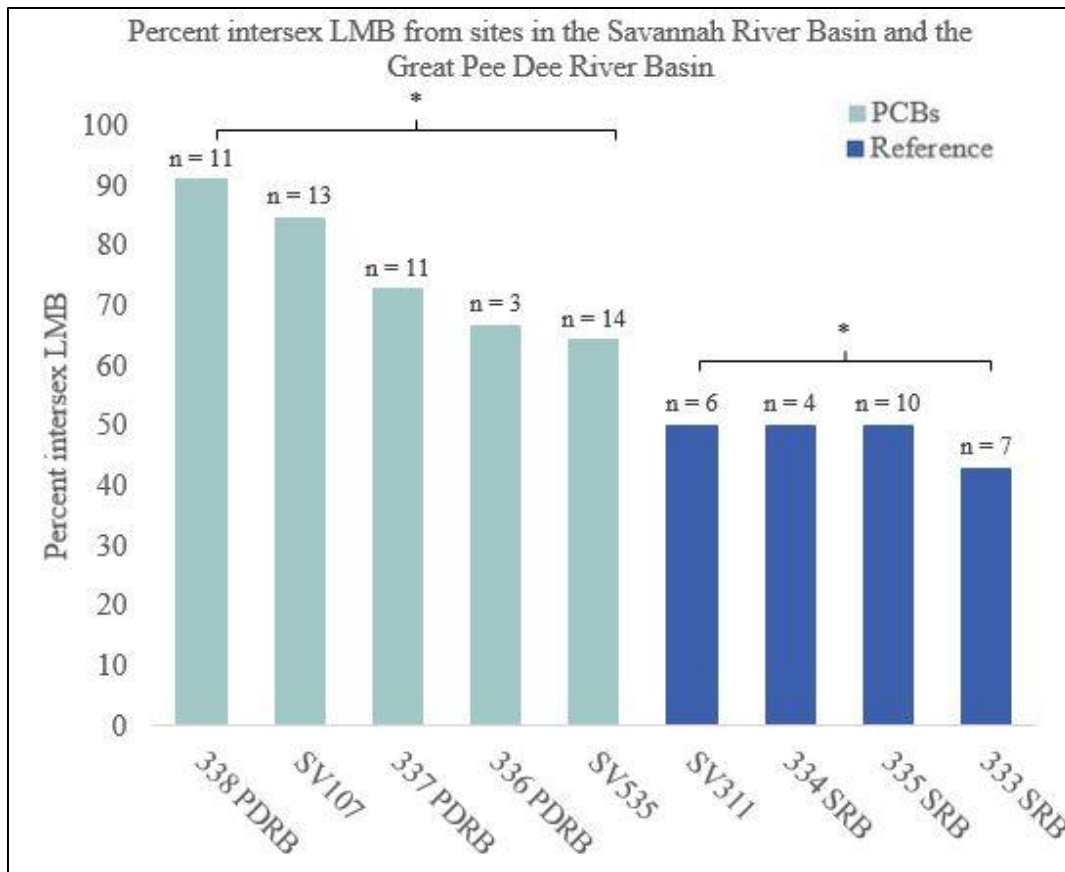


Figure 4.7: Percentage of intersex male LMB collected from sites with PCBs in fish tissue and sites with no PCBs. This data combines intersex prevalence rates from Hinck et al. (2009) and the current study.



Figure 4.8: Percentage of land use types comprising the average male VTG ($\mu\text{g/ml}$), average intersex severity score, and intersex prevalence at each site. The figure above shows the distribution of land use types (agricultural, urban / developed, and forest) at each site as a percentage of all land use in the watershed drainage for each site.

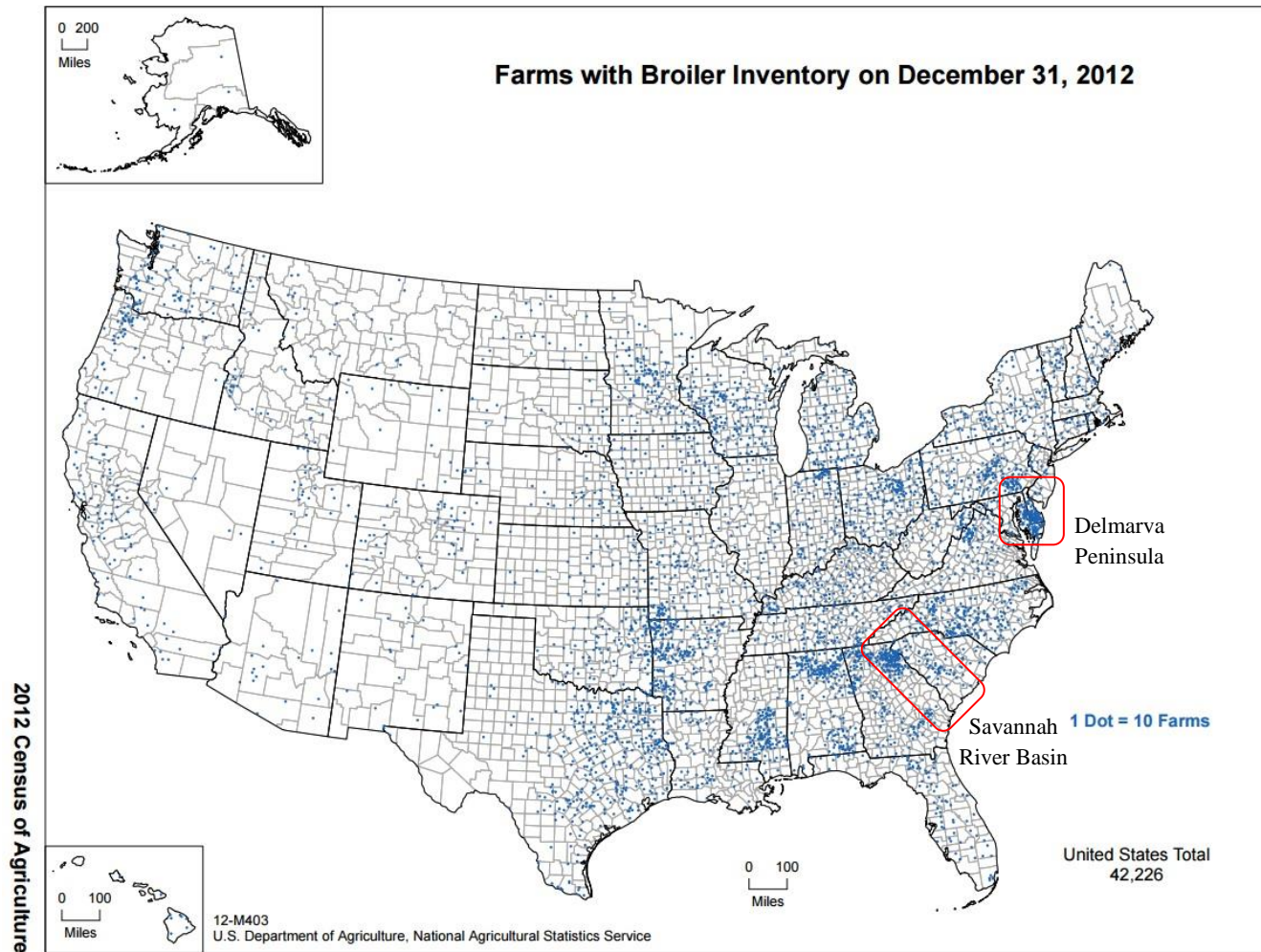


Figure 4.9: USDA 2012 Census of Agriculture map of farms with broiler inventory for United States. The map above shows a dot-density map of broiler farms in the United States. One dot is equal to 10 farms. There is a high concentration of broiler farms in the upper northeast corner of Georgia, which borders the Savannah River Basin. The other area with high density of broiler farms is in the Delmarva Peninsula, which has already seen evidence of endocrine disruption in fish from this area (Yonkos et al., 2010).

CHAPTER 5 CONCLUSIONS

Results of this study indicated that endocrine disruption in fish was measured in largemouth bass in Lake Hartwell and Lake Keowee in South Carolina as measured by plasma vitellogenin (VTG), intersex severity, and intersex prevalence. Findings indicated:

(1) Measurable levels of plasma VTG, an egg yolk precursor protein, were detected in male fish from all sites.

(2) Intersex was measured in male fish but there were no cases of intersex observed in females collected for this study.

(3) Intersex severity scores ranged from 0 to 2.72 on a scale from 0 to 4, with 4 as the most severe score for intersex. Average intersex severity scores were higher in males from PCB-contaminated sites (0.57 at SV-107 and 0.99 at SV-535) compared to the reference site (0.26 at SV-311).

(4) Intersex in males manifested as testicular oocytes (TO). Histological analysis indicated that testicular oocytes were present in 23 of 33 (69%) of male largemouth bass sampled and was found in fish from all three sites. Sites with PCB-contamination had intersex prevalence rates of 85% (SV-107) and 64% (SV-535) in male fish. The reference site had lower intersex prevalence in males compared to the PCB-contaminated sites and intersex prevalence was 50% (SV-311).

(5) Statistical analysis of these data suggest there may be a significant association between PCB concentration in fish tissue and endocrine disruption in the form of VTG production, intersex severity, and intersex prevalence in male largemouth bass in this region of South Carolina.

(6) Additional studies on detailed land use in the surrounding area and identification of other endocrine disrupting compounds potentially impacting these fish will better characterize the overall exposure to endocrine disrupting contaminants of fish from Lake Hartwell and Lake Keowee and provided further explanation for the observed endocrine disruption in male largemouth bass from these lakes.

REFERENCES

- Agency for Toxic Substances and Disease Registry (ATSDR). (2000). *Toxicological Profile for Polychlorinated Biphenyls (PCBs)*.
- Ahlborg, U.G., Becking, G.C., Birnbaum, L.S., Brouwer, A., Derks, H.J.G.M, ... Liem, A.K. (1994). Toxic equivalency factors for dioxin-like PCBs: Report on WHO-ECEH and IPCS consultation, December 1993. *Chemosphere*, 28(6), 1049-1067.
- Aldridge, E.C. III. *Pesticide and PCB Levels in Fish Tissue Collected from South Carolina Waters, 1974 – 1976*. SC DHEC, Biological Monitoring Section.
- Allen, J.R. and Barsotti, D.A. (1976). Effects of transplacental and mammary movement of PCBs on infant Rhesus-monkeys. *Toxicology*, 6(3), 331-340.
- Anderson, M.J., Cacula, D., Beltman, D., Teh, S.J., Okihiro, M.S., Hinton, D.E., ... Zelikoff, J.T. (2003). Biochemical and toxicopathic biomarkers assessed in smallmouth bass recovered from a polychlorinated biphenyl-contaminated river, *Biomarkers*, 8(5), 371-393.
- Ankley, G.T. and Johnson, R.D. (2004). Small fish models for identifying and assessing the effects of endocrine-disrupting chemicals. *ILAR Journal*, 45(4), 469-483.
- Baldigo, B.P., Sloan, R.J., Smith, S.B., Denslow, N.D., Blazer, V.S., Gross, T.S. (2006). Polychlorinated biphenyls, mercury, and potential endocrine disruption in fish from the Hudson River, New York, USA. *Aquatic Sciences*, 68(2), 206-228.
- Bateman, K.S., Stentiford, G.D., Feist, S.W. (2004). A ranking system for the evaluation

- of intersex condition in European flounder (*Platichthys flesus*). *Environmental Toxicology and Chemistry*, 23(12), 2831-2836.
- Berglund, O., Larsson, P., Ewald, G., Okla, L. (2001). Influence of trophic status on PCB distribution in lake sediments and biota. *Environmental Pollution*, 113(2), 199-210
- Berglund, O., Nyström, P. Larsson, P. (2005). Persistent organic pollutants in river food webs: influence of trophic position and degree of heterotrophy. *Canadian Journal of Fisheries and Aquatic Sciences*, 62(9), 2021-2032.
- Beyer J. (1996). *Fish biomarkers in marine pollution monitoring: evaluation and validation in laboratory and field studies*. (Unpublished master's thesis, University of Bergen, Norway).
- Billard, R. (1978). Testicular feed back on the hypothalamo-pituitary axis in rainbow trout (*Salmo gairdneri*. R.) *Ann. Biol. Anim. Biochim. Biophys*, 18(4), 813-818.
- Billard, R., Breton, B., Fostier, A., Jalabert, B., and Weil, C. (1978). Endocrine control of the teleost reproductive cycle and its relation to external factors: Salmonid and Cyprinid models. In P.J. Gaillard & H.H. Boer (Eds.), *Comparative Endocrinology* (pp. 37-48). Amsterdam: Elsevier.
- Bitman, J. and Cecil, H.C. (1970). Estrogenic activity of DDT analogs and polychlorinated biphenyls. *Journal of Agricultural and Food Chemistry*, 18(6), 1108-1112.
- Bjerregaard, L.B., Korsgaard, B., Bjerregaard, P. (2006). Intersex in wild roach (*Rutilus rutilus*) from Danish sewage effluent-receiving streams. *Ecotoxicology and Environmental Safety*, 64(3), 321-328.

- Bjerregaard, P. and Hansen, J.C. (2000). Organochlorines and heavy metals in pregnant women from the Disko Bay area in Greenland. *Science of the Total Environment*, 245(1-3), 195-202.
- Blazer, V.S. (2002). Histopathological assessment of gonadal tissue in wild fishes. *Fish Physiology and Biochemistry*, 26(1), 85-101.
- Blazer, V.S., Iwanowicz, L.R., Iwanowicz, D.D., Smith, D.R., Young, J.A., Hedrick, J.D., Foster, S.W., Reeser, S.J. (2007). Intersex (testicular oocytes) in smallmouth bass from the Potomac River and selected nearby drainages. *Journal of Aquatic Animal Health*, 19(4), 242-253.
- Blazer, V.S., Iwanowicz, L.R., Henderson, H., Mazik, P.M., Jenkins, J.A., Alvarez, D.A., Young, J.A. (2012). Reproductive endocrine disruption in Smallmouth Bass (*Micropterus dolomieu*) in the Potomac River basin: spatial and temporal comparisons of biological effects. *Environmental Monitoring and Assessment*, 184(7), 4309-4334.
- Blazer, V.S., Iwanowicz, D.D., Walsh, H.L., Sperry, A.J., Iwanowicz, L.R., Alvarez, D.A.,... Manning, R. (2014). Reproductive health indicators of fishes from Pennsylvania watersheds: association with chemicals of emerging concern. *Environmental Monitoring and Assessment*, 186(10), 6471-6491.
- Brown, D.P. (1987). Mortality of workers exposed to polychlorinated biphenyls – an update. *Archives of Environmental Health*, 42(6), 333-339.
- Buck, G.M., Sever, L.E., Mendola, P., Zielezny, M., Vena, J.E. (1997). Consumption of contaminated sport fish from Lake Ontario and time-to-pregnancy. New York State Angler Cohort. *American Journal of Epidemiology*, 146(11), 949-954.

- Burdick, G.E., Harris, E.J., Dean, H.J., Walker, T.M., Skea, J., Colby, D. (1964). The accumulation of DDT in lake trout and the effects on reproduction. *Transactions of the American Fisheries Society*, 93(2), 127-136.
- Campbell, C.M, Walsh, J.M., Idler, D.R. (1976). Steroids in the plasma of the winter flounder *Pseudopleuronectes americanus* W. A seasonal study and investigation of steroid involvement in oocyte maturation. *General and Comparative Endocrinology*, 29(1), 14-20.
- Carlsen E., Giwercman A., Keiding N., Skakkebaek N.E. (1992). Evidence for the decreasing quality of semen during the past 50 years. *British Medical Journal*, 305(6854), 609-613.
- Cooke, P.S., Sato, T., and Buchanan, D.L. (2001). Disruption of Steroid Hormone Signaling by PCBs. In L.W. Robertson & L.G. Hansen (Eds.), *PCBs: Recent Advances in Environmental Toxicology and Health Effects* (pp. 257-262), Lexington, KY: The University Press of Kentucky.
- Davis, J.T. and Lock, J.T. (1997). *Largemouth Bass Biology and Life History*. (Southern Regional Aquaculture Center (SRAC) Publication No. 200). Stoneville, MS.
- Denslow, N.D., Chow, M.C., Kroll, J.K., Green, L. (1999). Vitellogenin as a biomarker of exposure for estrogen or estrogen mimics. *Ecotoxicology*, 8(5), 385-398.
- Devlin, R.H. and Nagahama, Y. (2002). Sex determination and sex differentiation in fish: an overview of genetic, physiological, and environmental influences. *Aquaculture*, 208(3-4), 191-364.
- Duke Energy. (2014). *Keowee-Toxaway Project – FERC Project No. 2503 – Shoreline Management Plan*.

- Durhan, E.J., Lambright, C., Wilson, V., Buttersworth, B.C., Kuehl, D.W., Orlando, E.F., ... Ankley, G.T. (2002). Evaluation of androstenedione as an androgenic component of river water downstream of a pulp and paper mill effluent. *Environmental Toxicology and Chemistry*, 21(9), 1973-1976.
- Fein, G.G., Jacobson, J.L., Jacobson, S.W., Schwartz, P.M., Dowler, J.K. (1984). Prenatal exposure to polychlorinated biphenyls: effects on birth size and gestational age. *Journal of Pediatrics*, 105(2), 315-320.
- Fierens, S., Mairesse, H., Heilier, J.F., De Burbure, C., Focant, J.F., Eppe, G., ... Bernard, A. (2003). Dioxin/polychlorinated biphenyl body burden, diabetes and endometriosis: findings in a population-based study in Belgium. *Biomarkers*, 8(6), 529-534.
- Flood Control Act of 1950, 2 U.S.C. §201.
- Flouriou, G., Pakdel, F., Ducouret, B., Valotaire, Y. (1995). Influence of xenobiotics on rainbow trout liver estrogen receptor and vitellogenin gene expression. *Journal of Molecular Endocrinology*, 15(2), 143-151.
- Fritts, M.W., Deboer, J.A., Fritts, A.K., Kellock, K.A., Bringolf, R.B., Casper, A.F. (2016). Survey of Intersex Occurrence in Largemouth Bass (*Micropterus salmoides*) from the Upper Illinois River Waterway. *American Midlands Naturalist*, 176(1), 168-175.
- Gaymon, H. (1992). *Assessment of Polychlorinated Biphenyls (PCBs) and Priority Pollutants in Fish of Lake Hartwell – 1991. Anderson, Oconee and Pickens Counties, South Carolina* (Technical Memorandum No. 001, SCDHEC No. 002-92)

- Geyer, H.J., Rimkus, G.G., Scheunert, I., Kaune, A., Schramm, K.W., Kettrup, A.,...
Mackay, D. (2000). Bioaccumulation and occurrence of endocrine disrupting chemicals (EDCs), persistent organic pollutants (POPs), and other organic compounds in fish and other organisms including humans. In: B. Beck (Ed.), *The Handbook of Environmental Chemistry* (pp. 1-166), Berlin-Heidelberg: Springer-Verlag.
- Gladen, B.C., and Rogan, W.J. (1991). Effects of perinatal polychlorinated-biphenyls and dichlorodiphenyl dichloroethene on later development. *Journal of Pediatrics*, 119(1), 58-63.
- Glover, J.B., Domino, M.E., Altman, K.C., Dillman, J.W., Castleberry, W.S., Eidson, J.P., Mattocks, M. (2010). Mercury in South Carolina Fishes, USA. *Ecotoxicology*, 19(1), 781-795.
- Gronen, S., Denslow, N., Manning, S., Barnes, S., Barnes, D., Brouwer, M. (1999). Serum Vitellogenin Levels and Reproductive Impairment of Male Japanese Medaka (*Oryzias latipes*) Exposed to 4-*tert*-Octylphenol. *Environmental Health Perspectives*, 107, 385-390.
- Guillette, L.J., Brock, J.W., Rooney, A.A., Woodward, A.R. (1999). Serum concentrations of various environmental contaminants and their relationship to sex steroid concentrations and phallus size in juvenile American alligators. *Archives of Environmental Contamination and Toxicology*, 36(4), 447-455.
- Hauser, R., Chen, Z.Y., Pothier, L., Ryan, L., Altshul, L. (2003). The relationship

- between human semen parameters and environmental exposure to polychlorinated biphenyls and p,p'-DDE. *Environmental Health Perspectives*, 111(12), 1505-1511.
- Hayes, T.B., Anderson, L.L., Beasley, V.R., de Solla, S.R., Iguchi, T., Ingraham, H., ... Willingham, E. (2011). Demasculinization and feminization of male gonads by atrazine: Consistent effects across vertebrate classes. *Journal of Steroid Biochemistry and Molecular Biology*, 127(1-2), 64-73.
- Heaton, S.N., Bursian, S.J., Giesy, J.P., Tillitt, D.E., Render, J.A., Jones, P.D., ... Aulerich, R.J. (1995). Dietary exposure of mink to carp from Saginaw Bay, Michigan. 1. Effects on reproduction and survival, and the potential risks to wild mink populations. *Archives of Environmental Contamination and Toxicology*, 28(3), 334-343.
- Hemmer, M.J., Bowman, C.B., Hemmer, B.L., Friedman, S.D., Marcovich, D., Kroll, K.J., Denslow, N.D. (2002). Vitellogenin mRNA regulation and plasma clearance in male sheepshead minnows, (*Cyprinodon variegatus*) after cessation of exposure to 17 β -estradiol and p-nonylphenol. *Aquatic Toxicology*, 58(1-2), 99-112.
- Hinck, J.E., Blazer, V.S., Denslow, N.D., Echols, K.R., Gale, R.W., Wieser, C., ... Tillitt, D.E. (2008). Chemical contaminants, health indicators, and reproductive biomarker responses in fish from rivers in the Southeastern United States. *Science of the Total Environment*, 390(2-3), 538-557.
- Hinck, J.E., Blazer, V.S., Schmitt, C.J., Papoulias, D.M., Tillitt, D.E. (2009). Widespread

- occurrence of intersex in black basses (*Micropterus spp.*) from US rivers, 1995-2004. *Aquatic Toxicology*, 95(1), 60-70.
- Homer, C.G., Dewitz, J., Fry, J., Coan, M., Hossain, N., Larson, C. ... Wickham, J. (2015). Completion of the 2011 National Land Cover Database for the conterminous United States- Representing a decade of land cover change information. *Photogrammetric Engineering and Remote Sensing*, 81(5), 345-354.
- Huisman, M., Koopmanessenboom, C., Fidler, V., Haddersalgra, M., Vanderpauw, C.G., Tuinstra, L.G.M.T., ... Boersma, E.R. (1995). Perinatal exposure to polychlorinated-biphenyls and dioxins and its effect on neonatal neurological development. *Early Human Development*, 41(2), 111-127.
- Idler, D.R., Hwang, S.J., Crim, L.W., Reddin, D. (1981). Determination of sexual maturation stages of Atlantic salmon *Salmo salar* captured at sea. *Canadian Journal of Fisheries and Aquatic Sciences*, 38(4), 405-413.
- Ingram, D.R., Miller, D.L., Ingram, T.R., Tannehill, J.E. (2011). Intersex Condition of Shoal Bass in the Flint River, Georgia. *Journal of Aquatic Animal Health*, 23(4), 189-194.
- Iwanowicz, L.R., Blazer, V.S., Guy, C.P., Pinkney, A.E., Mullican, J.E., Alvarez, D.A. (2009). Reproductive Health of Bass in the Potomac, USA, Drainage: Part 1. Exploring the Effects of Proximity to Wastewater Treatment Plant Discharge. *Environmental Toxicology and Chemistry*, 28(5), 1072-1083.
- Iwanowicz, L.R., Blazer, V.S., Hitt, N.P., McCormick, S.D., DeVault, D.S., Ottinger,

- C.A. (2012). Histologic, immunologic, and endocrine biomarkers indicate contaminant effects in fishes of the Ashtabula River. *Ecotoxicology*, 21(1), 165-182.
- Jacobson, S.W., Fein, G.G., Jacobson, J.L., Schwartz, P.M., Dowler, J.K. (1985). The Effect of Intrauterine PCB Exposure on Visual Recognition Memory. *Child Development*, 56(4), 853-860.
- Jacobson, J.L. and Jacobson, S.W. (1996). Intellectual Impairment in Children Exposed to Polychlorinated Biphenyls in Utero. *New England Journal of Medicine*, 335, 783-789.
- Jacobson, J.L., Humphrey, H.E.B., Jacobson, S.W., Schantz, S, L., Mullin, M.D., Welch, R. (1989). Determinants of Polychlorinated Biphenyls (PCBs), Polybrominated Biphenyls (PBBs), and Dichlorodiphenyl Trichloroethane (DDT) Levels in the Sera of Young Children. *American Journal of Public Health*, 79,1401-1404.
- Jansen, H.T., Cooke, P.S., Porcelli, J., Liu, T.C., Hansen, L.G. (1993). Estrogenic and antiestrogenic actions of PCBs in the female rat – in vitro and in vivo studies. *Reproductive Toxicology*, 7(3), 237-248.
- Janz, D.M. (2000). Endocrine System. In: G.K. Ostrander (Ed.) *The Laboratory Fish. Handbook of Experimental Animals*. (pp. 189-217). San Diego, CA: Academic Press.
- Jobling, S., Sheehan, D., Osborne, J.A., Matthiessen, P., Sumpter, J.P. (1996). Inhibition of testicular growth in rainbow trout (*Oncorhynchus mykiss*) exposed to estrogenic alkyphenolic chemicals. *Environmental Toxicology and Chemistry*, 15(2), 194-202.

- Jobling, S., Nolan, M., Tyler, C.R., Brighty, G.C., Sumpter, J.P. (1998). Widespread sexual disruption in wild fish. *Environmental Science and Technology*, 32(17), 2498-2506.
- Jobling, S., Burn, R.W., Thorpe, K., Williams, R., Tyler, C. (2009). Statistical modeling suggests that antiandrogens in effluent from wastewater treatment works contribute to widespread sexual disruption in fish living in English rivers. *Environmental Health Perspectives*, 117(5), 797-802.
- Johansen, B.E. (2003). *The Dirty Dozen: Toxic Chemicals and the Earth's Future*. Westport, CT: Greenwood Publishing Group.
- Kellock, K.A., Trushel, B.E., Ely, P.C., Jennings, C.A., Bringolf, R.B. (2014). Survey of Intersex Largemouth Bass from Impoundments in Georgia USA. *Transactions of the American Fisheries Society*, 143(3), 565-572.
- Kester, M.H.A., Bluduk, S., Tibboel, D., Meinl, W., Glatt, H., Falany, C.N., ... Visser, T.J. (2000). Potent inhibition of estrogen sulfotransferase by hydroxylated PCB metabolites: a novel pathway explaining the estrogenic activity of PCBs. *Endocrinology*, 141(5), 1897-1900.
- Kidd, K.A., Blanchfield, P.J., Mills, K.H., Palace, V.P., Evans, R.E., Lazorchak, J.M., Flick, R.W. (2007). Collapse of a fish population after exposure to a synthetic estrogen. *PNAS*, 104(21), 8897-8901.
- Kobayashi, M., Sorensen, P.W., Stacey, N.E. (2002). Hormonal and pheromonal control of spawning behavior in the goldfish. *Fish Physiology and Biochemistry*, 26(1), 71-84.
- Larkin, P., Sabo-Attwood, T., Kelso, J., Denslow, N.D. (2002). Gene expression analysis

- of largemouth bass exposed to estradiol, nonylphenol, and p,p'-DDE.
- Comparative Biochemistry and Physiology Part B Biochemistry and Molecular Biology*, 133(4), 543-557.
- Larsson, D.G.J., Adolfsson-Erici, M., Parkkonen, J., Pettersson, M., Berg, A.H., Olsson, P.E., Forlin, L. (1999). Ethinylloestradiol – an undesired fish contraceptive? *Aquatic Toxicology*, 45(2-3), 91-97.
- Lavado, R., Thibaut, R., Raldúa, D., Martín, R. Porte, C. (2004). First evidence of endocrine disruption in feral carp from the Ebro River. *Toxicology and Applied Pharmacology*, 196(2), 247-257.
- Lavenda, N. (1949). Sexual differences and normal protogynous hermaphroditism in the Atlantic sea bass, *Centropristis striatus*. *Copeia*, 1949(3), 185-194
- Lazorchak, J.M., Griffith, M.B., Mills, M., Schubauer-Berigan, J., McCormick, F., Brenner, R., Zeller, C. (2015). Proof of concept for the use of macroinvertebrates as indicators of polychlorinated biphenyls (PCB) contamination in Lake Hartwell. *Environmental Toxicology and Chemistry*, 34(6), 1277-1282.
- Letcher, R.J., Bustnes, J.O., Dietz, R., Jenssen, B.M., Jørgensen, E.H., Sonne, C., ... Gabrielsen, G.W. (2010). Exposure and effects assessment of persistent organohalogen contaminants in arctic wildlife and fish. *Science of the Total Environment*, 408(15), 2995-3043.
- Lewis, W.M. and Flickinger, S. (1967). Home Range Tendency of the Largemouth Bass (*Micropterus salmoides*). *Ecology*, 48(6), 1020-1023.
- Longnecker, M.P., Rogan, W.J., Lucier, G. (1997). The human health effects of DDT

- (dichlorodiphenyl-trichloroethane) and PCBS (polychlorinated biphenyls) and an overview of organochlorines in public health. *Annual Review of Public Health*, 18(1), 211-244.
- Loomis, D., Browning, S.R., Schenck, A.P., Gregory, E., Savitz, D.A. (1997). Cancer mortality among electric utility workers exposed to polychlorinated biphenyls. *Occupational and Environmental Medicine*, 54(10), 720-728.
- Lubzens, E., Young, G., Bobe, J., Cerdá, J. (2009). Oogenesis in teleosts: How fish eggs are formed. *General and Comparative Endocrinology*, 165(3), 367-389.
- Luna, L. (1992). *Histopathologic methods and color atlas of special stains and tissue artifacts*. Downers Grove, IL: Johnson Printers.
- Lundkvist, U. (1990). Clinical and reproductive effects of Clophen A50 (PCB) administered during gestation on pregnant guinea pigs and their offspring. *Toxicology*, 61(3):249-257.
- Macek, K.J. (1968). Reproduction in brook trout *Salvelinus fontinalis* fed sublethal concentrations of DDT. *Journal of the Fisheries Research Board of Canada*, 25(9), 1787-1796.
- Matozzo, V., Gagné, F., Marin, M.G., Ricciardi, F., Blaise, C. (2008). Vitellogenin as a biomarker of exposure to estrogenic compounds in aquatic invertebrates: A review. *Environment International*, 34(4), 531-545.
- McDonald, D.G. and Milligan, C.L. (1992). Chemical Properties of Blood. In: W.S. Hoar, D.J. Randall, A.P. Farrell (Eds.) *Fish Physiology*, vol. XIIB (pp. 55-133), New York, NY: Academic Press.
- McKinney, J.D., and Waller, C.L. (1994). Polychlorinated biphenyls as hormonally

- active structure analogues. *Environmental Health Perspectives*, 102(3), 290-297.
- Mecozzi, M. (2008). Largemouth Bass – *Micropterus salmoides*. Wisconsin Department of Natural Resources – Bureau of Fisheries Management. PUBL-FM-709-08.
- Mendola, P., Buck, G.M., Vena, J.E., Zielesny, M., Sever, L.E. (1995). Consumption of PCB-contaminated sport fish and risk of spontaneous fetal death. *Environmental Health Perspectives*, 103(5), 498-502.
- Mendola, P., Buck, G.M., Sever, L.E., Zielesny, M., Vena, J.E. (1997). Consumption of PCB-contaminated freshwater fish and shortened menstrual cycle length. *American Journal of Epidemiology*, 146(11), 955-960.
- Miwa, S., Yan, L., Swanson, P. (1994). Localization of two gonadotropin receptors in the salmon gonad by in vitro ligand autoradiography. *Biology of Reproduction*, 50(3), 629-642.
- Moore, M., Mustain, M., Daniel, K., Chen, I., Safe, S., Zacharewski, T., ... Balaguer, P. (1997). Antiestrogenic activity of hydroxylated polychlorinated biphenyl congeners identified in human serum. *Toxicology and Applied Pharmacology*, 142(1), 160-168.
- Monteiro, M.S., Pavlaki, M., Faustino, A., Rema, A., Franchi, M., Gediell, L., ... Soares, A.M.V.M. (2015). Endocrine disruption effects of p, p'-DDE on juvenile zebrafish. *Journal of Applied Toxicology*, 35(3)253-260.
- Nadakavukaren, A. *Our Global Environment: A Health Perspective* (7th edition). Long Grove, IL: Waveland Press, Inc.
- National Research Council. (1999). *Hormonally Active Agents in the Environment*. Washington, DC: National Academy Press.

- Orlando, E.F., Kolok, A.S., Binzcik, G.A., Gates, J.L., Horton, M.K., Lambright, C.S. ... Guillette, L.J. (2004). Endocrine-disrupting effects of cattle feedlot effluent on an aquatic sentinel species, the fathead minnow. *Environmental Health Perspectives*, 112(3), 353-358.
- Oskam, I.C., Ropstad, E., Dahl, E., Lie, E., Derocher, A.E., Wiig, O. ... Skaare, J.U. (2003). Organochlorines affect the major androgenic hormone, testosterone, in male polar bears (*Ursus maritimus*) at Svalbard. *Journal of Toxicology and Environmental Health, Part A*, 66(22), 2119-2139.
- Papoulias, D.M., Noltie, D.B., Tillitt, D.E. (2000). An in vivo model fish system to test chemical effects on sexual differentiation and development: exposure to ethinyl estradiol. *Aquatic Toxicology*, 48(1), 37-50.
- Parker, G.A. (1992). The evolution of sexual size dimorphism in fish. *Journal of Fish Biology*, 41(1), 1-20.
- Pasmanik, M. and Callard, G.V. (1988). Changes in brain aromatase and 5 alpha-reductase activities correlate significantly with seasonal reproductive cycles in goldfish (*Carassius auratus*). *Endocrinology*, 122(4), 1349-1356.
- Patandin, S., Koopman-Esseboom, C., De Ridder, M.A.J., Weisglas-Kuperus, N. (1998). Effects of environmental exposure to polychlorinated biphenyls and dioxins on birth size and growth in Dutch children. *Pediatric Research*, 44(4), 538-545.
- Patandin, S., Lanting, C.I., Mulder, P.G.H., Boersma, E.R., Sauer, P.J.J., Weisglas-Kuperus, N. (1999). Effects of environmental exposure to polychlorinated biphenyls and dioxins on cognitive abilities of Dutch children at 42 months of age. *Journal of Pediatrics*, 134(1), 33-41.

- Patiño, R. and Sullivan, C.V. (2002). Ovarian follicle growth, maturation, and ovulation in teleost fish. *Fish Physiology and Biochemistry*, 26(1), 57-70.
- Persky, V.W. (2001). Health Effects of Occupational Exposure to PCBs. In L.W. Robertson & L.G. Hansen (Eds.), *PCBs: Recent Advances in Environmental Toxicology and Health Effects* (pp. 99-102). Lexington, KY: The University Press of Kentucky.
- Pinto, P.I., Estêvão, M.D., Power, D.M. (2014). Effects of Estrogens and Estrogenic Disrupting Compounds on Fish Mineralized Tissues. *Marine Drugs*, 12(8), 4474-4494.
- Purdom, C.E., Hardiman, P.A., Bye, V.J., Eno, R.C., Tyler, C.R., Sumpter, J.P. (1994). Estrogenic effects of effluents from sewage treatment works. *Chemistry and Ecology*, 8(4), 275-285.
- Rogan, W.J., Gladen, B.C., McKinney, J.D., Carreras, N., Hardy, P., Thullen, J., Tingelstad, J., Tully, M. (1987). Polychlorinated Biphenyls (PCBs) and Dichlorodiphenyl Dichloroethene (DDE) in Human Milk: Effects on Growth, Morbidity, and Duration of Lactation. *American Journal of Public Health*, 77, 1294-1297.
- Rohde, F.C., Arndt, R.G., Foltz, J.W., Quattro, J.M. (2009). *Freshwater Fishes of South Carolina*. Columbia, SC: The University of South Carolina Press.
- Rosenblum, P.M., Brandt, T.M., Mayes, K.B., Hutson, P. (1994). Annual cycles of growth and reproduction in hatchery-reared Florida largemouth bass, *Micropterus salmoides floridanus*, raised on forage or pelleted diets. *Journal of Fish Biology*, 44(6), 1045-1059.

- Rylander, L., Stromberg, U., Dyremark, F., Ostman, C., Nilsson-Ehle, P., Hagmar, L. (1998). Polychlorinated biphenyls in blood plasma among Swedish female fish consumers in relation to low birth weight. *American Journal of Epidemiology*, 147(5), 493-502.
- Safe, S.H. (1992). Toxicology, structure-function relationship, and human and environmental health impacts of polychlorinated biphenyls: Progress and problems. *Environmental Health Perspectives*, 100, 259-268.
- Sager, D.B. (1983). Effect of postnatal exposure to polychlorinated biphenyls on adult male reproductive function. *Environmental Research*, 31(1), 76-94.
- Schantz, S.L., Widholm, J.J., Rice, D.C. (2003). Effects of PCB exposure on neuropsychological function in children. *Environmental Health Perspectives*, 111(3), 357-376.
- Schlumberger Technology Corporation. (2014). Final Report – Lake Hartwell 2014 Fish and Sediment Study. (URS Project No. 20500689.00001). Sugar Land, TX.
- Schmitt, C.J., Dethloff, G.M., Hinck, J.E., Bartish, T.M., Blazer, V.S., Coyle, J.J. ... Tillitt, D.E. (2004). Biomonitoring of Environmental Status and Trends (BEST) Program: Environmental Contaminants and their Effects on Fish in the Rio Grande Basin (Scientific Investigations Report 2004 – 5108). Columbia, MO: USGS Columbia Environmental Research Center.
- Schreck, C.B. and Hopwood, M.L. (1974). Seasonal androgen and estrogen patterns in goldfish, *Carassius auratus*. *Transactions of the American Fisheries Society*, 103(2), 375-378.
- Schulz, R.W., de Franca, L.R., Lareyre, J.J., LeGac, F., Chiarini-Garcia, H., Nobrega,

- R.H., Miura, T. (2010). Spermatogenesis in fish. *Comparative Endocrinology*, 165(3), 390-411.
- Seegal, R.F. (1996). Epidemiological and laboratory evidence of PCB-induced neurotoxicity. *Critical Reviews in Toxicology*, 26(6), 709-737.
- Segner, H., Eppler, E., Reinecke, M. (2006). The Impact of Environmental Hormonally Active Substances on the Endocrine and Immune Systems of Fish. In M. Reinecke, G. Zaccane, B.G. Kapoor (Eds.), *Fish Endocrinology* (Vol. 1) (pp. 809-865). Enfield, NH: Science Publishers.
- Sharpe, R.M. and Skakkebaek, N.F. (1993). Are oestrogens involved in falling sperm counts and disorders of the male reproductive tract? *Lancet*, 341(8857), 1392-1395.
- Sinks, T., Steele, G., Smith, A.B., Watkins, K., Shults, R.A. (1992). Mortality among workers exposed to polychlorinated biphenyls. *American Journal of Epidemiology*, 136(4), 389-398.
- Simmons, S.L., Cummings, J.A., Clemens, L.G., Nunez, A.A. (2005). Exposure to PCB 77 affects the maternal behavior of rats. *Physiology & Behavior*, 84(1), 81-86.
- Sivarajah, K., Franklin, C.S., Williams, W.P. (1979). Some studies on the hepatic microsomal enzyme activities and steroid hormone levels in Carp, *Cyprinus carpio* exposed fish for six months. *Journal of Fish Biology*, 15(3), 249-253.
- South Carolina Department of Health and Environmental Control (SCDHEC). (2016). *South Carolina Fish Consumption Advisories*.
- Specker J.,L. and Sullivan, C.V. (1994). Vitellogenesis in fishes: status and perspectives.

- In K.G. Davey, R.E. Peter, S.S. Tobe (Eds.). *Perspectives in Comparative Endocrinology Invited Papers from XII International Congress of Comparative Endocrinology, Toronto, Ontario, Canada, 16-21 May 1993* (pp. 304-315).
Ottawa, Ontario: National Research Council of Canada.
- Stewart, P., Reihman, J., Lonky, E., Darvill, T., Pagano, J. (2000). Prenatal PCB exposure and neonatal behavior assessment scale (NBAS) performance. *Neurotoxicology and Teratology*, 22(1), 21-29.
- Stone, W.S., Gilliom, R.J., Ryber, K.R. (2014). Pesticides in U.S. Streams and Rivers: Occurrence and Trends during 1992 – 2011. *Environmental Science and Technology*, 48(1), 11025-11030.
- Strüssmann, C.A. and Nakamura, M. (2002). Morphology, endocrinology, and environmental modulation of gonadal sex differentiation in teleost fishes. *Fish Physiology and Biochemistry*, 26(1), 13-29.
- Tyler, C.R., Santos, E.M., Prat, F. (1999). Unscrambling the egg—cellular, biochemical, molecular, and endocrine advances in oogenesis. In B. Norberg, O.S. Kjesbu, G.L. Taranger, E. Andersson, S.O. Stefansson (Eds.). *Proceedings of the 6th International Symposium on the Reproductive Physiology of Fish, Bergen 1999* (pp. 273-280). Bergen, Norway: University of Bergen.
- United States Environmental Protection Agency (EPA). (1990). *EPA Superfund Record of Decision: Sangamo Weston, Inc./Twelve-mile Creek/Lake Hartwell PCB Contamination OUI*. EPA-ROD-R04-91-100.
- United States Environmental Protection Agency (EPA). (1994). *EPA Superfund Final*

- Record of Decision: Sangamo Weston, Inc./Twelve-mile Creek/Lake Hartwell PCB Contamination OU2. PB-94-964012.*
- United States Environmental Protection Agency (EPA). (1999). *Polychlorinated Biphenyls (PCBs) Update: Impact on Fish Advisories*. Office of Water. EPA-823-F-99-019.
- United States Environmental Protection Agency (EPA). (2000). *Guidance for Assessing Chemical Contaminant Data for Use in Fish Advisories*. Office of Water. EPA-B-00-008.
- Vajda, A.M., Barber, L.B., Gray, J.L., Lopez, E.M., Woodling, J.D., Norris, D.O. (2008). Reproductive disruption in fish downstream from an Estrogenic wastewater effluent. *Environmental Science & Technology*, 42(9), 3407-3414.
- Van Der Kraak, G., J., Munkittrick, K.R., McMaster, M.E., Portt, C.B, Chang, J.P. (1992). Exposure to bleached kraft pulp mill effluent disrupts the pituitary-gonadal axis of white sucker at multiple sites. *Toxicology and Applied Pharmacology*, 115(2), 224-233.
- Vandenberg, L.N., Colburn, T., Hayes, T.B., Heindel, J.J., Jacobs, D.R. Jr., Lee, D.-H., ... Myers, J.P. (2012). Hormones and endocrine-disrupting chemicals: low-dose effects and nonmonotonic dose responses. *Endocrine Reviews*, 33(1), 378-455.
- Van der Oost, R., Beyer, J., Vermeulen, N.P.E. (2003). Fish bioaccumulation and biomarkers in environmental risk assessment: a review. *Environmental Toxicology and Pharmacology*, 13(2), 57-149.
- Vega-Lopez, A.N., Martinez-Tabche, L., Dominguez-Lopez, M.L., Garcia-Latorre, E.,

- Ramon-Gallegos, E., Garcia-Gasca, A. (2006). Vitellogenin induction in the endangered goodeid fish *Girardinichthys viviparous*: Vitellogenin characterization and estrogenic effects of polychlorinated biphenyls. *Comparative Biochemistry and Physiology C – Toxicology & Pharmacology*, 142(3-4), 356-364.
- Walkowiak, J., Wiener, J.A., Fastabend, A., Heinzow, B., Kramer, U., Schmidt, E. ... Winneke, G. (2001). Environmental exposure to polychlorinated biphenyls and quality of home environment: effects on psychodevelopment in early childhood. *Lancet*, 358(9293), 1602-1607.
- Walters, D.M., Fritz, K.M., Johnson, B.R., Lazorchak, J.M., McCormick, F.H. (2008). Influence of Trophic Position and Spatial Location on Polychlorinated Biphenyl (PCB) Bioaccumulation in a Stream Food Web. *Environmental Science and Technology*, 42(7), 2316-2322.
- Weisglaskuperus, N., Sas, T.C.J., Koopmanesseboom, C., Vanderzwan, C.W., Deridder, M.A.J., Beishuizen, A. ... Sauer, P.J.J. (1995). Immunological effects of background prenatal and postnatal exposure to dioxins and polychlorinated-biphenyls in Dutch infants. *Pediatric Research*, 38(3), 404-410.
- Williams, J.E. (2000). The Coefficient of Condition of Fish. In J.C. Schneider (Ed.), *Manual of fisheries survey methods II: with periodic updates*. Ann Arbor, MI: Michigan Department of Natural Resources.
- Woodling, J.D., Lopez, E.M., Maldonado, T.A., Norris, D.O., Vajda, A.M. (2006). Intersex and other reproductive disruption of fish in wastewater effluent dominated Colorado streams. *Comparative Biochemistry and Physiology Part C: Toxicology and Pharmacology*, 144(1), 10-15.

Yamamoto, T.O. (1969). Sex differentiation. In W.S. Hoar and D.J. Randall (Eds.) *Fish Physiology* (vol. 3) (pp.117-175). New York, NY: Academic Press.

Yonkos, L.T., Fisher, D.J., Van Veld, P.A., Kane, A. S., McGee, B.L., Staver, K.W. (2010). Poultry litter–induced endocrine disruption in fathead minnow, sheepshead minnow, and mummichog laboratory exposures. *Environmental Toxicology and Chemistry*, 29(10), 2328-2340.

Yonkos, L.T., Friedel, E.A., Fisher, D.J. (2014). Intersex (testicular oocytes) in largemouth bass (*Micropterus salmoides*) on the Delmarva Peninsula, USA. *Environmental Toxicology and Chemistry*, 33(5), 1163-1169.

APPENDIX A – SITE DESCRIPTIONS AND LOCATIONS

Table A.1: Station names and descriptions for Lake Hartwell and Lake Keowee stations sampled and sites from Hinck et al. (2009).

Station	Description	Latitude	Longitude
<i>Lake Hartwell</i>			
SV107	Twelvemile Creek arm at SC 133	34°42'48.60" N	82°49'54.16" W
SV535	Andersonville Island vicinity near confluence of Tugaloo and Seneca Rivers	34°28'39.13" N	82°51'56.06" W
<i>Lake Keowee</i>			
SV311	At SC 188, Cane Creek arm 3.5 mi NW Seneca	34°43'52.44" N	82°58'26.39" W
<i>Savannah River Basin</i>			
333 (S1)	Savannah River, Augusta, GA	33°22'00.18" N	81°56'46.44" W
334 (S2)	Savannah River, Sylvania, GA	33°01'16.86" N	81°31'04.50" W
335 (S3)	Savannah River, Port Wentworth, GA	32°13'26.34" N	81°08'47.04" W
<i>Great Pee Dee River Basin</i>			
336 (P1)	Pee Dee River, Rockingham, NC	34°53'22.14" N	79°51'24.89" W
337 (P2)	Pee Dee River, Pee Dee, SC	34°21'23.22" N	79°41'35.19" W
338 (P3)	Pee Dee River, Bucksport, SC	33°42'18.09" N	79°11'24.00" W