# INVESTIGATION OF THE OCCURRENCE OF ENDOCRINE DISRUPTION IN FISH AND ASSOCIATED FACTORS FOR LENTIC WATERS OF GEORGIA, USA

by

KRISTEN A. KELLOCK

(Under the Direction of Robert B. Bringolf)

## ABSTRACT

Reports of intersex fish (males with oocytes in their testicular tissue) in water bodies around the world have stimulated widespread concern about the effects that chemicals are having in the environment. Estrogens and estrogen-like chemicals in the environment are known to induce intersex and other forms of endocrine disruption in fish. Intersex fish can have decreased sperm production, decreased sperm motility and decreased fertilization success compared to histologically ‘normal’ male fish. The aquatic environment may contain a host of endocrine disrupting chemicals. Complex chemical mixtures associated with wastewater effluent, agricultural and industrial run-off all pose a threat to fish from egg fertilization to spawning. Endocrine disruptors can mimic sex hormones in humans, terrestrial wildlife and aquatic organisms and endocrine disruptors of particular concern are the natural and synthetic estrogens, and estrogenic compounds. My experiments address hypotheses regarding the distribution and incidence of intersex largemouth bass in Georgia and possible factors contributing to this condition. Both lab and field based projects were performed to explore relationships among possible environmental influences, background rates of intersex and endocrine disruption in fish.

A survey of intersex largemouth bass, waterborne estrogens and estrogenic potency in impoundments across Georgia provided valuable information about the spatial distribution of the condition, the potential background rate of intersex in basses, and characteristics of waters associated with high rates of intersex. Laboratory-based experiments explored possible contributing factors to endocrine disruption in fish including sediment lifecycle exposures with sediment collected from high intersex impoundments. Hierarchical modeling with AICc for model selection was used for predicting the probability of an intersex fish present in a water body. Without conclusive evidence that estrogens or sediment-bound estrogens were playing a substantive role in endocrine disruptions, the ubiquitous pollutant nitrate was investigated as a potential causative factor as many of the smaller impoundments were highly eutrophic. Results from this project describe the distribution of intersex largemouth in Georgia impoundments. Intersex fish are most likely found in small, shallow impoundments where estrogenic exposure and other endocrine disrupting chemicals may or may not be heavily involved.

INDEX WORDS: intersex, estrogens, largemouth bass, nitrates, impoundments, hierarchical, sediment

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# DEDICATION

For all Kellocks great and small

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# BACKGROUND AND CHAPTER OBJECTIVES

## INTRODUCTION

Recent trends in human reproductive health such as declining sperm count in men in developed countries (50% decrease since 1900), increases in reproductive cancers (e.g., prostate, uterine, breast, testicular), increases in reproductive tract developmental deformities, and early-onset of puberty, particularly in pre-teen girls, have long been suspected to be linked to chemical contaminants (Toppari et al. 1996; Damgaard et al. 2002; Giwercman et al. 2007; Toppari 2008). The increases in abnormalities in human reproductive health have been associated with similar trends in fish and wildlife. A direct link between these reproductive abnormalities in humans and endocrine disrupting chemicals (EDCs) has not been established; however, the endocrine system is conserved among vertebrates and it is increasingly evident that many chemicals alter endocrine system structure and function in fish and wildlife (Colborn et al. 1993). These disturbing trends have stimulated widespread concern about the effects that chemicals are having in the environment.

The detrimental effects of EDCs on humans and wildlife have gained much notoriety since the 1990s (Kavlock 1999). Endocrine disrupting chemicals can mimic or antagonize particular sex hormones (i.e., estrogens or androgens) and may be causative agents in many ecotoxicological problems (Nilsson 2000). These chemicals disturb an organism's natural endocrine system activity which is responsible for normal developmental, growth, reproductive, metabolic and behavioral processes (Kavlock et al. 1996). Endocrine disrupting chemicals that act as estrogens by binding to and activating estrogen receptors are commonly referred to as “environmental estrogens” and have been intensely studied (Purdom et al. 1994; Harries et al. 1997; Jobling et al. 1998; Schultz 2003 et al.; Lange et al. 2009; Zhang et al. 2010). Environmental estrogens include natural and synthetic hormones as well as many anthropogenic compounds. These chemicals have varying degrees of estrogenic activity in humans, wildlife and aquatic organisms (Nilsson 2000; van Aerle et al. 2002). Some endocrine disruptors known as anti-estrogens bind but do not activate the estrogen receptor, effectively blocking the estrogen response. Certain PCB congeners, some phytoestrogens as well as taximofen are all examples of anti-estrogens (Connor et al. 1997; Collins et al. 1997; Paech et al. 1997). Androgens and anti-androgens are another group of EDCs and androgens, such as trenbolone, are often used as a growth promoter in beef cattle. Trenbolone and other hormones can enter aquatic systems as waste run-off and can alter development, behavior and reproductive activity of male and female fish (Ankley et al. 2003; Orlando et al. 2004). Anti-androgens block normal androgen action, resulting in a feminizing effect that has been observed in fish (Filby et al. 2007). More recently, nutrients, specifically nitrates, have been implicated as endocrine disruptors in aquatic vertebrates (Guillette and Edwards 2005; Edwards and Guillette 2007; Hamlin et al. 2008). Nitrate is a major (and ubiquitous) global pollutant and can be measured in eutrophic surface waters as well as aquifers (Sampat 2000; Guillette and Edwards 2005; Edwards et al. 2006a).

Fish, more so than other vertebrates, are particularly susceptible to the effects of EDCs exposure. Fish in EDC contaminated waters can presumably experience a lifecycle exposure because EDCs are found in the water column (Kolpin et al. 2002; Ternes et al. 2004), sediments (Peck et al. 2004; Sellin et al. 2010), and the food web (Rasmussen et al. 1990; Geyer et al.

2000). Most studies of fish endocrine disruption are focused on the hypothalamic-pituitary-gonadal axis. This axis controls sexual differentiation, development and reproduction. Specific hormonal triggers and responses are critical during periods of differentiation in the fish for normal developmental processes.

Environmental cues including photoperiod and temperature changes prompt the start of spawning season in fishes. The fish senses the environmental cues and the hypothalamus releases gonadotropin releasing hormone (GnRH) to act on the pituitary gland and trigger the release of gonadotropic hormones, follicle stimulating hormone (FSH) and luteinizing hormone (LH) into circulation. Receptors for FSH and LH are found in the gonads and in male fish gonadotropic hormones act on Leydig cell in the testes to release testosterone and 11-ketotestosterone (11-KT), the most potent androgens in fish (Evans and Claiborne 2006). 11-KT then stimulates surrounding Sertoli cells to release insulin-like growth factors necessary for spermatogonia development (Evans and Claiborne 2006). In fish and mammals sex steroids are synthesized from cholesterol in gonadal tissue. Entry of cholesterol into gonadal cell mitochondria is regulated by steroidogenic acute regulatory (StAR) protein before hormone synthesis begins. Once inside the mitochondria, cholesterol is modified by $P450_{SCC}$ (side chain cleavage) and converted to pregnenolone. Subsequent steps in steroid synthesis continue to further form sex hormones leading ultimately to testosterone and estradiol. Testosterone is converted to estradiol by aromatase and also converted to 11-KT by a dehydrogenase enzyme (Evans and Claiborne 2006).

In female fish, the binding of gonadotropic hormones trigger the release of estrogens, which also control vitellogenesis. 17-β estradiol stimulates production of vitellogenin by the liver. Vitellogenin, an egg yolk precursor protein necessary for normal oocyte maturation and development in oviparous vertebrates, is transported via blood to the developing ovaries. Male fish are genetically capable of producing vitellogenin but typically lack the natural estrogen signaling that initiates vitellogenin production (Folmar 2001). In the presence of exogenous estrogen males will synthesize vitellogenin and thus, production of vitellogenin by male and juvenile fish is presently the standard biomarker of exposure to estrogens (Sumpter and Jobling 1995). Vitellogenin is synthesized in a dose-dependent fashion with estrogen exposure and can be damaging to the liver and kidneys of developing fish (Hutchinson et al. 2006). Caged male rainbow trout *Oncorhynchus mykiss* exposed to sewage treatment effluent in UK rivers had upwards of 100,000-fold induction in of vitellogenin after only a two week exposure (Purdom et al. 1994). Consequences of long term vitellogenin induction in male fish are not well understood but at a minimum protein synthesis is energetically expensive. For example, Korte et al. (2000) induced vitellogenin production in male fathead minnows with a single estradiol injection of 0.5 mg/kg or 5 mg/kg and determined the effects of plasma and hepatic vitellogenin over a 3-week period. Evidence of an increase in vitellogenin in the male minnow was measured as early as 8 hours after the injection and was detectable for 6-days following exposure to estradiol (Korte et al. 2000).

### *Intersex Fish*

Feminized roach *Rutilis rutilis* caught in municipal wastewater treatment lagoons along the River Lea (a branch of the Thames River) in the United Kingdom were first identified by British anglers in the 1970s. Further inspection of the fish’s gonads by fish biologists working with the Thames River Water Company revealed male gonadal tissue infiltrated by primary oocytes, a strictly female tissue (Sumpter and Johnson 2008). Hermaphroditism is common in some fishes, but in gonochoristic fish species, which have separate sexes throughout life, the presence of testicular oocytes (intersex) is a pathological condition that is not routinely observed (Hecker et al. 2006). Roach is a gonochoristic species so the discovery of intersex individuals generated substantial public and scientific attention. The intersex condition of roach in the River Lea was demonstrated to be a result of exposure to a hormonally-active component of municipal wastewater effluent (Jobling et al. 1998). In the decade following this discovery, links between wastewater effluent and the intersex condition have been the subject of intense investigation around the world (Sweeting 1981; Jobling et al. 1998; Woodling et al. 2006; Blazer et al. 2007; Sumpter and Johnson 2008). Endocrine disruption in fish has been induced in laboratory studies with exposures to natural and synthetic hormones (Jobling et al. 2002a; van Aerle et al. 2002; Van den Belt et al. 2003; Parrott and Blunt 2005), which are routinely measured in treated municipal wastewater effluent and in surface waters (Desbrow et al. 1998; Kolpin et al. 2002).

The individual components of wastewater effluent that cause endocrine disruption are difficult to quantify because of the highly complex nature of wastewater, but the most routinely quantified endocrine disruptors include the natural and synthetic estrogens as well as industrial compounds (Larsson et al. 1999). Natural estrogens consist of estrone (E1), 17β-estradiol (E2) and estriol (E3). Ethinylestradiol (EE2) is a synthetic estrogen. Women excrete E2 at a rate of 2-12 μg/person/day and this hormone is approximately three times more soluble in water than synthetic counterparts (Ying et al. 2002). The synthetic estrogen, EE2, is found in oral contraceptives and in hormone replacement therapy for postmenopausal women. Women receiving hormone replacement therapy and menstruating women excrete approximately 2.3 μg/day and 3.5 μg/day of E2, respectively, while pregnant women can excrete approximately 259 μg/day of E2 (Ying et al. 2002). Estrone and estriol are also naturally metabolized and excreted from the human body and these hormones are also formed during the microbial breakdown of E2 occurring in a municipal WWTF (Lee and Liu 2002). Numerous studies have documented that these estrogens as well as a variety of pharmaceuticals are not removed by many wastewater treatment processes and are being released into rivers with treated wastewater (Lee and Peart 1998; Kolpin et al. 2002; Ternes et al. 2004; Auriol et al. 2006). A recent study that deployed caged fathead minnows below a wastewater facility in Boulder, CO showed that treatment facility upgrades from trickling filter to activated sludge increased removal efficiency of natural hormones from the final effluent (Barber et al. 2012). Other tertiary treatments in the wastewater treatment process, including activate sludge, carbon filtration, and ozonation remove hormones and a variety of EDCs to varying degrees but rarely with 100% removal (reviewed by Auriol et al. 2006)

In addition to natural estrogens, pharmaceuticals, agricultural waste, and industrial byproducts can all contribute to estrogenic potency of surface waters. Pharmaceuticals prescribed for birth control or depression-related symptoms enter wastewater through human excretion and notable pharmaceutical environmental estrogens include the previously mentioned EE2 (Länge et al. 2001; Parrott and Blunt 2005; Kidd et al. 2007) and the selective serotonin reuptake inhibitor fluoxetine (Foran et al. 2004; Mennigen et al. 2008). Agricultural areas, particularly those with concentrated animal feeding operations (CAFOs) for cattle, poultry or swine are often substantial source of androgens and estrogens (Lange et al. 2001; Soto et al. 2004). In agricultural settings, estrogens and other hormones are primarily released in animal waste and enter the aquatic environment thorough runoff from manure piles or via fields fertilized with manure (Finlay-Moore et al. 2000; Lange et al. 2002). Industrial compounds including bisphenol A and nonylphenol can also bind and activate the estrogen receptor (Gaido et al. 1997; Takayanagi et al. 2006). These and other industrial compounds mimic sex hormones and have led to reproductive and developmental problems in fish (Gray and Metcalfe 1997; Warner and Jenkins 2007).

The mechanisms responsible for intersex in fish are not known; however, a number of factors such as endogenous steroid hormones, temperature, pH, and EDCs can influence sex differentiation in fish (reviewed by Devlin and Nagahama 2002). Steroid hormones are critical during sexual differentiation and direct the gonad to develop into a testis or an ovary during gonadogenesis in gonochoristic fish (Piferrer 2001). Exposure to exogenous steroid hormones (such as estrogens or androgens) during the period before or during sex differentiation (the labile period) can result in sex reversal (Jobling et al. 1998; van Aerle et al. 2002; Lange et al. 2009). Sex reversal via hormone treatments has long been used in the aquaculture industry to produce fish of all one sex (Hunter and Donaldson 1983; Piferrer 2001). Intersex is not well understood but may be a condition of incomplete sex reversal. Occurrence of intersex in wild fish appears to fluctuate seasonally. Blazer et al. (2007) reported a higher prevalence of intersex in smallmouth bass *Micropterus dolomieu* from the Potomac River drainage basin (West Virginia, USA) during prespawn-spawning season than postspawn season. Blazer suggests seasonal differences may be attributed to testicular oocytes released with sperm during mating, so fewer testicular oocytes are observed in postspawn months.

The intersex condition has individual as well as potential population-level implications; intersex fish have been shown to have decreased sperm production, sperm motility and fertilization success compared to normal males (Jobling et al. 2002b). Harris et al. (2011) used DNA microsatellites of male intersex roach to assign parentage in breeding scenarios. Intersex male roach were able to breed with varying success, but the males with more severe intersex (i.e.

higher numbers of oocytes) had a decrease of up to 76% of reproductive performance when compared to normal males. Few studies have investigated the population-level effects of environmental estrogens but in a unique approach, Kidd et al. (2007) describe effects of EE2 addition in a whole-lake ecosystem. The study was conducted over a 7-year period in the Experimental Lakes Area of Ontario, Canada. The lake was dosed weekly with EE2 to maintain a target concentration of 6 ng/L. The experimental lake (Lake 260) was studied for two years prior to EE2 exposure, three years during the EE2 exposure and for two years after EE2 exposure had ceased. Kidd et al. (2007) reported the impact of EE2 on the sustainability of the fathead minnow population and in the first year, EE2 exposure resulted in feminization of male fathead minnows. Symptoms included vitellogenin induction, intersex gonads and loss of secondary sex characteristics such as breeding colors and tubercles. Within two seasons of the initiation of EE2 exposure, the fathead minnow population collapsed due to reproductive failure and increased mortality (Kidd et al. 2007). However, another cyprinid species (pearl dace, *Margariscus margarita*) also present in the Lake 260 study, showed a more stable population abundance following the EE2 exposure (Palace et al. 2006), leading to questions regarding differences in species sensitivity to EDCs.

Exposure to low concentrations of exogenous estrogens during sexual differentiation can result in feminization of genetically male fish and exogenous androgen exposure can lead to masculinization of female fish (Jobling et al. 1998). Feminization of male fish can include loss of secondary sex characteristics, induction of vitellogenin, decrease in gonadal somatic index (GSI), and absence of important reproductive behaviors (Sumpter and Jobling 1995; Länge et al. 2001; Parrott and Blunt 2005; Colman et al. 2009). Masculinization of female fish can include changes in behavior, altered GSI and changes in body morphology (Bortone and Cody 1999;

Parks et al. 2001; Leusch et al. 2006). Research investigating early life stage exposures to estrogens, male fathead minnows showed increased vitellogenin and ovarian duct formation in the testes. These results were induced by a low-dose EE2 exposure from 10 and 15 days post hatch (van Aerle et al. 2002). Länge et al. (2001) found that increased rates of gonadal abnormalities (i.e. intersex), increased vitellogenin and female-skewed sex ratios were present in fathead minnows exposed to EE2 as larvae. Decreases in egg fertilization rates and loss of male secondary sex characteristics in adults were also documented in male fathead minnows exposed to EE2 as larvae (Parrott and Blunt 2005). A more recent study by Anke Lange et al. (2009) identified putative sex reversed males in roach exposed to EE2 from fertilization for two years. The 4 ng/L EE2 exposure resulted in an all-female test population with a cohort (presumably sex reversed males) with a lower stage of ovarian development than the control group (Lange et al. 2009).

Early endocrine disruption studies consistently identified hormonal pollution associated with municipal wastewater effluent in the aquatic environment to be the cause of negative effects to developmental and reproductive processes in fish (Purdom et al. 1994; Tyler et al. 1998; Jobling et al. 1998; Jobling et al. 2002a). Incidence of intersex in roach ranged from 4 to 100% in rivers across a large geographic area of the United Kingdom and the condition was anecdotally linked with wastewater effluent (Jobling et al. 1998). In the US intersex has been observed in many water bodies throughout the country in a variety of fish species (Harshbarger et al. 2000; Folmar 2001; Woodling et al. 2006; Hinck et al. 2009; Ingram et al. 2011) and often coincided with wastewater effluent. The occurrence of intersex in white suckers *Catostomus sommersoni* was greater in Colorado streams dominated by wastewater effluent (Vajda et al. 2008) and the incidence of intersex in smallmouth bass in the Potomac River drainages has been associated with wastewater effluent as well (Blazer et al. 2007); however, background rates of intersex in these species are unknown. Jobling et al. (1998) suggested a background rate of intersex at approximately 4% in roach, and Komen et al. (1989) suggested a background rate of intersex of up to 5% in common carp *Cyprinus carpio*. A background rate of 0.5% in bream *Abramis brama* (Hecker et al. 2002) has been recorded and background rates as high as 55% have been reported in java tilapia, *Oreochromis mossambicus* (Marchand et al. 2010). However, Blazer et al. (2007) and Blazer et al. (2012) suggest that no level of intersex is 'natural' in gonochoristic species and questions remain about the potential impact of intersex at the population level for affected fish species.

In a recent review of wild intersex fish from 54 field studies, 37 different fish species from 17 families were identified as intersex males spanning the US, Mexico, Canada, Europe, Scandinavia, South America, Asia, South Africa and the Middle East (reviewed by Bahamonde et al. 2013). Furthermore, 15 of the 54 intersex field studies reported intersex in 'reference' sites. For example, Blazer et al. (2012) observed 11% intersex smallmouth bass from the Gauley River reference site in West Virginia, which had low surrounding human density, no input from wastewater, no input from animal feeding operations and few numbers of animals in the catchment basin. Intersex greenside darter *Etheostoma blenniodes* and rainbow darter *E. caeruleum* were reported with 33 and 50% intersex rates, respectively, from upstream of wastewater effluent and urban development reference sites from the Grand River in Ontario (Tetreault et al. 2011). In a survey of intersex in gudgeon *Gobio gobio*, Van Aerle et al. (2001) reported a 6% intersex rate in the United Kingdom for lakes selected as reference sites because they received no direct wastewater effluent. This leads to questions regarding a 'natural' rate of intersex as intersex is found in these relatively unimpacted reference areas.

Hinck et al. (2009) reported that intersex largemouth bass *Micropterus salmoides* were found in rivers across the US. Four of the 16 species examined had intersex individuals. Intersex was most common in largemouth bass in the southeastern US. Southeastern collection sites included the Apalachicola, Savannah and Pee Dee River Basins and male intersex rates were as high as 91% among certain sample sites. Causes for the intersex condition were not presented in this study and the authors did not analyze water samples for the presence of estrogens (natural or synthetic) or other hormones that have previously been associated with the intersex condition. Organochlorine residues, 2,3,7,8-tetrachlorodibenzo-p-dioxin-like activity (TCDD-EQ), total mercury, DDT metabolites, PCBs and *trans*-nonachlor were the most frequently detected chemicals among southeastern sample sites, but occurred regardless of the presence of intersex fish (Hinck et al. 2008; Hinck et al. 2009). Thus intersex among these fish may not be related to endocrine disrupting chemicals.

### *Research rationale*

The disproportionate incidence of intersex largemouth bass in the southeastern US (Hinck et al. 2009) provided preliminary information regarding the occurrence of intersex in southeastern river basins in the US, but did not establish a background rate of intersex among largemouth bass or other black basses. Further investigation of the extent and distribution of intersex in the Southeast is necessary to understand possible causes for the prevalence of this condition in waters of the region. Further sampling is also required to determine a background rate for intersex in largemouth bass. While environmental estrogens and other EDCs have been demonstrated to induce intersex and other forms of endocrine disruption in fish, causes of intersex in lakes and impoundments without municipal wastewater effluent or agricultural wastes are not well understood. Additional surveys focused on lakes and impoundments, in addition to the previous work in rivers, are critical for understanding background intersex rates for fish species and identifying specific factors that may be contributing to the pervasiveness of intersex in southeastern US waters.

This dissertation is divided into three research chapters focused on endocrine disruption and intersex in fish from Georgia impoundments and possible causative factors associated with these anomalies. Chapter 2 describes the distribution of intersex largemouth bass in impoundments across Georgia and has been submitted and accepted for publication by *Transactions of the American Fisheries Society*. Chapter 3 describes possible causative factors for the intersex condition in impoundment largemouth as well as determining the probability of individual fish being intersex from impoundments sampled in chapter 1and will be submitted to *Environmental Science and Technology* for publication. Chapter 4 investigates the role of nitrate as an endocrine disruptor in fish and will be submitted to *Environmental Toxicology and Chemistry* for publication.

### *Chapter 2 Overview*

Despite intensive research on environmental estrogens and wastewater effluent effects on fish, many questions still remain regarding the implications of EDC exposure during sensitive life stages and the effects at fish population levels. While intersex black basses have been observed in Georgia rivers (Hinck et al. 2009; Ingram et al. 2011) and intersex largemouth bass predominated in southeastern US waters, a more intensive survey of the extent and distribution of intersex largemouth bass in Georgia impoundments would provide valuable insight into patterns of intersex and sites where intersex is most prevalent. Determining a baseline rate of intersex is critical to provide additional evidence to determine if the intersex is exclusively linked to estrogenic substances in the water. Eleven impoundments containing largemouth bass of various sizes and multiple landuse patterns were selected among four different physiographic regions across Georgia in an effort to sample intersex in areas of least impact. Impoundments were free of wastewater effluent and received little agricultural input. My primary objective for this study was to establish a background intersex rate for largemouth bass as intersex is thought to be an abnormal condition in gonochoristic species. Understanding the extent and distribution of intersex fish in the environment is a critical first step toward developing a management strategy to address any abnormal conditions.

### *Chapter 3 Overview*

We discovered high rates of intersex in largemouth bass from many of the impoundments sampled across Georgia (Chapter 2); therefore, the objective of Chapter 3 was to investigate the potential factors contributing to high rates of intersex in Georgia impoundments. We examined the potential for sediment and water from the impoundments to cause endocrine disruption in fish. Sediment was collected from impoundments where the highest rates of intersex largemouth bass were observed and laboratory-based exposures with fathead minnows were used to determine effects of EDCs bound to sediment. This was of particular concern for largemouth bass as they are nest building fish and eggs will develop in direct contact with possibly contaminated sediments. Endpoints in this 9-month full lifecycle study were gonadal somatic index (GSI), growth, vitellogenin and 11-ketotestosterone (11-KT) for male fish only.

The second part of this study was to identify physical, chemical and environmental factors associated with the intersex largemouth bass from impoundments. We examined characteristics of the impoundments and the fish collected from them and used binomial mixed models to examine relationships between these variables and the incidence of intersex. Impoundment level factors included in the models were LC-MS data for natural and synthetic estrogens in the water, non-specific total estrogenic potency of the water, nitrate concentrations, surface area, depth and Secchi measurements. Fish level variables included length, GSI, liver somatic index (LSI), catch-per-unit effort and relative weight.

### *Chapter 4 Overview*

My final objective was to investigate nitrate as a potential factor contributing to endocrine disruption in fish. In Chapter 2 we discovered that intersex rates were highest in small, eutrophic impoundments and we hypothesized that nitrate may have been a factor. We conducted a full lifecycle nitrate exposure in the lab with fathead minnows to assess the physiological effects of nitrate on developing fish. Endpoints assessed at sexual maturity included survival, growth (weight), condition factor, vitellogenin production, 11-KT and intersex.

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# CHAPTER 2

# SURVEY OF INTERSEX LARGEMOUTH BASS *MICROPTERUS SALMOIDES* FROM IMPOUNDMENTS IN GEORGIA USA[1]

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[1] Kellock KA, Trushel, BE, Ely PC, Jennings CA and Bringolf RB. 2013. Submitted to *Transactions of the American Fisheries Society*, 3/29/13.

## ABSTRACT

Intersex fish are increasingly being reported in water bodies around the world, primarily in rivers that receive treated wastewater; few studies have investigated intersex in waters that do not receive wastewater. In a recent reconnaissance survey of intersex fish in North America, a high rate of intersex was reported for largemouth bass *Micropterus salmoides* in some southeastern US rivers; however, the spatial extent of intersex has not been well-described for impoundments on a statewide scale. Therefore, our objective for this project was to survey the occurrence of intersex largemouth bass in a variety of impoundment habitats across Georgia. Largemouth bass were collected from 11 impoundments without direct wastewater or agricultural inputs. Gonads from all male largemouth bass were evaluated for the incidence and the severity of the intersex condition based on presence and arrangement of testicular oocytes. Overall 48% of male largemouth bass collected from impoundments were intersex. Among largemouth bass from impoundments, incidence of intersex ranged from 0 - 82% and surface area of the impoundment was a strong predictor of intersex incidence (R = 0.88, p = 0.0003, n=11). Population-level effects of intersex and causative factors of endocrine disruption in the impoundments remain unknown, but the high incidence of intersex males in small impoundments suggests that factors other than municipal wastewater or agricultural effluents typically associated with intersex are involved.

# INTRODUCTION

Intersex fish (males with testicular oocytes) have recently been documented in rivers throughout the United States (Woodling et al. 2006; Vajda et al. 2008; Hinck et al. 2009) and Europe (Jobling et al. 1998; Allen et al. 1999; van Aerle et al. 2001). For gonochoristic fish, intersex is an abnormal histological condition (Hecker et al. 2006) and intersex in the US and Europe has often been linked with hormonally active compounds in surface waters such as natural and synthetic hormones. Treated municipal wastewater is commonly discharged into surface water and has been reported to contain a host of steroid hormones (estrogens and androgens), pharmaceuticals, industrial chemicals, and pesticides (Desbrow et al. 1998; Kolpin et al. 2002; Ternes et al. 2004). Many of these compounds are capable of interfering with normal endocrine function in wildlife and humans and are collectively known as endocrine disrupting chemicals (EDCs) (Colborn et al. 1993). Fish exposed to EDCs, including natural and synthetic hormones, at environmentally relevant concentrations in the laboratory have developed gonadal and reproductive abnormalities including intersex (Länge et al. 2001; Parrott and Blunt 2005; Lange et al. 2008). Early-life exposure to EDCs appears to be critical to inducing abnormalities that may not become apparent until sexual maturity. In most fishes, the period of sexual differentiation occurs shortly after hatch and is especially sensitive to alterations in the levels of androgens, estrogens or both in the developing fish and such alterations can result in feminization of males, intersex, or complete sex reversal (Jobling et al. 2002a; van Aerle et al. 2002; Lange et al. 2009). Jobling et al. (2002) reported that male fish with testicular oocytes had significantly lower sperm production, reduced sperm motility and lower fertilization success compared to normal males, and increased severity of intersex was correlated with a decrease in number of viable offspring produced.

Though not well-studied to date, the intersex condition has individual as well as population-level implications. In a unique 7-year whole-lake study, Kidd et al. (2007) dosed an experimental lake with an environmentally relevant concentration of 4-6 ng/L ethynylestradiol (EE2) and reported a collapse of the fathead minnow *Pimephales promelas* population by the third year of dosing. Recruitment failure among the fathead minnows was a result of impaired reproduction and increased mortality. Harris et al. (2011) reported that in competitive breeding scenarios, severely intersex wild roach *Rutilus rutilus* showed a 76% reduction in fertilization success compared to normal males. In addition to potential effects on population size, decreased reproductive fitness in intersex males may lead to reduced genetic diversity in populations because fewer individuals are contributing to the gene pool as well as decreased recruitment (Harris et al. 2011).

Recent reports of high (to 100%) intersex rates in male black bass *Micropterus* spp. from various sites across the southeastern (Hinck et al. 2009; Ingram et al. 2011) and eastern (Blazer et al. 2007; Iwanowicz et al. 2009) US have all involved fish from riverine environments. Recently, Blazer et al. (2012) described significant correlations between land-use characteristics (percent agriculture and animal density) and intersex prevalence in smallmouth bass *Micropterus dolomieu* in Potomac River catchments. Interestingly, Blazer et al. also reported up to 11% intersex among smallmouth bass collected from a reference river with low human density, no wastewater effluent and little agricultural input. A background rate of intersex occurrence in black basses has not yet been defined; however, Jobling et al. (1998) suggested that intersex may occur at approximately 4% in roach, and Komen et al. (1989) suggested a background rate of intersex of up to 5% in common carp *Cyprinus carpio*.

Despite the high intersex rates reported by Hinck et al. (2009) for some southeastern US rivers, to date, intensive intersex surveys of impoundments are lacking and intersex rates have not been reported for largemouth bass in lakes, ponds or impoundments. These types of habitats generally receive little or no direct discharge of municipal wastewater and may provide insight to background levels of intersex in largemouth bass and other species. Therefore, our objective was to intensively survey the spatial distribution and severity of intersex in largemouth bass from lentic waters in Georgia, USA.

## METHODS

### *Sampling and field procedures*

Largemouth bass were collected from 11 impoundments across Georgia (Figure 2.1) in February – June of 2010 and 2011. Impoundments ranged in surface area from approximately 1 to 18,200 hectares (ha) and spanned four physiographic regions (Table 2.1). Designated uses for impoundments included hatchery ponds for grow out of catchable size fish, public fishing areas, private fishing ponds, flood control, and multipurpose reservoirs.

At each site, we attempted to capture ≥15 adult (> 200 mm) male largemouth bass by boat electrofishing. Upon collection, fish were held (<5 hours) in aerated live-wells until processing. Fish were euthanized in the field with an overdose (100 mg/L) of buffered tricaine methane sulfonate. Total length (to nearest cm) and weight (to nearest g) were recorded and individual fish were placed in uniquely labeled plastic bags on ice and transported to the University of Georgia Aquatic Science Laboratory for necropsy. Fish were dissected for positive identification of sex and subsequent removal of gonads. Entire testes were removed, weighed, positioned longitudinally in tissue cassettes, and fixed in 10% neutral buffered formalin for histological processing and analysis. Gonadal somatic index (GSI) values, liver somatic index (LSI) values and relative weight (Wr) values were calculated for all males at all sample sites and categorized according to water body type: river or impoundment. GSI was calculated by:

$$\text{GSI} = (\text{gonad weight (g)} / \text{total weight (g)}) \times 100.$$

LSI was calculated by:

$$\text{LSI} = (\text{liver weight (g)} / \text{total weight (g)}) \times 100.$$

Relative weight was calculated from standard weight equations adapted from the Georgia Department of Natural Resources (Weaver 1981). Catch-per-unit effort (CPUE) was used as a measure of fish density and calculated by total number of fish caught divided by fishing time in hours.

### *Histological Analysis*

Confirmation of oocytes in testicular tissue can only be made microscopically, thus histological processing of testicular tissue was completed at the Veterinary Medicine Diagnostic Lab at the University of Georgia. Briefly, the preserved testes were dehydrated in alcohol, embedded in paraffin wax, sectioned at 5 µm, mounted on glass microscope slides, and stained with hematoxylin and eosin. Sectioned and stained testes were examined under a light microscope for count and arrangement of oocytes. Intersex was positively identified when at least a single oocyte was observed (Figure 2.2), and total number of oocytes was recorded for each intersex fish. A subsample of 40 randomly selected slides was analyzed by a second qualified reader for 98% agreement for the presence of oocytes in testicular tissue and 93% agreement for number of oocytes present in the entire longitudinal section. Severity of intersex was assigned a value (1-4) according to a modified version of the index reported by Blazer et al.

(2007). We examined an entire single longitudinal section of the testis, rather than multiple cross sections. All testes had a length of at least 5X diameter, which provided for surface area of a single longitudinal section that was at least equivalent to the surface area of 10 cross sections, for which Blazer et al. (2007) reported a > 90% probability of detecting oocytes, even in samples with few testicular oocytes (i.e., low severity). Briefly, severity rank 1 was a testis section with a single oocyte, severity rank 2 was a testis with multiple singular (i.e., not clustered) oocytes, severity rank 3 was defined by clustering of 2-5 oocytes, and severity rank 4 was identified by multiple clusters in multiple areas within the longitudinal section.

### *Statistical Analyses*

We used linear regression to evaluate the relationship between impoundment size and incidence and severity of intersex. The Kruskal-Wallis test was used to compare intersex severity among impoundments and to compare number of oocytes observed from impoundments where intersex was present. A paired t-test was used to compare normal male and intersex males among all impoundments for fish length, GSI values, LSI values and Wr. No transformations were required for analysis. We performed all statistical analyses with SAS Version 9.3 (SAS Institute, Cary NC) statistical analysis software. All statistical comparisons were made at $\alpha = 0.1$ level of significance.

## RESULTS

Among 11 impoundments sampled in Georgia, USA 157 male largemouth bass were collected. All fish appeared in good health; gross abnormalities or lesions were not observed on any fish. Histological evaluation indicated that all fish were undergoing active spermatogenesis and were in pre-spawn or spawning condition.

Intersex was observed in 76 of the 157 (48%) male largemouth bass collected from impoundments. Incidence of intersex varied substantially (0 to 82%) among impoundments (Figure 2.3) and was greatest in the smallest impoundments. For example, all impoundments < 8 ha had ≥ 67% intersex males while intersex was < 21% in all impoundments > 80 ha. Intersex incidence was strongly negatively correlated (R = 0.88, p = 0.0003, n=11) with impoundment surface area (Figure 2.4).

Intersex severity varied among fish from 0 (no intersex) to 4 (most severe) among impoundments (Table 2.2); however, no significant differences in severity were detected among intersex fish (df = 8, H = 11.72, p = 0.16). Oocyte numbers ranged from 1-401 among intersex fish. Mean number of oocytes varied from 3 - 73 across all impoundments where intersex occurred (Table 2.2), but variability in number of oocytes was high within an impoundment and there was no statistical difference in mean number of oocytes among impoundments (df = 8, H = 12.54, p = 0.12).

Mean (± standard deviation; SD) length of intersex fish from impoundments (28.3 ± 4.5 cm) was significantly lower (df = 8, t-value = 2.00, p = 0.08) than length of normal males from impoundments (31.3 ± 4.7 cm) (Figure 2.5). Intersex males were not differentiated from normal males by gonad size, liver size, or body condition. Mean (± SD) GSI values were not statistically different (n = 8, t-value = 0.18, p = 0.86) between normal (0.24 ± 0.03) and intersex (0.24 ± 0.04) males collected from impoundments. Mean LSI did not differ (n = 8, t-value = -0.98, p = 0.35) between normal (0.85 ± 0.05) and intersex (0.90 ± 0.08) males from impoundments. Lastly, mean Wr did not differ (n = 8, t-value = 0.40, p = 0.69) between normal (98 ± 2.5) and intersex (96 ± 2.9) males in impoundments.

## DISCUSSION

Intersex fish, including largemouth bass, have most often been associated with riverine habitats that receive wastewater effluent; however, this study demonstrates that intersex fish are also found extensively in some impoundments, even those that do not directly receive wastewater, runoff from confined animal feeding operations, or inputs from other types of intensive agriculture activities. The overall intersex rate of largemouth bass sampled from impoundments in this study was 48%, which is as high as or higher than recent reports of intersex largemouth bass collected from southeastern river basins (Hinck et al. 2009). Intersex rates ranged among the 11 impoundments ranged from 0-82%. Factors or potential stressors contributing to high rates of intersex largemouth bass from impoundments in the present study are not yet understood.

Few studies have reported intersex rates for fish from lentic habitats and none have reported intersex rates for black basses from lentic waters. Kavanagh (2004) reported 22-83% intersex in white perch *Morone americana* from lentic sites that received wastewater (Bay of Quinte, Lake St. Clair and Cootes Paradise in the lower North American Great Lakes region) but the same study found no intersex in the same species collected from a lentic reference site (e.g., without wastewater) at Deal Lake, New Jersey, USA. Biologists and resource managers have experienced difficulty determining background rates of intersex, but the absence of intersex in some impoundments in the present study suggests that any ‘background’ rate of intersex may be low for largemouth bass. Blazer et al. (2007; 2012) suggested that no level of intersex is ‘natural’ in gonochoristic species such as black basses and questions remain about the potential impact of intersex at the population level for black basses. Furthermore, not all fish species sampled from the same collection site will exhibit intersex. Baldigo et al. (2006) reported intersex largemouth bass and smallmouth bass, but no intersex was observed in brown bullheads *Ameiurus nebulosus* collected from the sites along the Hudson River in New York, USA.

Impoundments in the present study ranged in size and designated usage with no human habitations within 200 m of any of the smaller impoundments, thus hormones and other EDCs from leaky septic systems were unlikely to be a factor. All impoundments were located in largely forested areas with minimal agricultural influence and, although some small impoundment watersheds included pastures within 50 m of the shoreline. No impoundment allowed livestock direct access to the water; however, small impoundments located in agricultural areas may be subject to runoff that contains hormones from confined animal feeding operations, as well as pesticides and other endocrine disrupting substances as observed by Orlando et al. (2004), Kolodziej and Sedlak (2007) and Blazer et al. (2007) in previous studies. We reported CPUE rates for largemouth bass collected were highest in the smaller impoundments (Table 2.1), indicating that fish densities were greatest in those bodies of water. Fish, as well as all vertebrates, excrete natural estrogens in urine and feces, so the high fish densities may have led to relatively high concentrations of natural estrogens in the water, sediments, or both. Our ongoing work in the impoundments includes assessment of natural estrogens and total estrogenic activity in water and sediment, and other factors that may be associated with intersex.

To date, estrogenic compounds and EDCs have been frequently associated with intersex, and few efforts have explored other factors that may be involved in development of intersex.

Small impoundments, in particular, are characterized by unique biotic and abiotic conditions in that may contribute to the occurrence of intersex. Nitrate is a ubiquitous pollutant in small impoundments and has been implicated as a potential endocrine disrupting chemical in fish (Edwards and Guillette 2007; Hamlin et al. 2008). Nitrate levels were not quantified at the time of fish collections for the present study but several of the landowners/managers of the smaller impoundments reported that they added inorganic fertilizer to stimulate primary productivity and enhance fish growth, a common pond management technique in the southeastern US. Anecdotally, the smaller impoundments generally appeared eutrophic to hypereutrophic based on the appearance of algal blooms (greenish color of water). Additionally, common cyanobacteria *Microcystis* have been reported to produce an estrogenic compound that induced vitellogenin mRNA in male fish (Rogers et al. 2011). *Microcystis* and other cyanobacteria frequently bloom in several of the ponds where we observed high rates of intersex bass and, therefore, should be explored for a potential role in intersex. Hinck et al. (2009) speculated that intersex is likely influenced by factors other than EDCs such as species sensitivities, timing of exposure or multiple exposures, and unique environmental factors. Hinck et al. (2009) also reported that intersex rates were consistently higher in largemouth bass than other fishes (i.e., common carp, channel catfish *Ictalurus punctatus*). Additional research is warranted to determine the factors associated with high rates of intersex in largemouth bass from small impoundments and other habitats and to determine if other species in small impoundments have intersex rates similar to those of largemouth bass.

The present study has provided an extensive descriptive intersex fish data set at a statewide scale. Our results have generated many questions about the factors associated with intersex, particularly in impoundments, and further sampling and analyses are needed to better understand if the high intersex rates in small impoundments are unique to the southeastern US or if this is a widespread phenomenon. In a recent intersex review article 54 field studies including 37 different fish species were reviewed and 15 of the 54 reported intersex species were detected in reference sites, leading to questions regarding intersex being a ‘natural’ phenomenon or induced from environmental factors (reviewed by Bahamonde et al. 2013). Our future work will investigate individual fish-level, impoundment-level and landscape level factors that may be associated with the intersex condition. The relatively high catch rates of largemouth bass in small impoundments in the present study suggest that adverse population-level effects had not occurred; however, we currently do not know if intersex largemouth bass spawn successfully. Additionally, because this is the first description of intersex at any of these water bodies, nothing is presently known of the temporal or spatial trends. An increasing rate and severity of intersex may eventually result in decreased reproductive success from reduced sperm production, reduced sperm motility or decreased fertilization success. Given the 76% reduction in fertilization success reported for severely intersex roach (Harris et al. 2011), the high rates of intersex male largemouth bass in small impoundments and some rivers are worthy of additional study.

Black basses, especially largemouth and smallmouth bass, are important species for recreational angling and the fishing industry worldwide, and many black bass species such as guadalupe bass *Micropterus treculii*, shoal bass *Micropterus cataractae* and red eye bass *Micropterus coosae* are the focus of intensive conservation activities in North America. Thus, efforts to understand the occurrence, cause(s), and effect(s) of intersex are a high priority research topic that should be of interest for biologists and natural resource professionals charged with management of these and other species.

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Figure 2.1. Location of impoundment sites where largemouth bass were collected for intersex survey during spring 2010 - 2011 in Georgia USA. Impoundments are designated by circles.

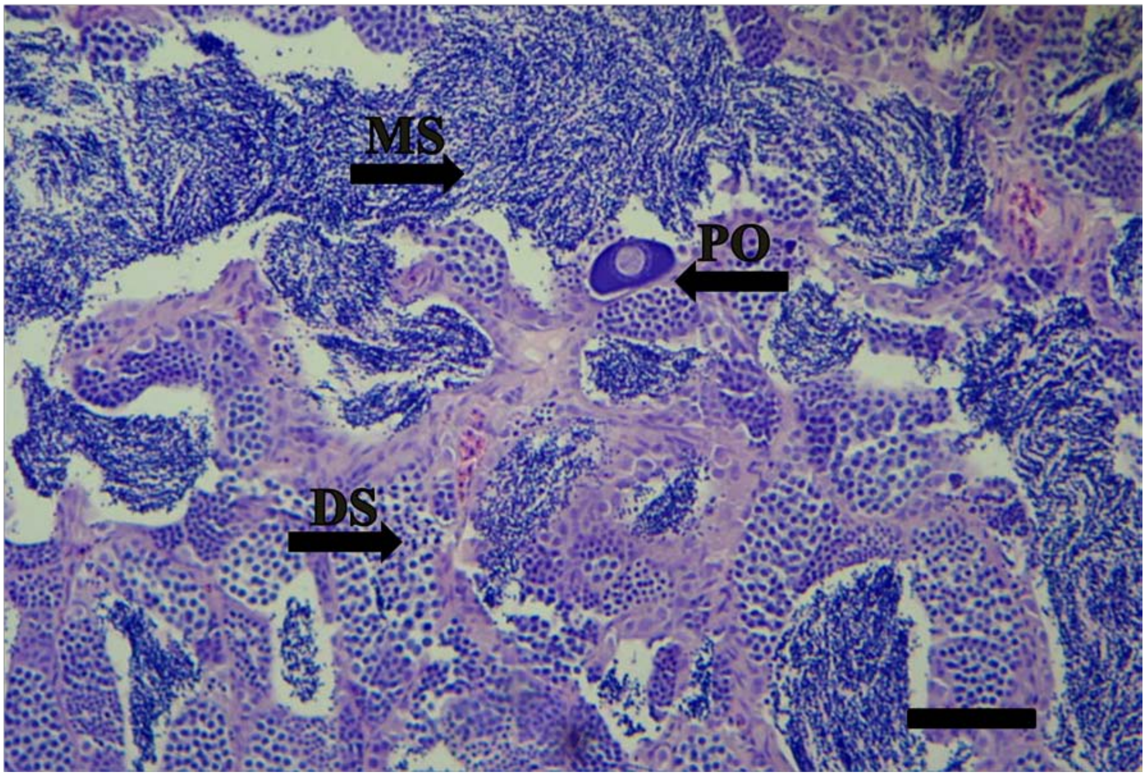

Figure 2.2. Histological confirmation of primary oocyte (PO) development in testicular tissue of a male largemouth bass from a private pond in Hancock County, GA collected in the spring of 2010. Mature sperm (MS) as well as developing spermatocytes (DS) are also observed within the testes. Entire longitudinal sections were read for oocytes. Scale bar = 100µm. H&E staining.

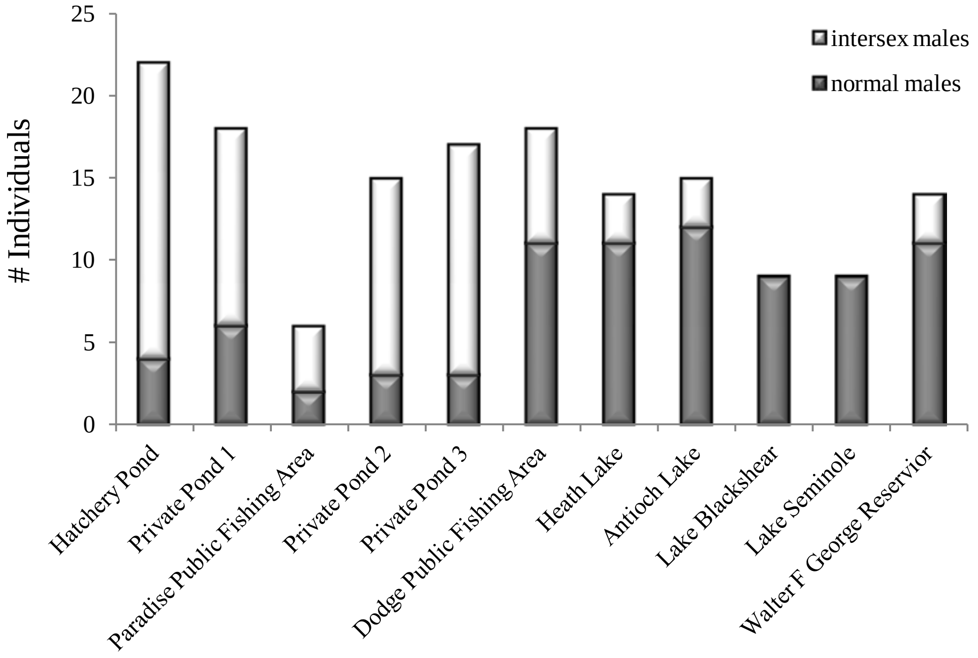

Figure 2.3. Total number of individual male largemouth bass separated into normal males and intersex males sampled at each impoundment site collected during the spring of 2010 - 2011 in Georgia, USA. Impoundment size increases left to right.

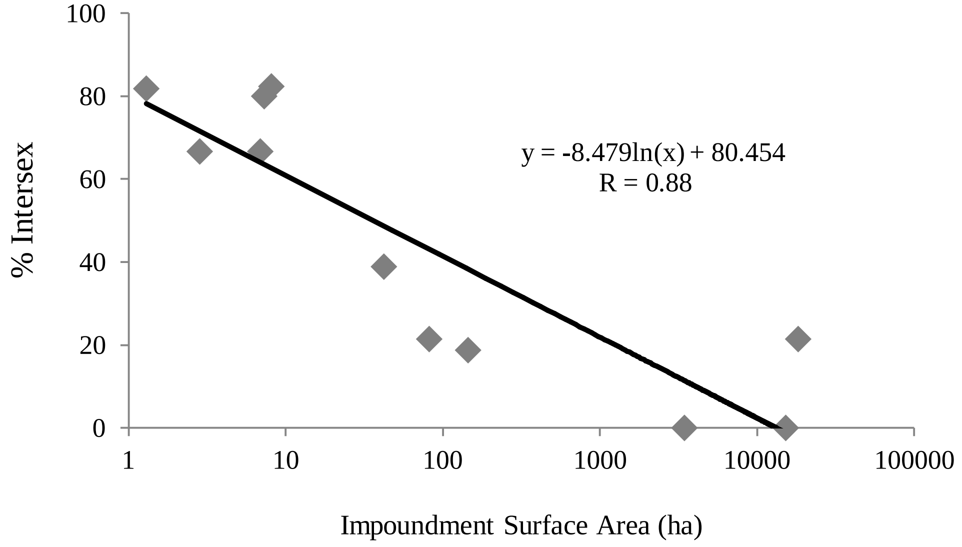

Figure 2.4. Log-linear regression analysis for incidence of intersex largemouth bass related to surface area in hectares (ha) for 11 impoundments sampled during 2010 and 2011 in Georgia, USA.

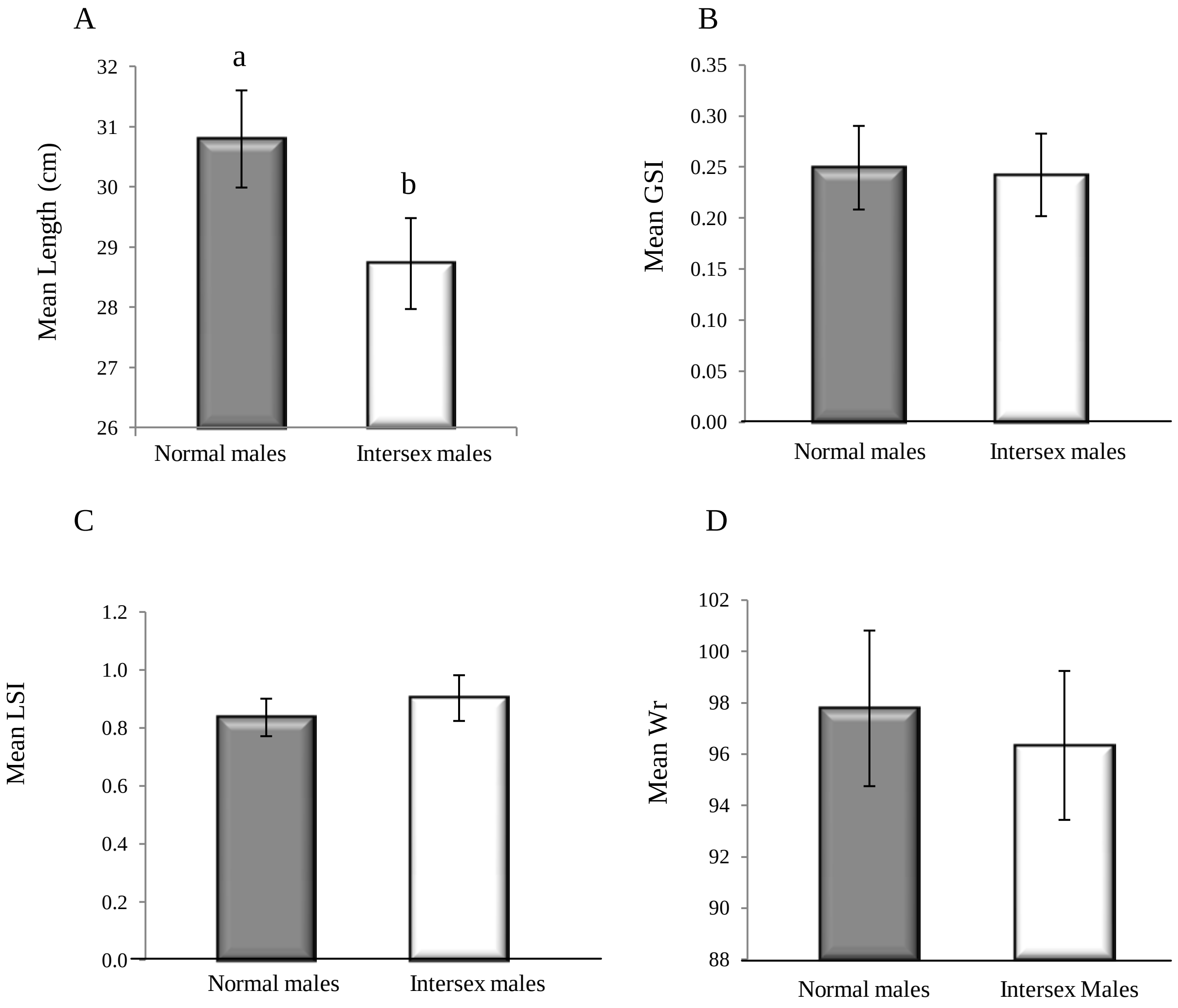

Figure 2.5. (A) Length (cm) (B) mean gonadal somatic index (GSI) (C) mean liver somatic index (LSI) and (D) mean relative weight (Wr) of normal and intersex male largemouth bass collected from 9 impoundments where intersex was observed during spring 2010 - 2011 in Georgia, USA. Intersex male n = 76; normal male n = 81. Error bars = standard error. Statistical differences between intersex and normal males are designated by unique letters. $\alpha = 0.10$

Table 2.1. Characteristics of impoundments sampled for intersex largemouth bass in Georgia, USA during spring 2010 and 2011.

| Impoundment | Size (ha) | County | Physiographic Region | CPUE[1] | Collection Date |
|---|---|---|---|---|---|
| Hatchery pond | 1.3 | Ben Hill | UCP[2] | 36 | 17-Feb-11 |
| Private pond 1 | 2.8 | Wilkes | Piedmont | 28 | 02-Apr, 08-Jun-2011 |
| Paradise PFA[3] | 6.9 | Berrien | UCP | 10 | 1-Apr-10 |
| Private pond 2 | 7.2 | Hancock | Piedmont | 20.7 | 12-Apr-10 |
| Private pond 3 | 8 | Wilkes | Piedmont | 21 | 11-Mar-11 |
| Dodge PFA | 42 | Dodge | UCP | 15 | 19-Apr-11 |
| Heath Lake | 82 | Floyd | Ridge & Valley | 7.5 | 19-May-11 |
| Antioch Lake | 145 | Floyd | Ridge & Valley | 21 | 18-May-11 |
| Lake Blackshear | 3440 | Crisp | UCP | 2.8 | 12-May-10 |
| Lake Seminole | 15,176 | Seminole | LCP[4] | 2.3 | 11-May-10 |
| Walter F George | 18,211 | Clay | UCP | 3.5 | 11-May-10 |

[1] CPUE = catch-per-unit effort

[2] UCP = upper coastal plain

[3] PFA = public fishing area

[4] LCP = lower coastal plain

Table 2.2. Number of intersex individual largemouth bass collected, median (and range) of intersex severity observed, and mean (and range) number of oocytes observed in testicular tissue for intersex males at impoundments where intersex occurred. Severity index of 4 is the most severe. All fish were collected in spring 2010 and 2011 in Georgia, USA.

| Water Body | Number of Intersex Individuals | Median Severity | Number of Oocytes |
|---|---|---|---|
| Hatchery Pond | 18 | 2 (1-4) | 18.2 (1-71) |
| Private Pond 1 | 12 | 2 (1-4) | 56.1 (1-297) |
| Paradise PFA | 4 | 3 (1-4) | 19.8 (1-61) |
| Private Pond 2 | 12 | 3.5 (2-4) | 72.9 (3-393) |
| Private Pond 3 | 14 | 3 (1-4) | 56.9 (1-401) |
| Dodge PFA | 7 | 2 (1-3) | 3.7 (1-9) |
| Heath Lake | 3 | 2 (1-3) | 9.7 (1-24) |
| Antioch Lake | 3 | 2 (1-3) | 16.7 (1-40) |
| Walter F George | 3 | 3 (2-4) | 15.0 (6-33) |

# CHAPTER 3

# FACTORS ASSOCIATED WITH INTERSEX LARGEMOUTH BASS FROM IMPOUNDMENTS IN GEORGIA USA[2]

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[2] Kellock KA, Hazelton PD, Irwin BJ and Bringolf RB. 2013. To be submitted to *Environmental Science and Technology*.

## ABSTRACT

Intersex fish reports are increasing worldwide, from both contaminated surface waters as well as reference sites. Endocrine disruption, including intersex, in fish is often associated with environmental estrogens in treated wastewater but few studies have explored other possible causative factors. We recently surveyed the occurrence of intersex largemouth bass *Micropterus salmoides* in 11 impoundments across Georgia, USA and among these 11 sites the incidence of intersex ranged from 0 to 82%. Our primary goal in this study was to explore potential factors associated with the occurrence of intersex largemouth bass in impoundments. Our first objective was to measure natural and synthetic estrogens as well as non-specific estrogenic potency from each impoundment. Second, we determined effects of sediment exposure to developing fish in a laboratory study to see if chemicals bound to sediments played a role in fish development. Lastly, we used a binomial mixed-model regression to estimate the probability of intersex for individual fish, and we explored potential predictor variables that varied at the level of individual fish, impoundment water quality, and watershed level. 17β-estradiol and estrone were measured in the impoundment and varied between 0.5-5.5 EEQ (estradiol equivalents) (ng/L). Similarly, measures of non-specific estrogenic potency ranged from 1.0 – 5.0 EEQ (ng/L). Lifecycle exposure to some impoundment sediment significantly increased vitellogenin and 11-ketotestosterone in male fathead minnows *Pimephales promelas*. Our model indicated that surface area and depth were the strongest predictors of intersex at the impoundment-level, with the highest probability of intersex in smaller and shallower impoundments, which corroborates observed intersex largemouth bass data among impoundments sampled in Georgia.

## INTRODUCTION

Reports of intersex (oocytes in testicular tissue) fish are increasing worldwide and are most often associated with river waters contaminated with endocrine disrupting chemicals (EDCs) (Jobling et al. 1998; van Aerle et al. 2001; Woodling et al. 2006; Blazer et al. 2007; Vajda et al. 2008; Hinck et al. 2009; Iwanowicz et al. 2009). Wastewater treatment discharge into rivers is a major source of estrogenic mixtures containing natural estrogens, xenoestrogens, pharmaceuticals and anti-androgenic chemicals (Kolpin et al. 2002; Aerni et al. 2004; Thorpe et al. 2006). Agricultural wastewater (e.g., effluent/runoff from confined animal feeding operations) has also been demonstrated to contain hormonally active compounds that cause reduced reproductive fitness of fish (Orlando et al. 2004; Kolodziej and Sedlak 2007; Sellin et al. 2010). Specific estrogens are often measured in water but estrogenic activity has also been reported at approximately 21 - 30 17β-estradiol equivalents (EEQ)/kg in sediments of rivers receiving wastewater effluent while estrogenic activity of overlying surface water was below detection limits (Peck et al. 2004). Sediment deposition of endocrine disrupting compounds may be of particular concern for nest building fishes which deposit eggs on the sediment.

Mechanisms that result in primary oocyte development in testicular tissue (intersex) are not yet known, but many factors can affect sexual differentiation in fish, including dissolved oxygen, pH, behavior, exogenous steroids, pollutants and EDCs (reviewed by Devlin and Nagahama 2002). The occurrence of intersex in fish has been associated with exposure to estrogenic effluents but non chemical factors such as species, season, fish age and length, as well as water quality and land use characteristics may also influence intersex rates (Blazer et al. 2007; Blazer et al. 2012). Oocytes in testicular tissue can range in number from a few to hundreds and are usually in the primary oocyte (cortical alveolar) stage of development and are visible only by microscopic examination. Gonochoristic fishes (e.g., basses, sunfishes, minnows, catfishes, etc.) are defined by genetically distinct, separate sexes (male or female) that differentiate shortly after hatch. Gonochoristic males do not normally contain eggs in their testicular tissue but some authors (Komen et al. 1989; Jobling et al. 1998) have suggested that 'background' rates of intersex may be 5% for some gonochoristic fish species. Blazer et al. (2007) suggested that carrying oocytes provides no evolutionary advantage to males; therefore, a 'normal' background intersex level does not exist for gonochoristic species.

Recently, high rates of intersex have been observed in black basses (largemouth bass, smallmouth bass *Micropterus dolomieu*, etc.) in the United States (Blazer et al. 2007; Hinck et al. 2009; Ingram et al. 2011) with the highest prevalence in southeastern US rivers (Hinck et al. 2009). Hinck (2009) reported an incidence of intersex male largemouth bass *Micropterus salmoides* ranging from 8-91% among riverine collections sites (n=111) across the US. Black basses generally had higher rates of intersex than other freshwater species such as channel catfish *Ictalurus punctatus,* common carp *Cyprinus carpio,* and white sucker *Catostomus commersoni* collected from the same sites but sample sizes were low so the authors recommended that caution must be exercised before drawing any conclusions about species sensitivity. Underlying factors for differences in intersex occurrence among species and locations are currently not well understood, but Blazer et al. (2007) suggested that human population and land use in the watershed of a site may be associated with intersex. Blazer et al. (2007) also reported that individual fish age and length were inversely correlated with occurrence of intersex. Younger and smaller smallmouth bass had higher intersex rates than older, larger individuals of the same species collected in the same areas at the same time. Blazer et al. (2007) also reported that intersex rates varied by season; intersex was more prevalent in spring and fall than in summer, a trend the authors suggested may have been due to shedding of the testicular oocytes during spawning activities.

Kellock et al. (in press 2013) recently reported an intensive statewide intersex survey for largemouth bass in Georgia USA. Unlike most previous intersex studies, Kellock et al. sampled impoundments and reported that male largemouth bass intersex rates ranged from 0 – 82% among the 11 impoundments sampled, even in those with no apparent inputs of municipal wastewater or agricultural effluents. Impoundments varied widely in size and physiographic region but all were in largely forested areas with little surrounding direct agricultural inputs. The purpose of this study was to explore factors associated with endocrine disruption and intersex fish in Georgia impoundments. We investigated waterborne natural and synthetic estrogens among the same 11 impoundments evaluated by Kellock et al. (in press 2013), used laboratory-based fish exposures with sediment collected from the impoundments with high intersex rates, and lastly, we developed a statistical model based on fish, water quality, and watershed factors to predict the probability of intersex for male largemouth bass from a given impoundment.

## METHODS

### *Largemouth bass collection and intersex determination*

Details of largemouth bass sampling, field and laboratory procedures have been previously described in Kellock et al. 2013 (in press). Intersex rates, fish length, GSI, LSI, relative weight and catch-per-unit effort rates for (n = 157) male largemouth bass collected by Kellock et al. 2013 from February – June 2010 and 2011 from 11 impoundments in Georgia, USA were used in this study.

### *Land Use Analysis*

Land use surrounding the 11 impoundments was classified into seven categories: barren land, forest, urban development, agriculture, herbaceous/scrub, open water, and wetland. Land cover was reported as a percentage for each catchment. Catchments were delineated for each impoundment with ArcHydro version 9 for ArcMap 9.3.1; briefly, we used digital elevation models (30-meter resolution) obtained from the National Elevation Dataset (USGS Elevation Dataset) and hydrologic units from the National Hydrology Dataset (USGS Hydrology Dataset) to determine catchment boundaries for a given water body.

### *Impoundment water analyses*

Water quality parameters (temperature, dissolved oxygen, pH) were recorded for all impoundments between 9 and 11am on the day of fish collection. Temperature among impoundments ranged from 14-19 °C, dissolved oxygen ranged from 7.06-8.01 mg/L and pH ranged from 6.66-8.01. One-liter water samples were collected in amber glass bottles from each of the 11 impoundments in March and April of 2013 and transported on ice to the University of Georgia Aquatic Science Laboratory. These dates were specifically chosen as this is when largemouth bass begin spawning and developing eggs/fry would be vulnerable to exogenous estrogens. Nitrate concentrations were measured in 10 ml subsamples with a nitrate kit (LaMotte 3689-SC) and colorimeter (Lamotte, SMART2). The remaining water was filtered through a 0.45µm Whatman filter to remove larger particulates, and then estrogens were extracted on a C-18 solid phase column (Supelco, Sigma-Aldrich Co.). A water blank consisting 1-L of ultrapure water from a MilliQ water purification unit was extracted in parallel with impoundment samples as a reference and a spiked 50 ng/L estradiol sample was included for estrogen recovery confirmation. Briefly, columns for solid phase extraction were prepared by passage of one volume (6 ml) of ethanol, followed by three volumes (6 ml X 3) of deionized water. The one liter sample was then extracted through the column at an approximate flow rate of 1 ml per minute and eluted once with 3 ml ethanol. A control sample (1-L) of MilliQ water was also extracted and eluted with ethanol. The eluted extracts were split into duplicate 1.5 ml subsamples that were used for analytical quantification of select estrogens and an overall measure of estrogenic potency.

Concentrations of the three natural estrogen steroid hormones (estrone (E1), estradiol (E2), and estriol (E3)) and the synthetic estrogen, ethinylestradiol (EE2), were quantified by liquid chromatography coupled with mass spectroscopy (LC-MS) (reviewed by Lopez de Alda et al. 2003) at the US EPA Office of Research & Development Laboratory in Athens, GA. Measures of total estrogenic potency were quantified using the estrogen receptor fluorescence polarization assay (FP assay), developed by and performed at the University of Florida. Briefly, the FP assay is a competitive binding assay using the human estrogen receptor alpha (ERα). A fluorescent tagged estrone molecule acts as the hot ligand in the assay. In the absence of an estrogenic competitor the fluorescent estrone is bound to ERα in solution causing a slow rotation of the large complex. When this fluorescent estrone is excited with polarized light it emits polarized light because of the slow rotation of the large complex. When a competitor is present the fluorescent estrone becomes free in solution and therefore spins faster. When the free fluorescent estrone is excited the fast spin results in depolarized light emission. Measurement of polarized and depolarized emission is used to then calculate the binding affinity for unknown samples. Concentrations of individual estrogens detected by LC-MS were converted to estradiol equivalents (EEQ) by multiplying the actual hormone concentration by the relative potency compared to E2, based on previous studies regarding estrogen receptor binding affinity (Coldham et al. 1997). For example, the relative potency of E2 is 1.0 and E1 has a relative potency (compared to E2) of 0.096.

### *Sediment collection*

Sediment was collected from each of three impoundments (Hatchery pond and Private ponds 1 and 3) in the fall of 2011 where high rates (> 67%) of intersex were previously reported for largemouth bass (Kellock et al. sin press 2013). One to two centimeters of sediment was carefully scraped from three areas of littoral zone habitat in each impoundment and pooled. A total of 10-L of sediment per impoundment was collected in glass vessels and transported on ice back to the University of Georgia

Aquatic Science Laboratory. Sediment was stored at 4°C overnight to settle and the following day excess water was decanted. Sediment from each impoundment was gently homogenized prior to use in fish assays.

*Fathead minnow sediment exposure*

Fathead minnow fry (<24 hours post hatch) were obtained from Aquatic Biosystems Inc. (Fort Collins, CO). For the first five weeks, fry were exposed to respective impoundment sediment in an automated intermittent flow-through apparatus. Fifty milliliters of sediment from each impoundment was added to four replicate 300 ml glass beakers fitted with screened weep holes to allow for water changes. Four water-only replicates were used as controls, reference sediment was not used. Approximately 200 ml of dechlorinated tap water was carefully overlaid on the sediment and the sediment was allowed to settle overnight. The following day, 15 larval fathead minnows were added to each replicate. Remaining sediment was stored in glass vessels at 4°C for later use. 100% of overlying water was renewed 3-4 times per day with the flow-through system and fry were fed live *Artemia* twice daily. Water quality including pH, temperature and dissolved oxygen were measured daily with a Hach HD40d water quality meter. Light cycle was maintained at 16:8 light:dark.

After 5-weeks in the flow-through apparatus the developing fish were transferred to 20-L glass aquaria containing 2-L of impoundment sediment for each of the three sampled impoundments. Four water-only replicate aquaria served as controls, and each of the three impoundment sediments had four replicate aquaria for a total of 16 experimental units with 15 fish per aquaria. Sediment was carefully overlaid with 15-L of dechlorinated tap water, allowed to settle and then aquaria were fitted with aquarium filters, prior to adding the fish. The juvenile fish were fed *Artemia* twice daily and transitioned to flake food (TetraMin) at approximately 3-months-old. Water quality including pH, temperature and dissolved oxygen were measured daily with a Hach HD40d water quality meter and fish were maintained on 16:8 hour light:dark cycle. Dead fish were removed from aquaria immediately. Fish remained in the 20-L aquaria until the exposure was terminated at 9-months.

*Fathead minnow processing*

Upon the termination of the sediment exposures, fish were euthanized by overdose with buffered MS-222 (Argent, Redmond WA). Lengths and weights were used to calculate Fulton's condition factor (K) using the formula:

$$K = (\text{weight (g)} / \text{Length (mm)}^3) \times 100{,}000.$$

Gonads were dissected and weighed for males and females to calculate gonadal somatic index (GSI) as calculated by:

$$GSI = (\text{gonad weight (g)} / \text{weight (g)}) \times 100.$$

For male fish, (n = 5 per replicate) blood was collected from the caudal vein into heparinized capillary tubes. Blood samples were pooled by replicate and were centrifuged (2000 rpm) for ten minutes to obtain plasma, which was transferred to a clean cryovial and stored at -80°C until hormone and vitellogenin analyses.

### *11-Ketotestosterone and vitellogenin analysis*

Plasma samples were allowed to thaw on ice and each was separated into duplicate glass vials. One duplicate sample was used for hormone analysis and the other for vitellogenin analysis. Plasma for 11-ketotestosterone (11-KT) analysis was extracted 3X with a 50:50 mixture of ethyl acetate:hexane equal to plasma volume (~75 µl for each 11-KT and vitellogenin) prior to beginning the assay. The extraction protocol followed the recommended protocol from the kit manufacturer (Cayman Chemical, Ann Arbor, MI). Plasma 11-KT was analyzed according to the protocol provided with the fish enzyme immunoassay 11-KT EIA kit (Cayman Chemical, Ann Arbor, MI). Samples were assayed in triplicate at two dilutions (1:10 and 1:100) and each plate contained a reagent blank in addition to 11-KT standards. All samples for each assay were run at the same time to minimize variance among plates. Absorbance (412 nm) was measured with a SpectraMax M2 plate reader (Molecular Devices, Sunnyvale, CA) and final 11-KT concentrations were determined with Softmax Pro 5 software (Molecular Devices, Sunnyvale, CA).

Vitellogenin was analyzed in plasma with a commercial enzyme immunoassay kit specific for fathead minnows (Cayman Chemical, Ann Arbor, MI), according to the protocol provided by the manufacturer. Plasma samples were analyzed in triplicate at two dilutions (1:50 and 1:5000). Duplicate samples of reagent blanks and vitellogenin standards were included on each 96-well plate. All plates were run simultaneously to minimize variance among plates. Absorbance (450nm) was measured with a SpectraMax M2 plate reader (Molecular Devices, Sunnyvale, CA) and final vitellogenin concentrations were calculated with Softmax Pro 5 software (Molecular Devices, Sunnyvale, CA).

### *Sediment Statistical Analyses*

Analysis of variance (ANOVA) and Dunnett's post hoc test were used to compare differences between control and treatment means for K, GSI, weight, 11-KT and vitellogenin among males. No transformations were required to achieve data normality. Statistical analyses were performed with SAS Version 9.3 (SAS Institute, Cary NC) statistical analysis software. All statistical comparisons were made at $\alpha = 0.05$ level of significance.

### *Statistical modeling*

The sampling approach used by Kellock et al. 2013 (in press) for largemouth bass resulted in a hierarchically structured data set, where individual fish responses were nested within a location grouping (i.e., the impoundments). We treated the 11 sampled impoundments as random effect intercepts as our intended scope of inference extends beyond these specific locations (Bolker et al. 2009). We examined variability in factors at the individual fish levels, water quality level and watershed level as they related to occurrence of intersex. Fish parameters included (GSI), liver somatic index (LSI; liver weight (g)/ fish weight (g) x 100), total length (cm), CPUE (as an index of fish density) and relative weight (Wr) determined by Kellock et al. 2013 (in press). Water quality parameters included nitrate concentration, LC-MS concentrations of estrogens (EEQ), total estrogenic potency measured by the FP assay (EEQ). Watershed level parameters used in models included land use (% forest or % agricultural), impoundment surface area (hectares), Secchi, and impoundment depth (m).

Six candidate models (Table 3.2) were created to represent hypotheses predicting the effects of factors on the incidence of intersex and included three global models, each incorporating all predictive variables for a given level (i.e., Global Fish Model, Global Water Quality Model, and Global Watershed Model). Data were analyzed using logistic regression analysis where occurrence of intersex was a binomial response variable for each fish. Data were standardized to a mean of zero and a standard deviation of one, thus unit changes in regression coefficients were equivalent to a change in one standard deviation of the predictor variable. To avoid mulitcollinearity, Pearson correlations (r) were performed on all pairs of predictor variables with a cutoff of $r = \pm 0.30$; no variables that were significantly correlated were included in the same model. All statistical analyses were conducted in SAS v9.3 (Statistical Analysis Software, Cary NC). To evaluate the relative quality of the models in relation to intersex occurrence, an information theoretic approach was utilized (Burnham and Anderson 2002). Akaike's Information Criteria (AIC) (Akaike 1973) with small sample adjustment (AICc) (Hurvich and Tsai 1989) was calculated for each model. The number of parameters used to estimate AICc values included fixed and random effects. The model with the smallest AICc value was considered the most plausible model. Akaike weights ($w_i$) were calculated to determine the relative importance of the models, with the best approximating possessing the highest $w_i$ value. Models with $w_i$ values within a 10% threshold of the best-approximating model were included as candidate models (Rieman et al. 2006). The general form of the estimation model was:

$$\text{Logit}\,(p_{ij}) = \alpha + \beta_1(\mathit{Surface\ Area}_j) + \beta_2(\mathit{Depth}_j) + R_j \qquad Rj \sim N(0, \sigma^2_R)$$

where: $p_{ij}$ is the probability of intersex for individual ($i$) from a given impoundment ($j$), $\alpha$ is the estimated intercept parameter, $\beta_1$ and $\beta_2$ are the estimated slope parameters for predictor variables: impoundment surface area (ha) and impoundment depth (m), and $R_j$ is the random effects of impoundment site. The site random effect followed a normal distribution with variance $\sigma^2_R$.

## RESULTS

### *Fish Data and Land Use Analysis*

In total, 76 of the 157 (48%) male LMB were intersex (Figure 3.1) and intersex occurrence varied from 0 – 82% across impoundments (Table 3.1) (see Kellock et al. in press 2013 for detailed results). Among impoundments, surface area ranged from 1.3 – 18,000 ha, watershed area ranged from 7.64 – 81,200,000 km$^2$, maximum depth ranged from 2 – 14.2 m, Secchi depth ranged from 0.3 – 1.5 m and largemouth bass CPUE ranged from 2.3 – 36 fish/hr (Table 3.1). Percent forested landuse was >40% among all sampled with agricultural areas ranging between 2-45% among all impoundments (Figure 2). Percent forested area and percent agricultural area for each impoundment were used in the statistical modeling.

*Water Analyses*

All impoundment water samples contained measurable (LC-MS) levels of E2 and E1; however, EE2 and E3 were not present in any of the water samples. Concentrations of E1 were converted to EEQ as previously discussed and summed with E2 to determine total EEQ for each impoundment, which ranged from 0.58-5.51 ng/L EEQ (Figure 3). Dodge Public Fishing Area (PFA) had the highest total EEQ (5.51 ng/L), yet this impoundment did not have the highest rate of intersex largemouth bass (39%). Hatchery pond and Private pond 3 were the two impoundments with highest rates of intersex (82%), and Hatchery pond had approximately 2.8 ng/L EEQ but Private pond 3 had only 0.5 ng/L EEQ. Lake Seminole had no intersex largemouth bass but its EEQ was among the highest of the impoundments at 4.0 ng/L, so estrogens are likely not affecting fish negatively in this water body. Estrogens were below detection limit (0.1 ng/L) for LC-MS water control (Figure 3.3) and estradiol recovery in spiked 50 ng/L samples was 118% among three samples with mean (±SD) estradiol concentration of 59.4 ± 0.87 ng/L. Overall, little or no relationship existed between incidence of intersex male largemouth bass and total LC-MS detected estrogens ($r^2 = 0.00004$) in the impoundments (Figure 3.4A).

The FP assay results provided the overall estrogenic potency of the water at the time of collection (Figure 3.3) and did not measure specific compounds. Measured concentrations ranged from 1.55-4.49 ng/L EEQ ng/L (Figure 3.3). Interestingly, the lowest estrogenic potency was from the hatchery pond, which had a moderate concentration of natural estrogens (as measured by LC-MS) and the highest rate of intersex (82%). Lake Blackshear had the highest estrogenic potency (4.49 EEQ ng/L) but no intersex fish were observed. Little to no relationship was observed between non-specific estrogenic potency quantified by the FP assay ($r^2 = 0.04$) of the impoundment water and incidence of intersex (Figure 3.4B).

*Sediment fathead minnow exposure*

In the sediment exposure, fathead minnow survival was > 90% in all control replicates and survival did not differ among treatments (df = 15, F = 2.53, p > 0.05). Throughout the 9-month exposure, water temperature was 23.6°C ± 0.80 (mean ± SD), pH was 7.6 ± 0.26 and dissolved oxygen was 8.5 ± 0.31 mg/L among all tanks. Across treatments no statistical differences were observed among males for K, GSI, and weight in exposed fathead minnow males (Figure 3.5 panels A, B and C). 11-KT was significantly increased (df = 15, F-value = 2.78, p = 0.05; Figure 3.5D) in fish exposed to Private pond 3 sediment compared to the control group. Increases in 11-KT did not appear to correspond to changes in overall growth (weight) or GSI in any of the exposed fish. Plasma vitellogenin was induced in male fathead minnows exposed to impoundment sediments, but statistical differences among treatment means were only observed between control males and males exposed to Hatchery pond sediment (df = 15, F-value = 3.87, p = 0.03; Figure 5E). Mean plasma vitellogenin was more than double (~116,000 ng/ml) in males exposed to Hatchery pond sediments compared to control males (~54,000ng/ml).

### *Statistical Modeling of Field Largemouth Bass Data*

Of the six models examined to predict incidence of intersex in male largemouth bass, the Impoundment Size model, containing surface area and depth of an impoundment, was the best approximating model (Table 3.3). This model was 7.2 times more likely to describe the relationship between intersex and watershed-level variables than the next best approximating, which was the Length and Density model that included length and CPUE (Table 3.3). The Length and Density model and the remaining four models fell below the 10% candidate model threshold, and were eliminated as plausible possibilities.

Based on the best plausible model, surface area and depth of an impoundment, intersex probability was negatively related to impoundment surface area, with a parameter estimate of -0.7757 (Figure 3.6) and a negative relationship also existed between impoundment depth and incidence of intersex, with a parameter estimate of -1.1472 (Figure 3.6) determined by the statistical software. Overall, smaller (ha) and more shallow (m) impoundments were more likely to have intersex largemouth bass based on our model.

## DISCUSSION

The goal of this study was to investigate possible causative factors contributing to the incidence of intersex and the probability of intersex largemouth bass from impoundments in Georgia. A definitive mechanism(s) for intersex in largemouth in small impoundments was not identified, but some testable hypotheses regarding impoundment characteristics emerged and were addressed in the present study. Collectively, the modeling results indicate that intersex male largemouth bass are most likely to be found in small, shallow impoundments. Natural estrogen concentrations were detectable in all impoundments and were not associated with land use (e.g., agriculture or urbanization), but may have been excreted naturally by the dense fish populations commonly found in small impoundments. This observation agrees with the highest CPUEs observed in the smallest impoundments. Furthermore, vitellogenin was induced in male fathead minnows exposed to sediments from smallest impoundments also having the highest intersex rates. Non-specific measures of total estrogenic activity (i.e., the FP assay) revealed variable measures of activity that were somewhat higher than natural estrogens detected by LC-MS in 7 of 11 impoundments, but overall the range of estrogenic potency was similar to the range of estrogen concentrations measured. These measures were a snap-shot of estrogen concentrations at a given time point, but estrogen levels likely vary throughout the year and systematic long-term monitoring is required to determine the extent of variation in estrogenic potency. Additional research is required to determine if this potency is related to the high intersex rates of largemouth bass from small impoundments. Notably, low (ng/L) concentrations of estrogens are routinely reported in municipal wastewater effluents (reviewed by Racz and Goel 2010). Further, Kidd et al. (2007) reported total reproductive failure of fathead minnows in a whole lake study with estrogen concentrations of approximately 4-6 ng/L and six of the 11 impoundments sampled in Georgia had estrogens ranging from approximately 4-6 EEQ ng/L, but largemouth bass population did not appear to be negatively affected at the time. Evidence from these studies and others suggests that estrogens may be an important factor influencing the probability of intersex in some impoundments.

In the present study, 11-KT and vitellogenin were generally elevated in fathead minnows exposed to sediment from impoundments with high intersex rates. If male fathead minnows interacted with sediment that has bioavailable estrogens, induction of vitellogenin would be expected (Panter et al. 1998; Länge et al. 2001; Palace et al. 2002); however, a reduction in 11-KT would have also be expected (Martinovic et al. 2007; Salierno and Kane 2009). It is possible organic matter in the sediments contributed nitrate ($NO_3$) to the aquaria, which has been shown to elevate sex steroids in Siberian sturgeon *Acipenser baeri* (Hamlin et al. 2008) and in fathead minnows (Kellock and Bringolf 2013; chapter 4). Hinfray et al. (2010) reported that sediment extracts collected from three river sites in France were contaminated with aromatase inhibitors resulting in decrease brain aromatase activity in rainbow trout *Oncorhynchus mykiss* microsomes. Aromatase is a critical enzyme required to convert testosterone to estradiol and inhibition of the enzyme would result in a higher ratio of androgens to estrogens. Plasma estradiol levels were not assessed in the current study, but would be useful to understand if increases in testosterone, and subsequently, estradiol and 11-KT are related. The partition coefficients ($K_{ow}$) of E1, E2, E3 and EE2 all range from 3.1 - 4.2, suggesting estrogens from the water column adsorb bed sediments; thus sediments may become a sink for estrogenic and other EDCs. Peck et al. (2004) suggested that while surface water concentrations of natural and synthetic estrogens generally vary between 1 - 5 ng/L, but bed sediments will sorb waterborne estrogens at higher concentrations. In the Peck et al (2004). study, two UK rivers receiving sewage treatment effluent were assessed for estrogenic chemicals present in the effluent, river water and sediments. The authors measured estrogenic activity of municipal wastewater effluent at 1.4-2.9 EEQ ng/L, while estrogenic activity of river water 1 km upstream and 1 km downstream of the effluent discharge was below detection limits. However, estrogenic activity of sediment in upstream and downstream sites ranged from 21.3-29.9 EEQ ng/L, which is approximately 10-fold higher than undiluted effluent in the river (Peck et al. 2004). Largemouth bass, like other sunfishes, are nest building fish, and 1000s of embryos are deposited directly on the sediment where they incubate for several days before hatching. The availability of sediment-bound estrogens to developing embryos in nests, newly hatched fry, or benthic organisms is not well-understood. The induction of vitellogenin in male fathead minnows exposed to sediments in the present study suggests that the estrogens are available to fish either through uptake across the gills or via dietary exposure. Elevated 11-KT in the male fish's plasma as we observed may also be an indicator that testosterone is also increased because it is converted to 11-KT as well as estradiol, which could contribute to increasing plasma estrogen levels in the fish.

A variety of natural and synthetic compounds such as plant borne estrogens (phytoestrogens), pesticides, pharmaceuticals, surfactants and other industrial compounds have been demonstrated to mimic natural estrogens by binding and activating the estrogen receptor (reviewed by Sonnenschein and Soto 1998). These and other possible compounds may also interfere with sex steroid synthesis, transport, and metabolism, thereby disrupting the circulating estrogen levels in the fish. None of these compounds were quantified in the present study but the watersheds were largely forested and land use was not likely a factor associated with intersex.

The small impoundments in the present study and in Kellock et al. (in press 2013) were largely managed for recreational fishing, not for agricultural use, and livestock did not have direct access to any of the impoundments, although runoff from adjacent pastures may have contributed some estrogens. Recently, Rodgers et al. (2011) and Sychrova et al. (2012) reported that common cyanobacteria (i.e., *Microcystis aeruginosa*) produce an unidentified compound with estrogenic activity. Although cyanobacteria and nutrients were not quantified in the present study, landowners/managers commonly fertilized the small impoundments with inorganic nitrogen as part of a pond management strategy to ultimately enhance fish populations for recreational fishing. The small impoundments appeared eutrophic (e.g., low Secchi depths and green coloration to water), so cyanobacteria may have played a role in contributing to the estrogen activity in the water. We did observe higher FP assay concentrations in 7 of 11 impoundments and cyanobacteria could account for this discrepancy between FP assay results and lower estrogen concentrations measured by LC-MS. We did not analyze for the presence of pesticides or legacy chemicals like DDT or PBCs, but they are highly persistent components and may be present in Georgia waters and could also contribute to overall impoundment estrogenicity.

Our modeling approach was useful for identifying some key factors at the watershed-levels that are associated with intersex, and although we did not determine a mechanism, we can with a reasonable degree of certainty predict the probability that a fish from a given impoundment (at least in Georgia, USA) will be intersex. Additional sampling and modeling efforts are needed to further explore fish, water quality, and watershed level variables that may be associated with intersex in largemouth bass. Isolation of watershed level factors that influence intersex was especially problematic in the present study because of the interconnectedness (autocorrelation) of many of the factors and underlying processes, but quantification and analysis of additional fish associated variables at the physiological or molecular level or unique watershed level variables may prove useful.

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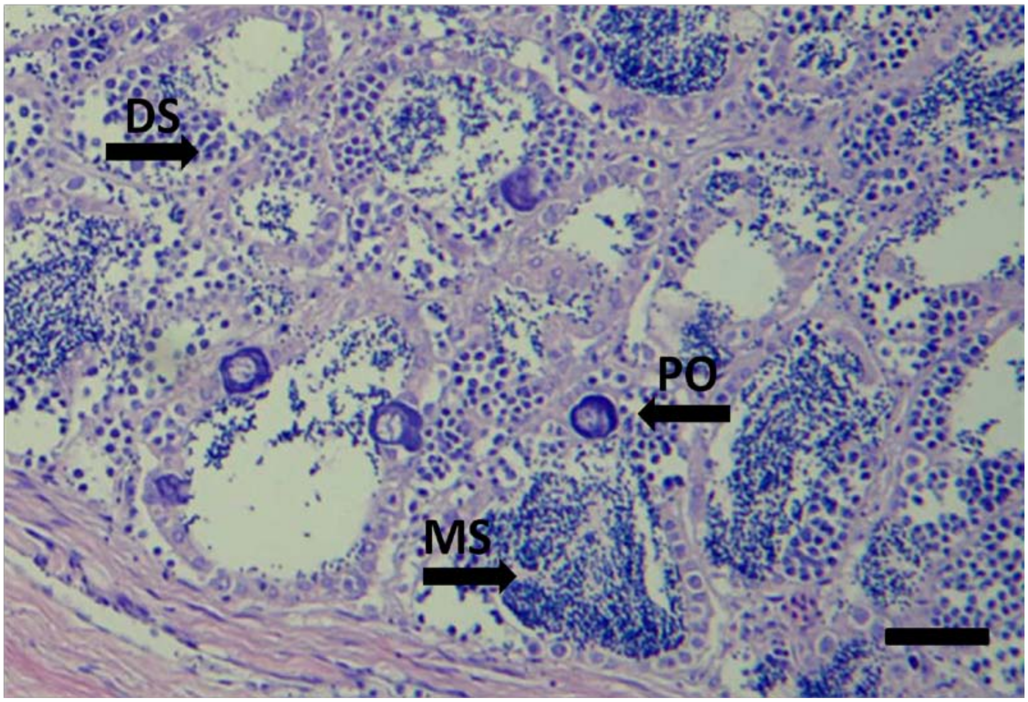

Figure 3.1. Histological confirmation of primary oocyte (PO) development in testicular tissue of a largemouth bass from a private pond in the Piedmont region of Georgia USA collected in the spring of 2010. Mature sperm (MS) as well as developing spermatocytes (DS) are also observed within the testes. Scale bar = 100µm. H&E staining.

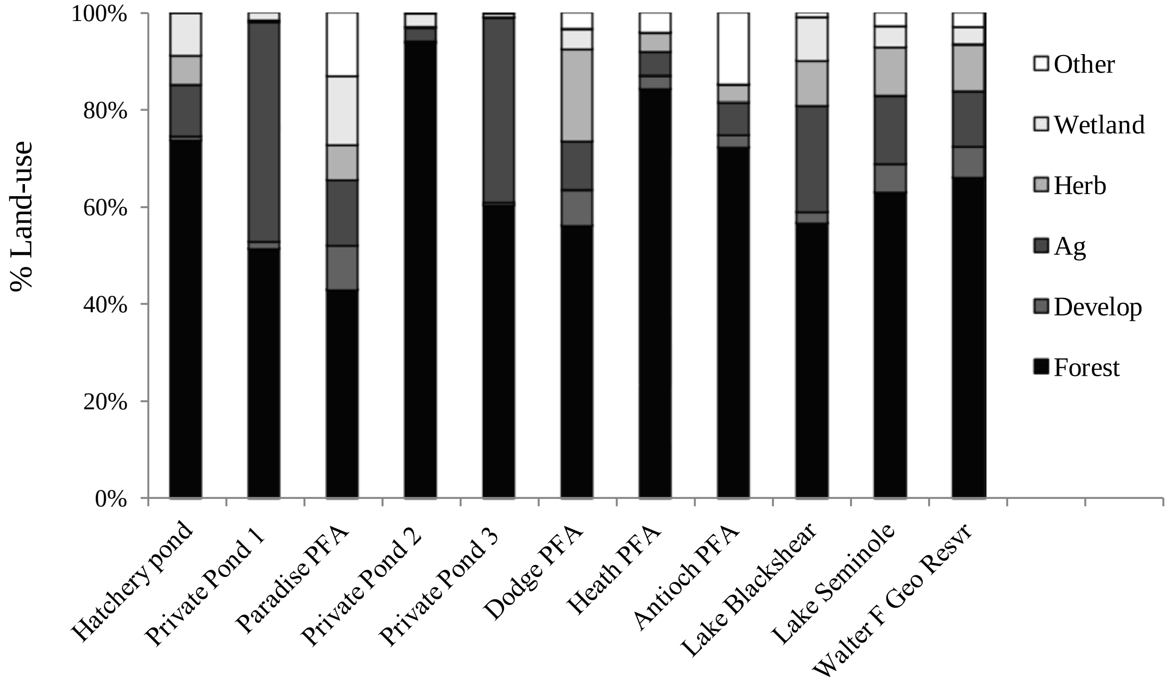

Figure 3.2. Land-use analysis for 11 impoundments in Georgia, USA sampled for intersex largemouth bass during spring 2010 and 2011.

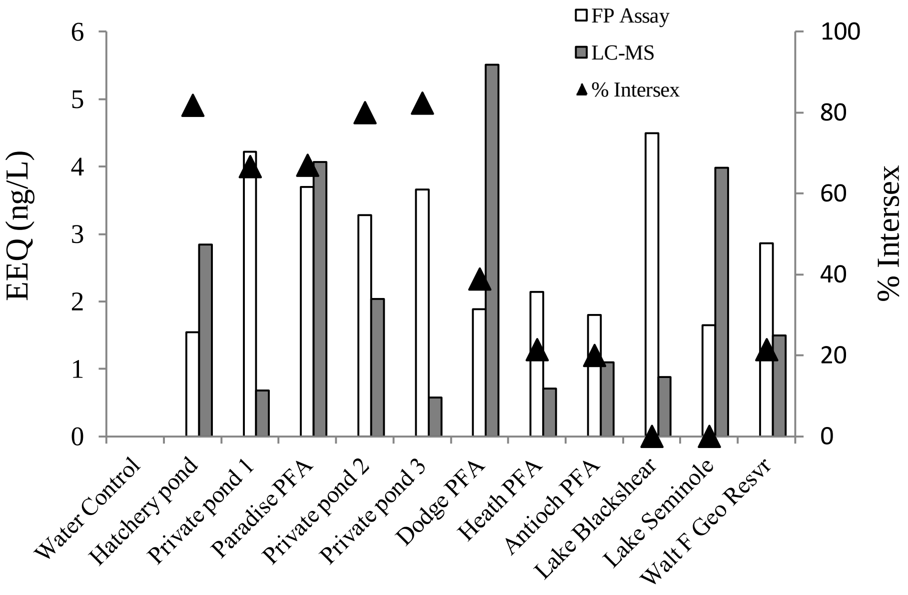

Figure 3.3. Estrogenic potency of an ultrapure water control and 11 impoundments sampled for intersex largemouth bass in Georgia during the spring of 2010 and 2011. Liquid chromatography mass spectrometry (LC-MS) analysis detected only estradiol and estrone, while the fluorescent polarization assay (FP Assay) measured total nonspecific estrogenic potency of the water. Both measurements were expressed as estradiol equivalents (EEQ) (ng/L). Estrogens in the water control were below detection limits. Secondary axis shows percent intersex observed from each impoundment.

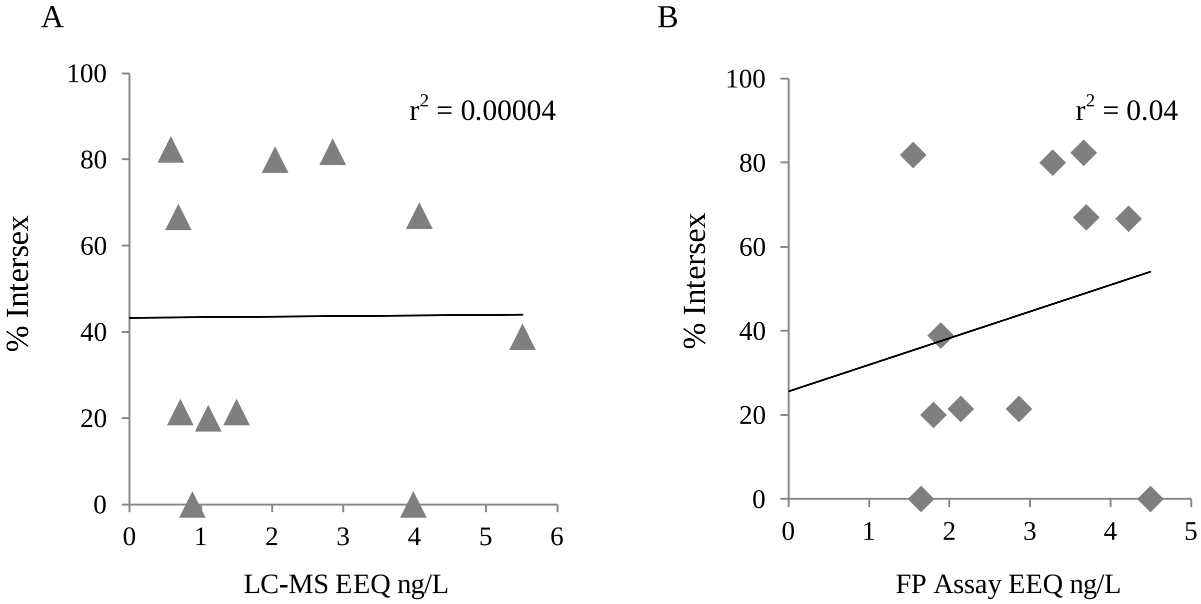

Figure 3.4. (A) Linear regression analyses for concentrations of natural estrogen detected by liquid-chromatography mass spectroscopy (LC-MS) and (B) by fluorescence polarization (FP Assay) related to intersex largemouth bass from 11 impoundments in Georgia. Measurements are in estradiol equivalents (EEQ) ng/L units.

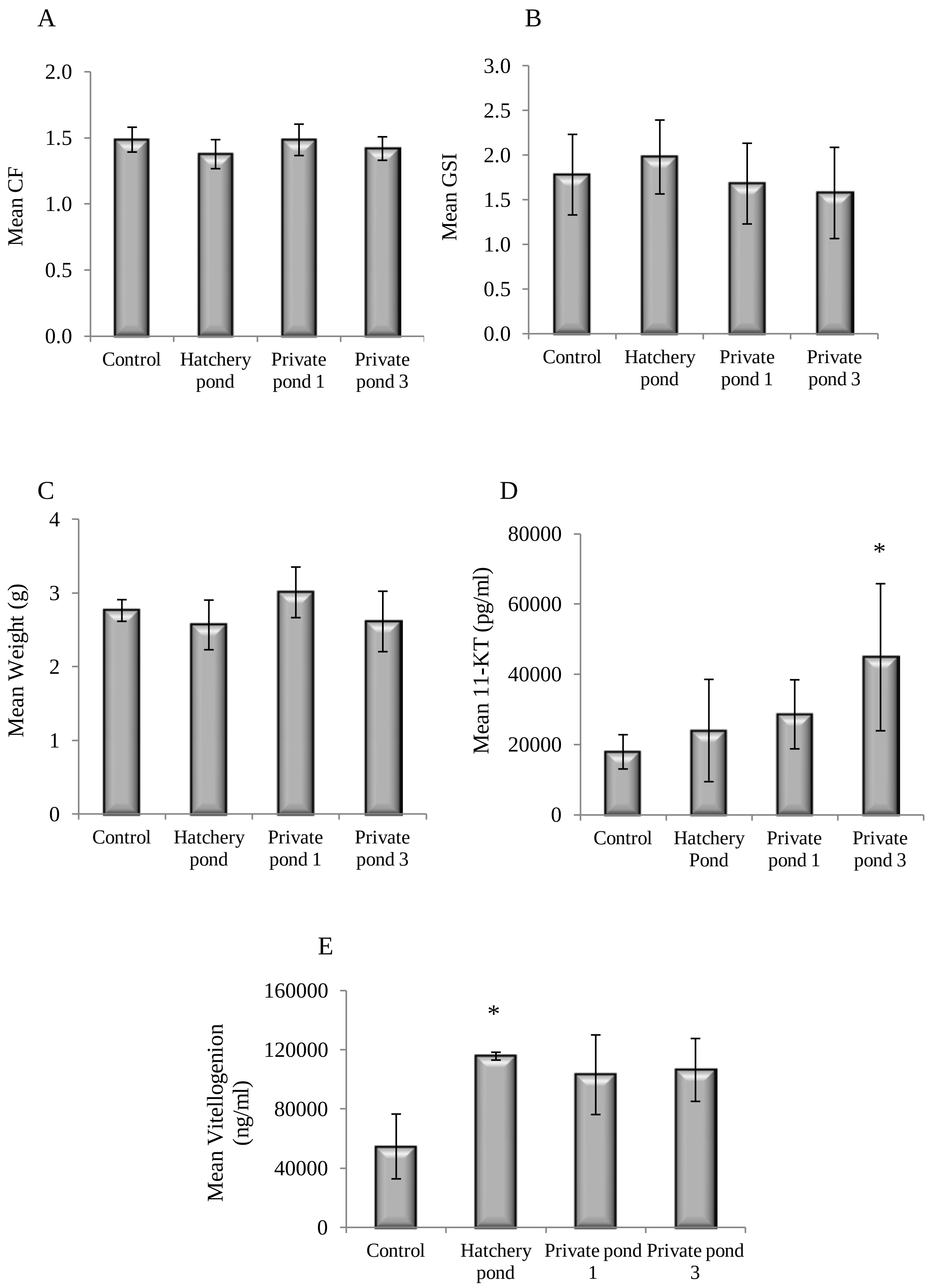
A
Mean CF
2.0
1.5
1.0
0.5
0.0
Control
Hatchery pond
Private pond 1
Private pond 3
B
Mean GSI
3.0
2.5
2.0
1.5
1.0
0.5
0.0
Control
Hatchery pond
Private pond 1
Private pond 3
C
Mean Weight (g)
4
3
2
1
0
Control
Hatchery pond
Private pond 1
Private pond 3
D
Mean 11-KT (pg/ml)
80000
60000
40000
20000
0
*
Control
Hatchery Pond
Private pond 1
Private pond 3
E
Mean Vitellogenion (ng/ml)
160000
120000
80000
40000
0
*
Control
Hatchery pond
Private pond 1
Private pond 3

Figure 3.5. (A) Mean condition factor (CF), (B) mean gonadal somatic index (GSI), (C) mean weight, (D) mean 11-ketotestosterone (11-KT) and (E) mean plasma vitellogenin among male fathead minnows following a lifecycle exposure to sediment collected from impoundments in Georgia USA with high (> 67%) largemouth bass intersex rates collected in spring 2011. Statistical differences (ANOVA, Dunnett's test) between control and treatments are noted by (*). Error bars are standard deviation. $\alpha = 0.05$

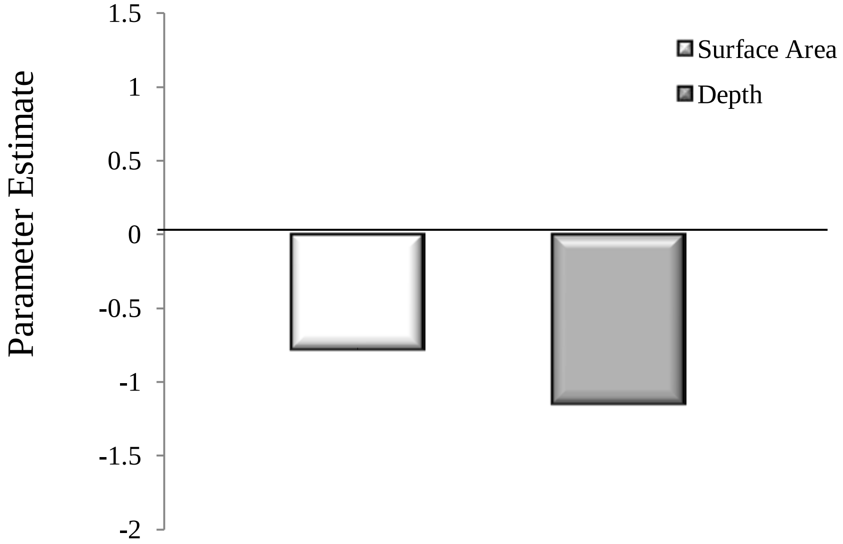

Figure 3.6. Parameter estimates for the most plausible model (impoundment size model) used for predicting the probability of an individual fish is intersex. Graphs shows the negative relationship with probability that an individual fish will be intersex.

Table 3.1. Selected descriptive values for 11 impoundments sampled for LMB[1] in Georgia, USA.

| Impoundment | County | Surface Area (ha) | Physiographic Region | CPUE[2] | Males Sampled (n) | Intersex Observed (%) |
|---|---|---|---|---|---|---|
| Hatchery Pond | Ben Hill | 1.3 | LCP[3] | 36 | 22 | 82 |
| Private Pond 1 | Wilkes | 2.8 | Piedmont | 26 | 18 | 67 |
| Paradise PFA[4] | Berrien | 6.9 | UCP[5] | 10 | 6 | 67 |
| Private Pond 2 | Hancock | 7.3 | Piedmont | 20.7 | 15 | 80 |
| Private Pond 3 | Wilkes | 8.1 | Piedmont | 21 | 17 | 82 |
| Dodge PFA | Dodge | 42.1 | UCP | 15 | 18 | 39 |
| Heath PFA | Floyd | 81.7 | RV[6] | 7.5 | 14 | 21 |
| Antioch PFA | Floyd | 144.5 | RV | 21 | 15 | 13 |
| Lake Blackshear | Crisp | 3439.9 | UCP | 2.8 | 9 | 0 |
| Lake Seminole | Seminole | 15175.9 | LCP | 2.3 | 9 | 0 |
| Walter F George | Clay | 18211.1 | UCP | 3.5 | 14 | 21 |

[1] Largemouth bass (*Micropterus salmoides*)
[2] Catch per unit effort (LMB/hr)
[3] Lower Coastal Plain
[4] Public Fishing Area
[5] Upper Coastal Plain
[6] Ridge and Valley

Table 3.2. Mixed models to evaluate relative importance of fish associated, water quality and watershed level variables affecting probability of intersex fish in Georgia impoundments.

| No. | Model name | Hypothesis | Models |
|---|---|---|---|
| 1 | Global Fish | All main effects at fish level will change response parameter | y = length + GSI + LSI + Wr + CPUE + $Rj^1$ |
| 2 | Impoundment Size | Response is dependent on impoundment size | y = $SA^2$ + depth + $Rj$ |
| 3 | Length and Density | Response is dependent on fish length and density | y = length + CPUE + $Rj$ |
| 4 | Global Watershed | All main effects at landscape level will change response parameter | y = SA + %forest + depth + LC-MS + $Rj$ |
| 5 | Agricultural Use | Response is dependent on surrounding agricultural activities | y = SA + %ag + nitrate + depth + $Rj$ |
| 6 | Global Water Quality | All main effects at water level will change response parameter | y = SA + depth + secchi + $FP^3$ + nitrate + $Rj$ |

[1] $Rj$ = random effect of site $Rj \sim N(0, \sigma^2_R)$

[2] SA = impoundment surface area

[3] FP = FP Assay

Table 3.3. Akaike information criteria with small sample bias adjustment (AICc); number of parameters (K), ΔAICc, Akaike weights (*w*) for candidate models (*i*) relating fish, water quality and watershed level parameters to incidence of male intersex largemouth bass. Impoundment size model is the best model as it has the lowest AICc score.

| Candidate Model | Model No. | K | AICc | ΔAICc | $w_i$ | % $w_i$ |
|---|---|---|---|---|---|---|
| Impoundment Size | 2 | 4 | 740.95 | 0.00 | 0.85 | 100.0 |
| Length and Density | 3 | 4 | 744.91 | 3.96 | 0.12 | 13.8 |
| Global Watershed | 4 | 6 | 749.17 | 8.22 | 0.01 | 1.6 |
| Agriculture Use | 5 | 6 | 749.22 | 8.27 | 0.01 | 1.6 |
| Global Water Quality | 6 | 7 | 753.08 | 12.13 | 0.00 | 0.2 |
| Global Fish | 1 | 7 | 760.82 | 19.87 | 0.00 | 0.0 |

# CHAPTER 4

# CHRONIC NITRATE EXPOSURE ALTERS REPRODUCTIVE PHYSIOLOGY IN FATHEAD MINNOWS[3]

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[3] Kellock KA and Bringolf RB. 2013. To be submitted to *Environmental Toxicology and Chemistry*.

# ABSTRACT

Nitrate is a ubiquitous aquatic pollutant that is commonly associated with eutrophication and dead zones in estuaries. At high concentrations nitrate is toxic to aquatic life but it has also been purported at environmental concentrations as an endocrine disruptor in fish. To investigate the potential for nitrate to cause endocrine disruption in fish, we conducted a full lifecycle study with fathead minnows *Pimephales promelas* exposed to nitrate (0, 50 and 250 mg/L) from <24 hr post hatch to sexual maturity. Incidence of intersex, body condition factor, gonadal somatic index, growth, 11-keto testosterone (11-KT; males only) and vitellogenin induction were determined in mature male and female fish. In nitrate exposed male fatheads both 11-KT and vitellogenin were significantly induced when compared with controls. However, intersex was not observed and significant differences were not found in condition factor, GSI or growth among males in response to nitrate. Females exposed to nitrate had significant increases in vitellogenin compared to control fish but no significant differences among condition factor, weight or GSI in nitrate exposed groups. Our findings suggest that environmentally relevant nitrate levels may disrupt steroid hormone synthesis or metabolism in male and female fish and may have implications for watershed management and nutrient pollution.

## INTRODUCTION

Endocrine disrupting chemicals (EDCs) including steroid hormones, pharmaceuticals, and pesticides are commonly detected in rivers that receive municipal wastewater effluent (Desbrow et al. 1998; Kolpin et al. 2002; Ternes et al. 2004) and can disrupt estrogenic, androgenic or thyroid hormone-dependent mechanisms including reproduction, metabolism, growth and development. A sizeable and growing body of literature describes effects of EDCs including endpoints such as testicular oocytes (intersex) (Jobling et al. 1998; Folmar 2001; Vajda et al. 2008), induction of vitellogenin (Purdom et al. 1994; Folmar 2001), decreased sperm count and motility (Jobling et al. 2002b; Jobling et al. 2002a), decreased fertilization success (Harris et al. 2011), reduced gonad size (Tetreault et al. 2011) and altered circulating sex hormone concentrations (Folmar 2001; Tetreault et al. 2011). As a result of these and other findings, the effects of EDCs on aquatic organisms have received considerable attention in popular media as well as scientific literature (Colborn et al. 1993; Colborn et al. 1996; Blazer et al. 2007; Guillette Jr and Edwards 2008; Hinck et al. 2009). The list of documented and purported EDCs continues to grow and currently includes nitrate ($NO_3$), one of the most ubiquitous aquatic pollutants worldwide. Widespread uses of organic and inorganic fertilizers, municipal wastewater effluents, and other anthropogenic activities have contributed to high levels of nitrate in the environment. Nitrate in surface waters draining agricultural areas can be as high as 264 mg/L-$NO_3$ (McCoy 1972) and has been reported at concentrations of 4.4 - 167.2 mg/L-$NO_3$ in artesian springs in agricultural areas (Katz 1999). Nitrate toxicity was reported in humans as early as the 1940s with incidence of methemoglobinemia caused by drinking nitrate-contaminated well water (Avery 1999). The U.S. Environmental Protection Agency (USEPA) later established a safe limit for nitrate in drinking water of 45 mg/L-$NO_3$ (US EPA 1986: Water Quality). Although the mechanism of action (interference with hemoglobin binding to oxygen) is the same for both humans and animals (Scott and Crunkilton 2000; Camargo et al. 2005), no such criteria has been established for protection of aquatic organisms.

Between 2006 and 2012 approximately 42,000 water ways in the U.S. were categorized as impaired under Section 303(d) of the Clean Water Act (US EPA: 303(d)). Of these 42,000 impaired waters, nutrient pollution is the cause of approximately 17% and is only surpassed by pathogens and metals among the top three causes of water impairment in the U.S. Eutrophication is a prominent cause of degraded water quality in freshwater systems (Sampat 2000) and is often the result of increased nutrient (nitrate and phosphorous) input.

Among fish, nitrate toxicity varies considerably by species. Salmonids such as larval Chinook salmon *Oncorhynchus tshawytscha*, rainbow trout *Oncorhynchus mykiss* and cutthroat trout *Oncorhynchus clarkii* are among the more sensitive fish species with mortality reported at concentrations of 10.12 - 33.44 mg/L-$NO_3$ in 30-day exposures (Kincheloe et al. 1979). Largemouth bass *Micropterus salmoides* can tolerate nitrate levels up to 422.4 mg/L-$NO_3$ with no apparent effect on growth or feeding over a 164-day exposure (Knepp and Arkin 1973).

Although nitrate toxicity to fish and other aquatic organisms is well-documented (see (Camargo et al. 2005; Camargo and Alonso 2006) it was first suggested as a potential EDC only recently by Guillette & Edwards (2005), who hypothesized that nitrate may have altered circulating steroid hormone levels in juvenile American alligators *Alligator mississippiensis* sampled from highly eutrophic lakes in Florida (Guillette and Edwards 2005). Since 2005, nitrate has been implicated as an EDC in several aquatic species including fish and amphibians. Mosquito fish *Gambusia holbrooki* exposed up to 22 mg/L-$NO_3$ of nitrate in Florida springs had decreased sperm count and increased testicular weight, but no reduction in spermatogenesis

(Edwards and Guillette 2007). Northern Leopard frogs *Rana pipiens* exposed to 10 mg/L-$NO_3$ during early developmental stages had testicular oocytes, female skewed sex-ratios, and less-developed testicular tissue (Orton et al. 2006). Southern toad tadpoles *Bufo terretris* exposed to nitrate levels of 132 mg/L-$NO_3$ in spring water showed reduced growth and altered thyroxine concentrations (Edwards et al. 2006b). Results of these studies generally support the hypothesis that nitrate is an EDC and may alter steroid hormone concentrations or activity.

We used a model freshwater fish species, the fathead minnow, to further investigate the endocrine disrupting effects of chronic nitrate exposure by examining reproductive physiology of males and females following full lifecycle nitrate exposure.

## METHODS

### *Study design*

Stock nitrate solutions were prepared weekly from reagent grade $NaNO_3$ (J.T. Baker, Center Valley, PA) dissolved in deionized water. Four replicate aquaria were used for each of the four treatments (control water, 50 mg/L $NO_3$, 250 mg/L-$NO_3$ and 50 ng/L estradiol) for a total of 16 experimental units. Temperature, pH and dissolved oxygen were measured daily with a Hach HQ40d water quality meter. Nitrate levels were measured weekly with a Lamotte nitrate test kit (3689-SC) and Lamotte colorimeter (model SMART2).

Fathead minnow embryos were purchased from Aquatic BioSystems, Inc. (Fort Collins, CO) and allowed to hatch during transport. Fifteen < 24 hour old larval fathead minnows were transferred into each 5-L beaker with 2-L of aerated dechlorinated tap water. The respective beakers were then spiked with appropriate volumes of stock to maintain appropriate nitrate levels. Fish were fed *ad libitum* with *Artemia* nauplii twice daily. A 90% water change was completed in the beakers every 48 hours to maintain target nitrate concentrations (50mg/L or 250mg/L-$NO_3$) and consistent water quality. Fish were maintained in the 5-L beakers for the first 60 days of the exposure, after which they were transferred to 20-L glass aquaria with 15-L of water. At this point the fish were transitioned from *Artemia* to flake food (TetraMin), fed *ad libitum* twice daily. Aquaria were aerated and equipped with charcoal aquarium filters. Nitrate levels increase naturally over time as ammonia in fish waste is converted to nitrite and nitrate, so a 50% water change was conducted weekly while the fish were in the 20-L aquaria to maintain target nitrate levels in all treatments. Fish remained in the 20-L aquaria within a 3500-L water bath to maintain a target temperature of 25°C until exposure termination (7-months total).

### *Fish Processing*

Upon termination of exposure, fish were euthanized by overdose (100 mg/L) with neutral buffered MS-222 (Argent, Redmond WA). Lengths and weights were recorded for six randomly selected males and six females per tank. All males in nitrate and control groups displayed breeding coloration and nuptial tubercles. Weights were averaged per experimental unit for each treatment and used as a measure of growth. Lengths and weights were used to calculate Fulton's condition factor (K) by the formula:

$$K = (\text{Weight (g)} / \text{Length}^3\text{ (mm)}) * 100{,}000$$

Males and females were bled from the caudal vein into heparinized glass microcapillary tubes (Fisher, #22-362-566) and blood from the six males or females in each replicate was pooled. Pooled blood samples were centrifuged (2000 rpm) for ten minutes to obtain plasma, which was transferred to a clean vial and stored at -80°C until hormone and vitellogenin analyses for males and vitellogenin analysis for females.

Gonads of males and females were dissected and weighed to calculate gonadal somatic index (GSI). GSI, calculated by dividing gonad weight (g) by total fish weight (g) and multiplying by 100, was averaged among replicates. Testes from three random males per treatment group were placed longitudinally into histology cassettes and fixed in 10% neutral buffered formalin for histological analysis. Histological processing of gonads was completed at the Veterinary Medicine Diagnostic Lab at the University of Georgia. Briefly, the preserved testes were dehydrated in alcohol, embedded in paraffin wax, sectioned at 6µm, mounted on glass microscope slides, and stained with hematoxylin and eosin. A single longitudinal tissue section allowed for examination of maximal surface area of testicular tissue. Sectioned and stained testes were examined by an experience reader under a light microscope for presence of oocytes. A subset (10%) of the sections was examined by a second reader to assess quality control.

### *11-Keto testosterone and vitellogenin analysis*

Plasma samples were allowed to thaw on ice and each sample was separated into two glass tubes. One duplicate sample was used for hormone analysis and the other for vitellogenin analysis. Plasma from male fish only was used for 11-KT analysis. Plasma was extracted three times with a 50:50 mixture of ethyl acetate/hexane. Plasma 11-KT was analyzed according to the protocol provided with the commercial enzyme immunoassay 11-KT kit for fathead minnows (Cayman Chemical, Ann Arbor, MI). The detection limit for the 11-KT assay was approximately 1.3 pg/ml. Samples were run in triplicate at two dilutions (1:10 and 1:100) and each plate contained a reagent blank. Standards were run in duplicate. All samples were run simultaneously to minimize variance among plates. Absorbance was measured with a

SpectraMax M2 plate reader (Molecular Devices, Sunnyvale, CA) and final concentrations were determined with Softmax Pro 5 software based on an 11-KT standard curve.

Vitellogenin analysis was conducted via commercial enzyme immunoassay kit (Cayman Chemical) for fathead minnow plasma samples. Male and female samples were run in triplicate, at two dilations (1:50 and 1:5000) and blanks were added to each plate. Standards were run in duplicate. All plates were run simultaneously to minimize variance among plates. Absorbance was measured with a SpectraMax M2 plate reader (Molecular Devices, Sunnyvale, CA) and final concentrations were determined with Softmax Pro 5 software based on a fathead minnow vitellogenin standard curve. The detection limit for the vitellogenin assay was 0.1 ng/ml.

### *Statistical Analyses*

Statistical analyses were performed with SAS Version 9.3 (SAS Institute, Cary NC) statistical analysis software. All statistical comparisons were made at $\alpha = 0.05$ level of significance. Analysis of variance (ANOVA) and Dunnett's test were used to compare differences between controls and treatment means for K, GSI, weight, 11-KT and vitellogenin among males. 11-KT data required a square root transformation to achieve normality. For females, ANOVA and Dunnett's test were also used to compare differences between controls and treatment means for K, GSI, weight and vitellogenin. No transformations were necessary to achieve normality.

## RESULTS

Survival was >80% in all tanks and did not differ among treatments ($F = 3.87$, $df = 15$, $p = >0.05$). Mean (± standard deviation) temperature was 24.6°C ± 0.48, pH was 7.6 ± 0.31 and dissolved oxygen was 7.2 ± 0.71 mg/L among all tanks during the exposure (n = 16). Throughout the exposure period mean (± standard deviation) nitrate concentration was 6.36 ± 2.4 mg/L-$NO_3$ in the control treatments while the 50 mg/L-$NO_3$ exposure was 52.6 ± 6.1 mg/L-$NO_3$ and the 250 mg/L-$NO_3$ treatment was 250.1 ± 14.4 mg/L-$NO_3$. Nitrate was 7.0 ± 2.5 mg/L-$NO_3$ in the 17β-estradiol (E2) positive control tanks.

No intersex males were observed among the control, 50 mg/L or 250 mg/L-$NO_3$ treatments; however, two of 12 males (16.7%) exposed to 50 ng/L estradiol contained oocytes and were identified as intersex. No significant differences were found between the control group and any treatment group for K (df = 15, F-value = 4.57, $p > 0.05$; Figure 4.1A). No significant differences were detected among control mean GSI and mean treatments for GSI (df = 15, F-value = 2.79, $p > 0.05$; Figure 4.1B). Mean growth (measured as weight at the end of the study) was significantly lower in the E2 exposed and nitrate-exposed fish than in control fish (df = 15, F-value = 9.47, p = 0.002; Figure 4.1C).

Vitellogenin in male fathead minnows was significantly increased in all treatments when compared to the control group (df = 15, F-value = 9.65, p = 0.002; Figure 4.1D). Vitellogenin was increased approximately 28 and 24-fold for the 50 and 250 mg/L-$NO_3$ exposures, respectively. 11-KT concentration was significantly higher (df = 15, F-value = 8.13, p = 0.003) in the 250 mg/L-$NO_3$ treatment when compared to control fish (Figure 4.1E) with an approximate 6-fold induction. Although 11-KT from the 50 mg/L-$NO_3$ treatment was not significantly different from the control there is an apparent increasing trend with increasing nitrate exposure concentration.

Condition factor (df = 15, F-value = 1.57, p = 0.28) and growth (df = 15, F-value = 1.56, p = 0.25) did not differ statistically among exposed females (Figure 4.2A and 4.2C). GSI was significantly lower in E2 exposed females compared to control females (df = 15, F-value = 4.68, p = 0.02; Figure 4.2B), but GSI was not statistically significant among nitrate treatments. Vitellogenin was significantly increased (1-fold) compared to the control in both the 50 and 250 mg/L-$NO_3$ exposures (df = 15, F-value = 3.75, p = 0.04; Figure 4.2D).

## DISCUSSION

Low levels of nitrate may occur naturally in aquatic systems, produced from nitrification of ammonia released as fish waste, decaying plant material, etc.; however, high concentrations of nitrate result from many anthropogenic activities such as agriculture (e.g., animal feedlots, inorganic and organic fertilizers), municipal wastewater effluent, leaky septic systems, and industrial effluents. We investigated the potential for nitrate to cause endocrine disruption and found that chronic exposure with one of the most ubiquitous global pollutants altered fish reproductive physiology. Specifically, 11-KT and vitellogenin were significantly induced in males and vitellogenin concentrations were also significantly higher in females exposed to nitrate.

We included a treatment of 17-β estradiol as a positive control for a compound known to interfere with reproductive physiology in fish (Miles-Richardson et al. 1999; Bjerselius et al. 2001; Brion et al. 2004). Except for induction of vitellogenin, nitrate did not appear to act as an estrogen. Growth, body condition and GSI were not significantly altered in either sex by nitrate exposure in the present study although GSI and condition factor are often negatively affected when fathead minnow (male or female) are exposed to estrogens during chronic exposures (Pawlowski et al. 2004; Parrott and Blunt 2005). Estrogens are also known to suppress 11-KT in male fish (Ankley et al. 2001; Martinovic et al. 2007; Salierno and Kane 2009) whereas 11-KT was induced by nitrate exposure in our study.

Vitellogenin induction in male fish is a hallmark of estrogen exposure (Le Guellec et al. 1988; Sumpter and Jobling 1995). Interestingly, the increases in male fish plasma vitellogenin with both nitrate treatments were comparable to the induction in the E2 treatment group. To the best of our knowledge, this is the first report of vitellogenin induction following nitrate exposure, although in human breast cancer cell lines nitrite and nitrate have been shown to bind and activate the estrogen receptor-α (Veselik et al. 2008). Therefore, one reason vitellogenin may have been induced in the male fathead minnows was as a result of direct interaction of nitrate with the estrogen receptor, which has been reported to bind to a wide variety of compounds (Routledge and Sumpter 1996; Desbrow et al. 1998; Sanseverino et al. 2005).

Significantly elevated levels of 11-KT were observed in an increasing trend corresponding to increased nitrate levels, while 11-KT was effectively suppressed by E2 exposure. In steroid hormone synthesis, androstenedione is a precursor for both testosterone and 11-KT and because testosterone is a precursor of 17β-estradiol, increased androgen production could lead to increased levels of circulating estradiol in male fish. Therefore, high endogenous estrogen levels may be a second possible reason for induction of vitellogenin in the male fatheads exposed to nitrate. Unfortunately we did not measure plasma estradiol levels as blood volumes were insufficient to do so following 11-KT and vitellogenin analysis. Typically increased vitellogenin in males occurs concurrently with decreases in androgens. For example, Martinović et al. (2007) reported increased vitellogenin in male fathead minnows and decreased 11-KT following exposure to sewage treatment effluent and an E2 waterborne exposure. The estrogen-exposed males also experienced a reproductive failure when having to compete with control males for females in a breeding scenario. We did not attempt to breed the fathead minnows exposed to nitrate in the present study but reproductive assays would lend insight into the mode of action for nitrate and help determine if nitrate exposure and the resulting increase in 11-KT in males would result in more aggressive behavior in a breeding scenario, even with increased levels plasma vitellogenin also present.

A study of endocrine disruption in juvenile American alligators *Alligator mississippiensis* from eutrophic lakes by Guillette et al. (1999) showed an inverse relationship between total nitrogen in the lake water and plasma testosterone in juvenile alligators, but a positive relationship was observed for estradiol in juvenile males. Three mechanisms have been proposed for nitrate that all result in disruption of steroid hormone synthesis and a reduction in hormone concentrations (reviewed by Guillette and Edwards 2005). Our results demonstrated a clear increase in male fish 11-KT and vitellogenin. Consistent with our findings, Hamlin et al. (2008) reported elevated testosterone levels in Siberian sturgeon *Acipenser baeri* exposed to nitrate (50.6 and 250.8 mg/L-$NO_3$) for 30 days. Hamlin et al. (2008) also reported increases in plasma 17β–estradiol levels, but did not investigate effects on vitellogenin. Edwards and Guillette (2007) reported no change in steroidogenesis in male mosquito fish exposed to 22 mg/L-$NO_3$ and an increase in testicular weight was noted. Similar to Edwards and Guillette (2007), we observed slight (but non-significant) increases in GSI and condition factor for males and females exposed to nitrate. Therefore, the current body of research does not seem to support the original hypothesis of suppressed steroidogenesis reviewed by Guillette and Edwards (2005). The 11-KT increases observed in this study may be explained by an upregulation of steriodogenesis caused by nitrate, or possibly by inhibition of steroid catabolism. Quantification of estradiol and aromatase activity would also be useful to determine the fate of excess testosterone induced by nitrate. Hamlin et al. (2008) proposed that increased nitrate levels can affect the liver so that circulating testosterone is not efficiently cleared and builds over time. We did not measure liver somatic index in our study. Regardless of the mechanism(s), increased 11-KT and induction of vitellogenin in the present study, are consistent with androgen induction in Siberian sturgeon (Hamlin et al. 2008), suggesting that nitrate alters endocrine function in fish.

Intersex fish have commonly been associated with exposure to municipal wastewater effluent (Jobling et al. 1998; Woodling et al. 2006; Tetreault et al. 2011). Municipal effluents often contain a variety of environmental estrogens but also contain a variety of other chemicals including nitrate. Lab studies with estrogens have confirmed that intersex can be induced by estrogens (Miles-Richardson et al. 1999; Länge et al. 2001; Ankley and Johnson 2004; Parrott and Blunt 2005) but recently high rates of intersex have been reported in largemouth bass from small, eutrophic impoundments with low estrogen inputs (Kellock et al. 2013 in press). Based on the potential for nitrate to cause endocrine disruption (Guillette and Edwards 2005), Kellock et al. (2013 in press) hypothesized that nitrate may also play a role in intersex in the largemouth bass in small impoundments. Although intersex was not induced by nitrate in the present study, fathead minnows may not be a suitable species in which to study causes of intersex because they are apparently not prone to develop testicular oocytes, even with full-lifecycle estrogen exposure (Sohoni et al. 2001; Länge et al. 2001) and are fairly tolerant to nitrate toxicity with an LC50 of 5,781 mg/L-$NO_3$ (Scott and Crunkilton 2000) compared to largemouth bass. Additional research is needed to determine if nitrate may play a role in the high frequency of intersex in black basses (Blazer et al. 2007; Hinck et al. 2009; Ingram et al. 2011) and other species (e.g., Roach, *Rutilus rutilus*) (Jobling et al. 1998) for which intersex has been commonly reported.

To the best of our knowledge, this is the first full lifecycle exposure investigating the endocrine disrupting effects of nitrate in fish and further study is necessary to fully understand long-term effects of nitrate on fish endocrine function. While in the present study nitrate altered 11-KT and directly or indirectly induced vitellogenin in male fathead minnows, effects of nitrate on reproductive fitness and population level parameters such as recruitment remain unknown. Future work is necessary to expose possible molecular mechanisms involved in 11-KT increase and vitellogenin induction and possible reproductive consequences for fishes following exposure to nitrate, a ubiquitous global pollutant.

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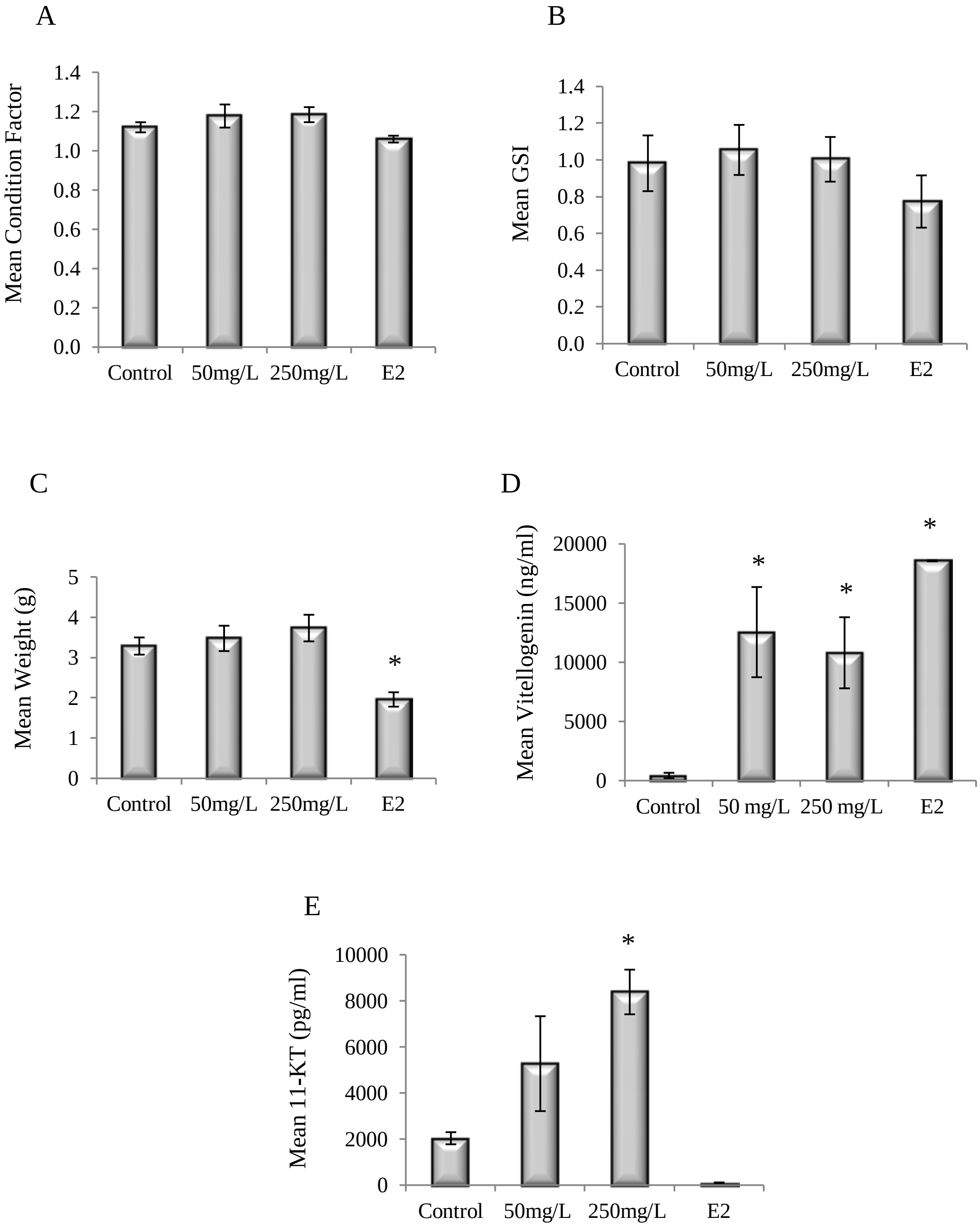
A
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Mean GSI
1.4
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Mean Weight (g)
5
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20000
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10000
5000
0
*
*
*
Control
50 mg/L
250 mg/L
E2
E
Mean 11-KT (pg/ml)
10000
8000
6000
4000
2000
0
*
Control
50mg/L
250mg/L
E2

Figure 4.1. (A) Mean condition factor, (B) mean gonadal somatic index (GSI), (C) mean weight, (D) mean plasma vitellogenin (ng/ml) and (E) mean concentration values of 11-ketotestosterone (11-KT) (pg/ml) from male fathead minnows exposed to nitrate (0, 50, or 250 mg/L $NO_3$) or 17β-estradiol (E2) at 50 ng/L for nine months from hatch to sexual maturity. Statistical differences (ANOVA, Dunnett's test) between control and treatments are noted by (*). Error bars are standard error. $\alpha = 0.05$.

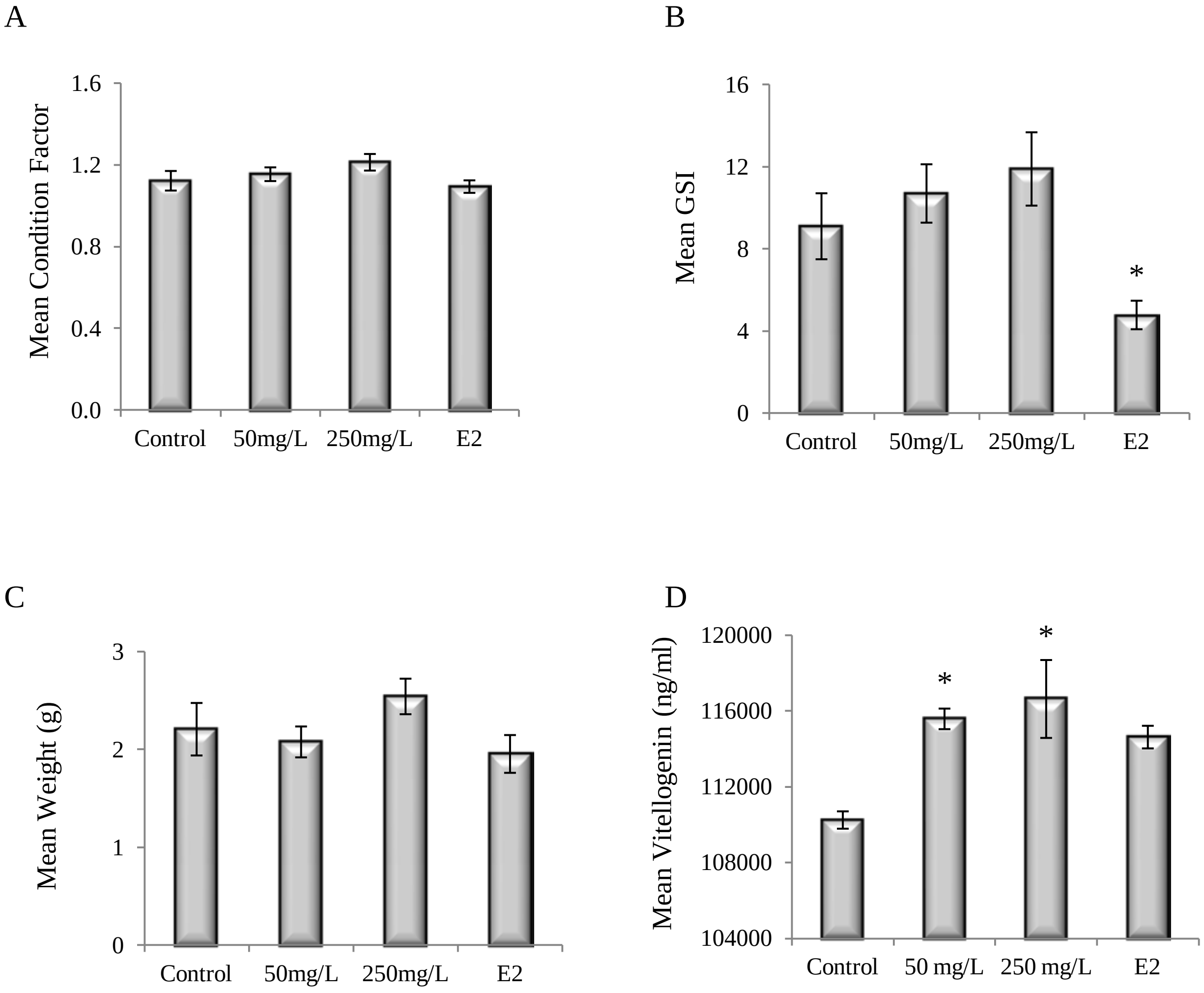

Figure 4.2. (A) Mean condition factor, (B) mean gonadal somatic index (GSI), (C) mean weight, (D) mean plasma vitellogenin (ng/ml) from female fathead minnows exposed to nitrate (0, 50, or 250 mg/L $NO_3$) or 17β-estradiol (E2) at 50 ng/L for nine months from hatch to sexual maturity. Statistical differences (ANOVA, Dunnett's test) between control and treatments are noted by (*). Error bars are standard error. $\alpha = 0.05$.

# CHAPTER 5

# SYNTHESIS AND CONCLUSIONS

## INTRODUCTION

Endocrine disruption in fishes and other aquatic organisms has been intensely studied for decades and began as an accidental finding of a hermaphroditic wild roach *Rutilus rutilus* in the River Lea in England in the late 1970s. These first observations of what are now known as intersex fish were associated with the effects of wastewater treatment facility effluent in internal Thames River reports by in 1981 (Sweeting 1981). Little concern was initially given to local fisheries, but more concern was given to human health effects. In a scientific article years later, links between estrogenic effects of wastewater and the occurrence of endocrine disruption (including intersex) in fish from British waterways was widely publicized (Jobling et al. 1998). Even in this Jobling et al. (1998) early work, intersex roach were observed in lakes and canals used as reference sites and Jobling suggested a low ‘natural’ background rate of intersex in roach is likely. Some 54 intersex field studies worldwide have followed since Jobling’s 1998 study. These have revealed intersex is occurring globally and in a variety of fish species from different families (reviewed by Bahamonde et al. 2013). A ‘hotspot’ for intersex for largemouth bass *Micropterus salmoides* has been identified in southeastern US rivers (Hinck et al. 2009). Hinck et al. (2009) emphasized the need to establish background rates of intersex for gonochoristic fish species to determine if intersex is a ‘natural’ phenomenon or results from anthropogenic sources.

Clearly, discharge of wastewater effluent (and its associated endocrine disrupting chemicals) into surface waters, particularly rivers, plays a substantive role in fish endocrine abnormalities (Folmar et al. 2001; Jobling et al. 2002; Baldigo et al. 2006; Blazer et al. 2007; Tetreault et al. 2011). However, little is known about causes of endocrine disruption, particularly intersex, in surface waters that do not receive wastewater from municipalities, industry or agriculture. In this dissertation, my studies examined incidence of intersex and endocrine disruption in fish from impoundment waters. I also investigated possible factors contributing to these conditions in impoundment fish. In chapter 2, I focused on the distribution of intersex largemouth bass in a statewide survey across Georgia impoundments. In chapter 3, I addressed impoundment-specific factors including waterborne estrogens, sediments and predictive models of intersex occurrence. Lastly, chapter 4 investigated the possibility that intersex and endocrine disruption may be induced by exposure to one of the most commonly occurring global pollutants, nitrate. My hope is that information from this combined body of work can be used by resource managers, fish biologists, ecotoxicologists etc. to identify factors contributing to intersex and ultimately develop strategies to reduce anthropogenic impacts that contribute to endocrine disruption.

## SYNTHESIS

Fish are excellent models for studying endocrine disruptors in the aquatic environment as they are subject to full lifecycle exposures and have been widely used for developmental and reproductive study (Kime 1998). High numbers of intersex male black basses *Micropterus* spp. in eastern (Blazer et al. 2007; Iwanowicz et al. 2009) and southeastern (Hinck et al. 2009; Ingram et al. 2011) US rivers have been observed, but no attempt to elucidate a background incidence for intersex black bass had been reported. My results from chapter 2 identified substantial numbers of intersex male largemouth bass from impoundments across Georgia, similar to rates reported by Hinck et al. (2009) in southeastern US rivers. Among the 11 impoundments we sampled, 157 male largemouth bass were collected for study and approximately half of those apparent male fish were intersex (~48%). Frequencies of intersex within impoundments ranged from 0 to 82%. These results were quite unexpected as the impoundments sampled appeared less impacted by anthropogenic activities when compared to rivers; impoundments were free of wastewater effluent and industrial waste, did not receive heavy agricultural input and were in largely forested areas. Even more surprising was the disproportionate prevalence of intersex males from smaller impoundments (<50 hectares).

The finding of high incidence of intersex in some impoundments was of concern because in a previous whole lake study, Kidd et al. (2007) observed reproductive failure and a population crash in fish exposed to environmentally relevant concentrations (4-6 ng/L) of a synthetic estrogen. In Chapter 2, fish feeding at lower trophic levels (i.e., bluegill *Lepomis macrochirus*, redbreast *Lepomis auritus*, and redear *Lepomis microlophus* sunfishes) in the impoundments were not investigated for endocrine disruption. The smaller impoundments I sampled, where the highest rates of intersex were discovered, were all effectively ‘bass crowded’ (characterized by dense populations of stunted fish) so at present, detrimental population effects do not appear to be occurring in these impoundments. Populations of other fish species in the impoundments were not investigated. However, this study is the first to examine intersex rates in impoundments and continued monitoring will be essential to understanding how the incidence of intersex and possible population level effects change over time. Although intersex does not appear to limit population size in the impoundments we examined, effects of intersex on reproduction and genetic diversity are currently unknown. If the relatively few ‘normal’ males are contributing the majority of the spawning effort, genetic diversity will be greatly reduced over time.

Chapter 2 was an initial attempt to uncover a background rate of intersex in largemouth bass. I had anticipated a small percentage of intersex males overall for a possible ‘natural’ background rate of intersex among these fish, but this was not the case. The high variability of intersex rates across sampled impoundments, with highest rates among smaller impoundments lead to more questions regarding impoundment specific characteristics that contribute to intersex.

Chapter 3 of this dissertation addressed hypotheses developed from chapter 2 results. I started with the question, ’what is unique among impoundments that yielded high rates of intersex largemouth bass?’ Initial steps to address this question included collecting water samples from each impoundment for quantification of natural and synthetic estrogens as well as measurement of non-specific estrogenic potency. Only two natural estrogens (estradiol and estrone) were detected by liquid chromatography mass spectrometry (LC-MS) and generally comparable estrogenic levels were measured for non-specific estrogenic potency. Furthermore, neither estrogen concentration nor non-specific estrogenic potency was closely related to incidence of intersex in any of the respective impoundments. Natural and synthetic estrogens are most often detected in surface waters and are a common culprit for feminizing male fish (Desbrow et al. 1998; Ternes et al. 1999; Snyder et al. 2001) but timing of exposure during sexual differentiation (Lange et al. 2001; van Aerle et al. 2002; Parrott and Blunt 2005; Länge et al. 2009) appears critical to severity of effects. I sampled sexually mature fish, and measured estrogen concentrations (or estrogenic potency); however estrogen/estrogen mimic concentrations are unknown for the period of sexual differentiation for these fish. I reported high fish densities (higher CPUE values) in smaller impoundments so the detected natural estrogens (and at least some of the estrogenic activity) may be attributed to natural waste excreted from female fish inhabiting the impoundments.

The log octanol/water partition coefficients ($K_{ow}$) for natural and synthetic estrogens are approximately 3-4 (Peck et al. 2004; Auriol et al. 2006) and sediment is a known sink for estrogenic (Legler et al. 2002; Peck et al. 2004) and anti-androgenic (Urbatzka et al. 2007) compounds in rivers receiving wastewater effluent. With no clear relationship in the present study between intersex and concentrations of estrogens/estrogen mimics in the impoundments, I considered sediment as a possible source of exposure to estrogens or estrogen mimics. I collected sediment from the impoundments that had >67% incidence of intersex largemouth bass and exposed fathead minnows to these sediments from <48 hours post hatch until sexual maturity (approximately nine months). Fathead minnows exposed to impoundment sediments demonstrated physiological effects compared to control treatments but overall, vitellogenin and 11-ketotestosterone (11-KT) were induced. Concurrent increases in both vitellogenin and 11-KT in these exposed male fish was unexpected. This induction of vitellogenin in male fish exposed to sediments suggests that the estrogens are available to fish either through uptake across the gills, dietary exposure or by conversion from testosterone via aromatase. Induction of vitellogenin in the male fish from exogenous sediment bound estrogens may have resulted in an upregulation of sex steroid production. Increasing circulating estrogens in the fish would have been synthesized from testosterone, which also is converted to 11-KT. This increase in estrogens in male fish would increase vitellogenin and subsequently increase 11-KT.

The last section of chapter 3 focused on modeling intersex in the impoundments. I explored factors associated with the probability that an individual fish from any impoundment was intersex. The impoundment level parameters of surface area and depth were the best predictors of the probability that individual impoundment fish will be intersex (at least in Georgia, USA). Specific mechanisms for induction of intersex were not elucidated, but we identified key characteristics of impoundments where high intersex rates occur.

With no definitive evidence for potential causes (i.e., no estrogen compounds or estrogen mimics) of intersex in impoundments, but clear trends of high intersex in small, shallow impoundments, I began considering other characteristics of the small impoundments that may have caused endocrine disruption in fish. Nitrate ($NO_3^-$) is a prolific environmental pollutant commonly found in high concentrations in eutrophic impoundments, and has recently been considered an endocrine disrupting chemical (Guillette and Edwards 2005). Other studies involving nitrate were focused on acute and chronic toxicity testing with invertebrates (reviewed by Camargo et al. 2005) and fish (Knepp and Arkin 1973; Kincheloe et al. 1979; Scott and Crunkilton 2000) but more recently, reproductive abnormalities have been documented in fish exposed to nitrate. Changes in secondary sex characteristics were reported in both male and female mosquito fish *Gambusia holbrooki* exposed to nitrate (Edwards et al. 2006; Edwards and Guillette 2007) and sex steroid hormone disruptions were noted in Siberian sturgeon *Acipenser baeri* exposed to nitrate (Hamlin et al. 2008). Several of the small impoundments (farm ponds and public fishing areas) I sampled for chapter 2 were fertilized with inorganic nitrogen (nitrate) to stimulate phytoplankton and zooplankton blooms to support dense fish populations so I hypothesized that nitrate was associated with endocrine disruption in fish from the impoundments.

Chapter 4, my final research chapter, investigated the putative endocrine disrupting effects of elevated nitrate to fish. Nitrate altered reproductive physiology in male fathead minnows by causing increased 11-KT and induction of vitellogenin when compared to control fish. Nitrate significantly induced vitellogenin and increased 11-KT at a concentration of 50 mg/L-$NO_3$, which, interestingly, is approximately equal to the maximum nitrate concentration (45 mg/L-$NO_3$) allowable in drinking water (US Environmental Protection Agency 1986). My results were consistent with sex steroid patterns observed in Siberian sturgeon exposed to nitrate (Hamlin et al. 2008) and my study was the first report of vitellogenin induction for male fish following increased nitrate exposure. Sex hormone levels and vitellogenin were not measured in largemouth bass collected from impoundments in Chapter 2, but would be informative to measure in future investigations. Intersex was not induced by nitrate exposure in fathead minnows but intersex has not been commonly reported with fathead minnows, even after long term exposures to estrogens, so this species may not be well suited for studying intersex. The role of nitrate in intersex in Georgia impoundments remains unknown but nitrate does appear to cause endocrine disruption. Future intersex studies should be conducted with species known to be sensitive to intersex, such as black basses.

## CONCLUSIONS

In this dissertation I have discussed interrelated topics focused on endocrine disruption in impoundment fish. This project is the first to extensively survey impoundments for the presence of intersex largemouth bass. Most field investigations regarding intersex fish over the past 20-30 years have focused on lotic environments, with occasional use of a lake as a relatively unimpacted reference water body. Among the most pertinent findings from my work are that relatively unimpacted sites can still be ‘hotspots’ for intersex fish even without direct input from well-known endocrine disrupting chemical sources like wastewater effluent or agricultural activities. I also demonstrated that multivariate modeling approaches can be very informative for identifying factors associated with intersex and creating hypotheses to test, but additional work is needed to understand the mechanisms by which intersex occurs in small impoundments. The role of non-traditional endocrine disrupting chemicals such as nitrate in intersex is still unclear but is definitely worthy of additional research. My hope for this dissertation is that it will be a resource to other ecotoxicologists and fisheries scientists researching endocrine anomalies in fish models and it will be useful for finding a cause or mechanism for intersex induction and other manifestations of endocrine disruption in fishes.

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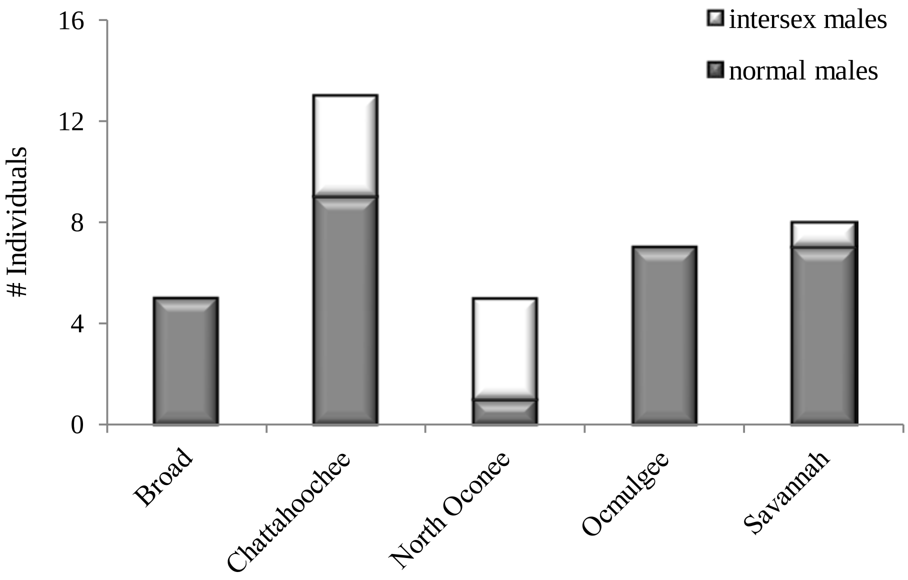
## APPENDIX A

## LARGEMOUTH BASS RIVER DATA



Total number of individual male largemouth bass separated into normal males and intersex sampled at river sites in Georgia, USA. All fish were sampled during Feb-June 2010 and 2011 by boat-mounted electroshocking at sites within 1 km of municipal wastewater discharges, except for the Broad River which does not receive municipal waste.