BIOTIC AND ABIOTIC FACTORS INFORMING

ADVERSE OUTCOME PATHWAYS FOR FEMINIZED FRESHWATER FISH

by

MATTHEW L. URICH

(Under the Direction of Robert Bringolf)

ABSTRACT

Exposure to estrogenic contaminants may pose a substantial threat to freshwater ecosystems worldwide by eliciting physiological and morphological effects in fish and other organisms that may contribute to population-level declines, yet more information regarding adverse outcome pathways is needed to assess risks. Evidence suggests that reproductive effects associated with estrogen-induced feminization of male fish may result in reproductive failure – an alarming prospect, considering the near-ubiquitous existence of estrogenic contaminants in the environment and increasing awareness of the widespread occurrence of feminized fish around the world. Estrogen exposure in aquatic environments is commonly linked to the widespread occurrence of testicular oocytes, an intersex condition characterized by the development of oocytes in the testis, and adverse reproductive effects associated with intersex have raised concerns about population-level effects. Furthermore, effects of global climate change may exacerbate existing effects of estrogens (or vice versa) via interactions with toxicodynamic and toxicokinetic processes, which could heighten risks for the foreseeable

future. Despite concerns, large gaps exist in our understanding of adverse outcome pathways. Key events leading from estrogen exposure to potential population-level effects in wild fish are poorly understood, precluding our ability to assess risk, identify areas of concern, and monitor ecosystem health. To better understand relationships between environmental factors, estrogen exposure, and physiological effects in fish, and to inform adverse outcome pathways for feminized fish, in the present work, a series of experiments investigating biotic and abiotic factors associated with the feminization of male fish were conducted.

INDEX WORDS: Ecotoxicology, Adverse outcome pathway, Intersex, Estrogens, Endocrine disruption, Largemouth bass, Model selection, Climate change, Metabolomics, Biomarkers

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DEDICATION

To the continuous determination of humankind to obtain truthful information about ourselves, and our interactions with the environment, in order to enact positive change in the world. And, to my loving fiancée, best friend, and eternal companion, Sarah.

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CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

Introduction

Feminizing Effects of Estrogens in Freshwater Fish

Widespread contamination of aquatic systems with endocrine disrupting chemicals (EDCs) has led to concerns about ecosystem health and population sustainability. A wide variety of compounds from various anthropogenic sources are known to elicit estrogenic sub-lethal effects on fish and other aquatic organisms, often at minute (low ng/L) concentrations, including abnormal gonad growth and function, sex reversal, and reproductive failure. Complex mixtures of EDCs including steroid hormones, pharmaceuticals, personal care products, pesticides, and industrial chemicals, with known or suspected effects on aquatic organisms, have been identified in close proximity to nesting fish populations (Kolpin et al., 2013). EDCs interfere with sensitive ratios of endogenous hormones that regulate the hypothalamic-pituitary-gonadal (HPG) axis in fish (Ankley et al., 2009), important for the timing and magnitude of numerous vital biological functions involved in development, metabolism, behavior, immunity, and reproduction. Under normal conditions, the HPG axis in fish relies on environmental cues such as photoperiod and temperature to initiate signaling cascades that regulate development and reproduction. EDC-induced dysregulation of the HPG axis can result in lasting or even permanent organizational (e.g., morphological) and activation-associated (e.g., hormone signaling) effects (Silbergeld et al., 2002). In fact, long-term sub-lethal effects of EDCs are more likely than acute effects

(Ankley and Johnson, 2004), and epigenetic effects of EDCs may even span generations (Anway and Skinner, 2006). Such effects have attracted considerable attention from ecotoxicologists and federal/state regulators where and small fish models (i.e., fathead minnows [*Pimephales promelas*], Japanese medaka, and zebrafish [*Danio rerio*]) are primarily employed to assess effects in other species (Ankley and Johnson, 2004).

Estrogenic EDCs, also referred to as environmental estrogens, or xenoestrogens, are a class of EDCs that can induce a variety of effects via activation of the estrogen receptor (ER) and related pathways. Environmental estrogens include both synthetic and natural compounds which originate from a wide variety of municipal, industrial, and agricultural sources and chemical classes. Though usually present at low concentrations in the environment, the relative potencies of estrogenic EDCs vary widely, and the combined effects of these compounds can exhibit an additive nature (Thorpe et al., 2003). Individually innocuous concentrations of estrogenic chemicals may induce considerable estrogenic activity when simultaneously present in mixtures (Brian et al., 2005; Jordan et al., 2012). Many EDCs, including estrogens, preferentially bind to sediment (Holthaus et al., 2002; Lai et al., 2000), which may serve as a persistent source of exposure in aquatic environments. Most natural estrogens (e.g., 17β-estradiol, E2), are rapidly degraded under aerobic conditions (Jürgens et al., 2002), but constant influxes can result in prolonged exposures. Agricultural runoff from concentrated animal feeding operations (CAFOs) containing animal waste plus pastures and crops fertilized with manure can release a considerable amount of natural estrogens into the aquatic environment (Ciparis et al., 2012; Jenkins et al., 2009; Lange et al., 2002; Lee et al., 2014; Soto et al., 2004; Yonkos et al., 2010), and pulses of estrogens from repeated application of manures and heavy rain events may continually elicit effects on fish.17α-ethinylestradiol (EE2), a synthetic estrogen found in oral

contraceptives and hormone therapy drugs, are not fully metabolized by humans and are excreted in the urine. EE2 and other EDCs are not fully removed by conventional wastewater treatment practices, and thus, are often present in effluents from wastewater treatment facilities (Auriol et al., 2006; Kolpin et al., 2002; Ternes et al., 2004). Synthetic compounds like EE2 are far more resistant to degradation than natural estrogens (Jürgens et al., 2002) and are also far more potent in fish (Metcalfe et al., 2001). The term 'pseudo-persistence' has been used to describe the constant influx of estrogens in aquatic systems from anthropogenic land use practices (Sumpter and Johnson, 2008), and organisms inhabiting estrogen-polluted waters may face chronic or repeated intermittent exposures throughout their lives.

By mimicking endogenous hormones or otherwise altering their activity, synthesis, or metabolism, environmental estrogens and other EDCs interfere with normal function of the HPG-axis, which can have a variety of physiological and morphological effects. Effects in fish have been documented for both sexes, but males are particularly sensitive, especially during early developmental stages. Environmental estrogens can have a variety of effects on various biological functions related to the HPG-axis, but reproductive effects in male fish are well-documented, and may have the greatest potential for ecological impact. Complex mixtures of environmental estrogens including steroid hormones, pharmaceuticals, personal care products, pesticides, and industrial chemicals, with suspected or documented estrogenic effects on aquatic organisms, have been identified in close proximity to nesting fish populations (Kolpin et al., 2013). There is growing concern among researchers and natural resource managers that current levels of environmental estrogens may pose a significant challenge to the integrity of exposed fish populations (Shved et al., 2008). In controlled laboratory exposures, estrogen concentrations at or below those observed in the environment have resulted in various effects in fish, including

pericardial/yolk sac edema (Johns et al., 2011), changes in sex ratio (Länge et al., 2001), reduced egg fertilization (Parrott and Blunt, 2005), induction of the egg-yolk precursor lipoprotein vitellogenin (Vtg) in male fish (Rodgers-Gray et al., 2000), abnormalities in gonad morphology (Brion et al., 2004), complete sex reversal (Lange et al., 2009), and total reproductive failure (Harris et al., 2011; Kidd et al., 2007; Nash et al., 2004). Similar effects have been documented in field surveys, in which reproductive (Jobling et al., 1998), immunological (Blazer et al., 2010), and morphological (Hinck et al., 2009) abnormalities were observed in wild fish populations from estrogen-contaminated habitats.

Individual components that contribute to estrogenic responses in aquatic systems are difficult to quantify due to their diverse properties and the complexity of their environmental distribution. Therefore, *in vitro* bioassays capable of measuring ER binding activity can be useful for characterizing the total estrogenic activity (estrogenicity) of environmental samples. Using an effects-based approach, estrogenicity can be measured and used as an indicator of an early biological response to estrogenic compounds, which can be used to predict/explain effects in apical endpoints. Such bioassays provide powerful insight to the combined response of organisms exposed to complex chemical mixtures, but they are often expensive and time consuming (Anderson et al., 1996; Legler et al., 2002). Yeast-based bioassays can be quickly utilized to screen environmental samples for potential estrogenic activity (Balsiger et al., 2010; Cespedes et al., 2005). Variations, like the bioluminescent yeast-estrogen screen (BLYES) assay exploit the estrogen-specific bioluminescent response of a recombinant strain of yeast (Saccharomyces cerevisiae) as indication of the presence of environmental estrogens in environmental samples (Alvarez et al., 2009). Human ERs in the cells bind to estrogenic compounds, which leads to the activation of the enzyme luciferase, and ultimately the emission

of light (Sanseverino et al., 2005). Bioluminescence occurs in a concentration-response continuum that can be used to assess total estrogenic activity of the sample, and potential hazard to exposed fish (Bergamasco et al., 2011). Luciferase transactivation bioassays have also been developed using human breast cancer cell lines that may offer advantages over similar bioassays. Chemically-activated luciferase expression (CALUX) cell bioassays such as the VM7Luc4E2 cell bioassay (Brennan and Tillitt, 2018), use similar mechanisms to measure of estrogenicity, but may offer increased sensitivity and affordability, compared to other assays. Measuring the estrogenicity of fish habitats may help to explain the distribution and extent of estrogen-like effects observed in fish populations. For example, estrogenicity bioassays have been used for modeling intersex in wild fish from EDC-contaminated environments (Arlos et al., 2018).

Concern Over Widespread Reports of Intersex Fish

Over the last few decades, increasingly frequent reports of intersex have raised awareness of the widespread occurrence of intersex among wild freshwater teleost fishes. Intersex, the simultaneous presence of male and female gonadal tissue, is known to occur in a broad range of vertebrate taxa, including reptiles (Guillette et al., 1994), and amphibians (Hayes et al., 2002), and is particularly prevalent among fish. Fish exhibit a wide diversity of genetic, physiological, and environmental mechanisms for sex differentiation (Devlin and Nagahama, 2002), and some marine species naturally change sex as part of their normal life-histories – for example, sequential hermaphrodites. Some have speculated that intersex in freshwater teleosts may occur, at least in part, as a natural phenomenon (Bahamonde et al., 2013), but intersex among gonochoristic (fixed-sex) fish species is largely considered to be an abnormal condition associated with exposure to estrogenic EDCs.

Intersex has been reported in a wide diversity of species throughout the world (Bjerregaard et al., 2006; Blazer et al., 2007; Jobling et al., 1998; Viganò et al., 2001), including at least 37 fish species from 17 families reported in 54 field survey studies from 24 countries (Hinck et al., 2009). In North America, reports of intersex are predominantly among members of the family Centrarchidae (i.e., sunfish, centrarchids), and intersex prevalence in these taxa appears to be particularly high in the Southeastern United States (Hinck et al., 2009). Some populations of black bass (e.g., largemouth bass, *Micropterus salmoides*, smallmouth bass, *Micropterus dolomieu*, and shoal bass, *Micropterus cataractae*) have up to 100% prevalence of intersex among males (Hinck et al., 2009; Ingram et al., 2011). Testicular oocytes (TO) is by far the most commonly observed form of intersex, especially among centrarchids, and is characterized by the presence of immature egg cells (oocytes) within the testis. Implications of locally high levels of intersex among certain regions, species, and/or populations are mostly unknown, but reports have caused concern for ecologically, commercially, and culturally important fish populations.

Intersex is associated with adverse physiological and morphological effects in fish, and understanding the nature of these effects may be crucial for assessing fish health. Intersex may be associated with increased parasite load in wild fish populations (Blazer et al. 2010), suggesting that intersex fish may exhibit immune suppression. Especially concerning are the adverse reproductive effects associated with intersex, which may lead to population-level effects. Though evidence is scarce, research suggests that individuals with severe levels of intersex have reduced reproductive fitness. In a study of the wild common roach (*Rutilus rutilus*) sampled from polluted streams in the United Kingdom, individuals with severe TO had reduced sperm motility and fertility compared with ‘normal’ male conspecifics lacking TO, and severity of intersex was

correlated with a decrease in viable offspring (Jobling et al., 2002). Harris et al. (2011) reported a 76% reduction in reproductive success among intersex male common roach compared to normal males. Fuzzen et al. (2015) also reported a reduction in fertilization success in wild rainbow darters (*Etheostoma caeruleum*) with severe intersex. However, it is likely that associated reproductive effects vary among taxa, since different forms of intersex occur in various taxa (Abdel-moneim et al., 2015). However, most reports of adverse reproductive effects have been associated with severe forms of intersex, in which entire regions of the testis are affected. Testicular oocytes, such as those common in black bass species, are typically focally distributed and pre-vitellogenic (Blazer et al., 2007), and the relevance of associated effects reported in other species with more severe forms of the condition is not known. Teleost fishes exhibit tremendous diversity in sex differentiation and gonad development, and relevant information is limited or unknown for many species in which intersex is common. Therefore, it is difficult to draw meaningful conclusions that can be extrapolated to all intersex fish based on results from a limited number of species. However, current evidence regarding adverse reproductive effects or intersex has alarming ecological implications.

The ultimate concern regarding adverse reproductive effects is the potential for population-level effects. Presumably, if intersex is associated with adverse reproductive effects, this may ultimately lead to a decline in population recruitment and eventually threaten loss of genetic diversity and population declines. Therefore, a major priority for intersex fish research is understanding potential connections between intersex and population-level effects. Evidence of population-level effects associated with intersex extremely limited, in part because of the scale and complexity of population-level experiments. For example, intersex fathead minnows were found among a population that experienced reproductive failure following long-term estrogen

exposure (Kidd et al., 2007). In this case, it is unknown whether intersex was a causative factor in the resulting population crash, or perhaps only a benign side-effect of estrogen exposure. Aside from this study, direct evidence of population-level effects of intersex is limited practically non-existent. More information is needed to assess ecological risk associated with intersex, and given its widespread occurrence in the environment, understanding causative factors is critical to identifying areas or populations of concern and recognizing the scope of potential ecological harm. From a toxicological perspective, understanding the role of intersex within the framework of adverse outcome pathways (AOPs) may be an important aspect to assessing risk of EDCs.

Efforts to Understand Causes of Intersex

After decades of research investigating potential causes of intersex in fish, it is apparent that under certain conditions, intersex can be induced by exposure to EDCs. Intersex can clearly be induced by EDC exposure, as repeatedly demonstrated in small fish models, such as Japanese medaka (*Oryzias latipes*) and zebrafish (*Danio rerio*; Abdel-moneim et al., 2015). In the environment, exposure to complex chemical mixtures is likely to play a role in many cases, but other factors such as temperature, population density, and genetics may also be involved. Field studies often compare results of intersex surveys with environmental factors of interest to elucidate causes. A major problem is that these surveys require lethal sampling of large numbers of fish. Recently, efforts have been made to develop non-lethal laparoscopy-based methods, (MacLeod et al., 2017) but histological evaluation of gonadal tissues remains the only widely-accepted method for identification of TO in fish. With strong evidence that intersex is related to EDC exposure, geographic variation in intersex prevalence is frequently attributed to various sources of anthropogenic land use. Intersex downstream of wastewater treatment facilities may likely be caused by exposure to chemical cocktails of pharmaceuticals and other known or

suspected EDCs known to exist in industrial and municipal effluents (Bjerregaard et al., 2006; Blazer et al., 2007, 2012; Hinck et al., 2008; Pollock et al., 2010; Vajda et al., 2008). In some cases, intersex appears to be related to non-point sources of pollution, specifically agricultural land use (Blazer et al., 2007, 2014; Ciparis et al., 2012; Iwanowicz et al., 2009). However, measured levels of contaminants often fail to fully explain intersex variability (Kolpin et al., 2013), suggesting that important influences may be overlooked. Nitrate, which often co-occurs with suspected EDCs is also suspected as an endocrine disruptor (Edwards and Hamlin, 2018, 2018; Poulsen et al., 2018), but is rarely investigated for associations with intersex. Perhaps most surprisingly, intersex has been reported at high levels even in protected areas considered to be relatively pristine (Iwanowicz et al., 2016), and can also occur in laboratory control and reference populations, a phenomenon which may be underreported (Grim et al., 2007). These findings, inconsistent with conventional EDC-exposure hypotheses, have exposed gaps in our understanding of intersex in fish, and may suggest the involvement of non-chemical factors. Physical environmental factors such as water temperature and dissolved oxygen, as well as social factors like population density, may also play a role. A recent study of intersex LMB in Georgia (USA) impoundments found a negative association between intersex and impoundment surface area (Bringolf et al., 2015), a factor unavoidably linked with surface water temperature and dissolved oxygen levels in warmer climates. In addition, biotic factors such as fish body size (Blazer et al., 2007) have been suggested to vary in intersex fish, introducing an additional source of variability typically unaccounted for in investigations. In short, intersex among wild freshwater fish appears to be a complex and widely circumstantial phenomenon, depending on a variety of known and unknown interdependent factors, and many questions regarding potential causes remain unanswered.

Metabolomics in the Study of Fish Feminization

Recent technological developments in analytical biochemistry have allowed researchers to simultaneously assess multiple biochemical variables to evaluate specific phenotypic responses to various environmental factors. 'Ecotoxicogenomics,' which includes the sub-fields of genomics, transcriptomics, proteomics, and metabolomics, can be used to assess sub-cellular effects of environmental contaminants in fish (Miracle and Ankley, 2005). Much of this field relies on the detection of specific genes, mRNA transcripts, and proteins, which may only be applicable to particular species, and ultimately, may not reflect changes in physiology. However, metabolomics offers the unique ability to simultaneously evaluate numerous biochemical responses that represent downstream effects of changes in endogenous metabolic pathways. Levels of low molecular weight metabolites are indicative of essential endogenous processes required for growth, cell maintenance and normal function (Dunn et al., 2005). The 'metabolome,' a complete collection of all metabolites, is the final downstream product of genomic, transcriptomic, and proteomic regulations that occur in response to various environmental stimuli. Metabolomics is a field presenting a tremendous amount of potential to advance our understanding of responses to environmental stressors, changes to specific cellular processes, and toxicant mechanisms of action (MoAs) in aquatic organisms (Bundy et al., 2009; Jones et al., 2013). Importantly, variables measured with these techniques are largely conserved among vertebrates, and are found in common across taxa, allowing for inter-species comparisons (Werner and Connon, 2013).

Biomarkers associated with feminization in male fish due to estrogen exposure, including reduced gonadosomatic index (GSI) and plasma vitellogenin levels, lack the sensitivity and specificity needed to differentiate between intersex and cohabiting estrogen-exposed male fish

without TO (Bahamonde et al., 2013). Induction of the gene ovarian structure protein-1 (OSP-1), is strongly associated with TO in Japanese medaka (*Oryzias latipes*; Abdel-Moneim et al., 2015), but analysis of OSP-1 relies on a species-specific sequence for detection. To date, most studies investigating intersex on a sub-cellular level has been limited to gene expression investigations of laboratory-reared species such as the fathead minnow (Feswick et al., 2016). Gene expression assays conducted on wild intersex fish have also provided valuable information (Bahamonde et al., 2015), but these techniques are limited to select wild fish species for which relevant RNA primers exist, and the relevance of results to unrelated species of interest is unknown. Responses of the fish metabolome can be extremely sensitive to EDC exposure. In one laboratory study, urinary metabolite levels of fathead minnows exhibited clear responses when exposed to EDCs individually as well as in mixtures (Collette et al., 2010). Another study demonstrated metabolic effects from exposure to a mixture of EDCs that were not predicted from exposure to the compounds individually (Jordan et al., 2012). This suggests that the additive nature of effects of some compounds that act through similar modes of action may be measured as an integrated biological response of the metabolome. Davis et al. (2013) demonstrated the utility of field-based exposure monitoring using metabolite profiles of caged fathead minnows exposed *in situ* to EDCs in the environment. This study demonstrated the ability to detect sex-specific responses to EDC exposure in metabolite profiles. Sex-specific responses were also reported in the skin mucus of fathead minnows exposed to Bisphenol-A (Ekman et al., 2015), a compound previously shown to induce TO in fish (Kang et al., 2002). These data suggest that minimally invasive techniques may be used to characterize the feminization of male fish, even in transient biofluids. Similar techniques may be used to examine biological matrices of wild fish for biomarkers of TO.

An important aspect of any potentially harmful biological condition or disease state relates to the mechanisms associated with its incidence. Identifying biochemical changes within the affected tissue may yield information relevant to molecular initiating events, as well as associated adverse physiological states. Analytical techniques applied in the field of metabolomics have provided important information about physiological conditions associated with diseased states, and are commonly used to study complex diseases such as cancers. Measuring levels of endogenous metabolites, collectively referred to as the metabolome, can yield important information regarding pathogenesis and related physiological effects of a condition. In a toxicological setting, metabolomics is used to elucidate mechanisms of toxicity to better understand how effects of chemical exposure are elicited on a biochemical level. Investigation of the molecular physiology within an intersex gonad could yield important information relevant to the induction of testicular oocytes and provide insight into biochemical mechanisms of intersex induction resulting from chemical exposure. Furthermore, in the case of intersex, it is necessary to preserve most of the testicular tissue for histological confirmation of the condition. Using metabolomics, valuable information could be gained by analyzing metabolite profiles of very small (~50mg) gonadal tissue samples, while still providing for adequate tissue for gonad histology. Biochemical activity within the intersex testis may be indicative of physiological and reproductive endpoints critical to assessing ecological risk. More information regarding the relationship of TO with potential adverse effects is needed to anchor the condition into structured ecotoxicological frameworks such as ecological risk assessments and AOPs.

The Upper Coosa River Basin: an Ecosystem Under Threat of Feminization

The Upper Coosa River (UCR) Basin in northwestern Georgia and southern Tennessee (USA) is among the most bio-diverse systems in the world, but within the past few decades, dramatic declines in aquatic biota have caused concern. Evidence of EDC contamination and intersex fish within imperiled regions of the UCR are a prime example illustrating the importance of understanding complex relationships between EDC exposure, anthropogenic land use, the feminization of fish, and adverse population-level effects. Seine surveys conducted from 1996 to 2012 in the Conasauga River, one of three major tributaries of the UCR suggested a dramatic decline in the abundance of several rare and sensitive fish species (Golder Associates, Inc., 2010; Hagler and Freeman, 2012). However, causes of these declines are largely unknown, since areas experiencing the greatest decline are generally upstream of major municipal or industrial sources of waste, and analyses of current use pesticides, PAHs (Sharpe and Nichols, 2007), and glyphosate (Lasier, 2012) have not indicated levels of concern. However, potentially harmful levels of estrogens have been measured in Conasauga River sediments and surface waters (Lasier et al., 2016), and Jacobs (2013) detected intersex among ten of 13 (76.9%) male sunfish (family Centrarchidae) collected from Conasauga River tributaries, potentially implicating estrogens in the feminization of fish in the region. Aside from this brief survey, comprehensive evidence regarding intersex in this region is lacking to better understand causative factors and potential implications with observed population declines. Understanding the extent of intersex throughout the region could be useful to elucidate causative factors of intersex of concern, and may also help to explain possible implications of intersex in population declines within the UCR and in comparable regions.

Research Rationale

Current knowledge is insufficient to pinpoint causes or assess risks associated with the feminization of male fish, despite concerns regarding the potential for adverse effects. To better understand ecological implications of environmental estrogens and related effects in fish, connections among factors of interest need to be established. Comprehensive analyses accounting for a broad scope of biotic and abiotic factors to investigate feminizing effects of estrogens such as intersex in fish are lacking. Specifically, toxicological, biochemical, geo-spatial, temporal, morphological, and taxonomic factors associated with environmental estrogens, and their effects, warrant further investigation.

Evidence that the intersex condition may indicate or mediate adverse reproductive physiology in fish is not well understood. To infer about potential population-level effects in fish, more information regarding the physiology underlying the intersex condition is needed to assess ecological risk and to understand the impetus of intersex toward adverse outcomes. Efforts to determine the extent and mechanisms of ecological risk associated with TO are severely limited by lethal histological methods of TO detection. Histological evaluation of large numbers of sexually mature male fish is required to make meaningful statistical comparisons, but lethal sampling is not prudent or practical for populations at risk of decline. Further, histological methods disallow for repeated sampling of individuals, hindering the investigation of temporal aspects of TO development. Thus, the development of non-lethal methods of TO identification is necessary to broaden the scope of investigations into causes and effects of TO.

Metabolomics offers a considerable advantage for studying intersex and other effects of EDCs in that techniques do not require a sequenced genome, and thus could be used to develop methods capable of interspecies comparisons. Identification of metabolite biomarkers of intersex,

particularly from non-lethally collected biological samples, would greatly expand the diagnostic ability of scientists to evaluate TO among fish species of interest. Molecular endpoints have been found in association with TO in fish, but most still require invasive or lethal sampling, and none display specificity or reproducibility required to reliably indicate TO. Transcript and protein markers such as OSP-1 may be useful for laboratory-based investigations, but their current utility is currently limited to organisms with well-characterized genomes. Simultaneous assessment of a variety of biochemical markers could provide valuable insight into the systems and pathways affected in wild intersex fish.

Chapter 2 Overview

The objectives of this study were to: (1) evaluate long-term effects of early life-stage estrogen exposure on endogenous hepatic metabolism, (2) to compare the utility of polar and nonpolar metabolite profiling for assessment of long-term effects of exposure, and (3) to provide insight to biochemical pathways associated with long-term physiological effects resulting from estrogen exposure in male fish.

Chapter 3 Overview

To better understand the relationships between intersex and biotic and abiotic factors in the UCR, we produced predictive models for intersex incorporating a broad range of variables including fish morphometrics, water quality, land use, seasonal sediment estrogenic activity, and sediment estrogen concentrations at sites representing a gradient of forested, agricultural, and urban development within the Upper Coosa River Basin. The primary objectives of this study were to: (1) determine the prevalence, severity, and geographic and taxonomic distribution of intersex among large-bodied fishes of the Upper Coosa River Basin, and (2) to determine the

relationship of variables associated with and/or predictive of the occurrence of intersex in the region.

Chapter 4 Overview

In this study, we evaluated the utility of LC/MS-based metabolomics for the identification of metabolite biomarkers associated with TO in liver and blood plasma from wild male LMB with varying TO counts. The objectives of this study were to: (1) identify candidate analytes for development of biomarkers of TO among wild LMB, and (2) elucidate biochemical mechanisms associated with the incidence of TO.

Chapter 5 Overview

Chapter 4 demonstrated that liver and blood plasma are potential sources for metabolic biomarkers of TO in wild fish. These matrices were useful for determining organism-level effects and mechanisms, but a better understanding of TO-associated mechanisms may be gained by examining the levels of metabolites directly from the affected tissue. Therefore, in Chapter 5, we evaluated the gonad metabolite profiles of wild intersex LMB to elucidate information relevant to molecular initiating events of TO and associated adverse physiological effects within the gonad. In addition, blood plasma biochemical profiling was employed to better understand organismal responses associated with TO and to evaluate its utility for classification of individuals with different levels of TO. Methods used in this study could be used to investigate intersex and other gonadal abnormalities in various species and to compare incidences of intersex occurring under different circumstances to better explain variability observed in the field and in laboratory studies.

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CHAPTER 2

NONPOLAR LIVER METABOLITE PROFILES OF ADULT FISH INDICATE LONG-TERM EFFECTS OF EARLY LIFE-STAGE EXPOSURE TO 17β-ESTRADIOL[1]

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Abstract

Long-term biochemical effects of estrogen exposure are poorly understood, despite evidence of association with prolonged, ecologically relevant endpoints in fish. In the present study, alterations in liver metabolite profiles of adult Japanese medaka (*Oryzias latipes*) exposed as juveniles to 128 ng/L 17β-estradiol (E2) were determined using LC/MS-based metabolite profiling (i.e., metabolomics). Exposures were conducted for 3 weeks (beginning at ~8 weeks post-hatch), followed by a 4-month grow-out/depuration period. Livers were dissected and extracted following depuration, and both polar and nonpolar phases were extracted and analyzed separately. Exposure resulted in prolonged effects in males detectable in the nonpolar phase, which were associated with testicular fibrosis detected histologically; whereas, effects in the polar phase were not apparent. Metabolite profiles of control females were distinct in both phases, and nonpolar profiles of exposed males showed similarities to profiles of females. Differences in phospholipid metabolism were apparent in exposed males, and elevated synthesis of peptides rich in nonpolar amino acid residues was also observed. These results suggest that exposure to estrogen at an early life stage may have long-term effects on nonpolar metabolite profiles indicative of physiological abnormalities in fish. Furthermore, prolonged effects in nonpolar profiles may offer advantages over polar profiles for ecological monitoring purposes.

Introduction

Widespread contamination of aquatic systems with endocrine disrupting chemicals (EDCs) has led to concerns about fish reproductive health and population sustainability. A wide variety of compounds from various anthropogenic sources are known to elicit estrogenic effects on male fish, including abnormal gonad growth and function, sex reversal, and reproductive failure, on fish and other aquatic organisms. Complex mixtures of EDCs including steroid

hormones, pharmaceuticals, personal care products, pesticides, and industrial chemicals with known or suspected effects on aquatic organisms have been identified in close proximity to nesting fish populations (Kolpin et al., 2013). Such compounds interfere with sensitive ratios of endogenous hormones in fish, important for regulating the timing and magnitude of numerous vital physiological functions involved in development, metabolism, behavior, immunity, and reproduction. There is growing concern among researchers and natural resource managers that current levels of environmental estrogens may pose a significant challenge to fish populations (Shved et al., 2008).

Early developmental stages of fish are the most sensitive to EDC exposure, and lasting or even permanent organizational (e.g., morphological) and activational (e.g., hormone signaling) effects may result (Silbergeld et al., 2002). In fact, EDCs are more likely to cause long-term sub-lethal effects rather than acute effects (Ankley and Johnson, 2004), and epigenetic effects of EDCs may even span generations (Anway and Skinner, 2006). Such effects have attracted considerable attention from ecotoxicologists and regulators; therefore, small fish models (i.e., fathead minnows [*Pimephales promelas*], Japanese medaka, and zebrafish [*Danio rerio*]) are primarily employed to assess effects in other species (Ankley and Johnson, 2004). Laboratory studies have reported a variety of morphological and physiological abnormalities resulting from exposure to estrogenic EDCs, including altered gene expression, pericardial/yolk sac edema (Johns et al., 2011), changes in sex ratio (Länge et al., 2001), reduced egg fertilization (Parrott and Blunt, 2005), induction of the egg-yolk components such as vitellogenin (Vtg) (Rodgers-Gray et al., 2000), gonadal abnormalities (Brion et al., 2004), and complete sex reversal (Lange et al., 2009). Similar effects have also been reported in wild fish populations from estrogen-contaminated habitats, including reproductive (Jobling et al., 1998), immunological (Blazer et

al., 2010), and morphological (Hinck et al., 2009) abnormalities. Of major concern is that effects on individuals have the potential for population-level implications, as suggested by studies reporting total reproductive failure of fish populations following exposure to estrogenic compounds (Harris et al., 2011; Kidd et al., 2007; Nash et al., 2004). Wild fish may experience fluctuating exposure regimes or intermittent exposures due to migrations within freshwater systems and/or fluxes of environmental EDC concentrations or bioavailability. The physiological effects of such exposures, particularly during early life-stages, may persist throughout life. Yet, little is known about how long-term biochemical changes may reflect or contribute to lasting adverse physiological changes.

Estrogen exposure has been reported to result in numerous biochemical changes in the liver of fish (Ekman et al., 2008; Sangiao-Alvarellos et al., 2005; Sehgal and Goswami, 2001; Whiting and Wiggs, 1978), primarily in enzyme activities and polar analytes like carbohydrates and amino acids. Effects on lipids have also been reported (Cakmak et al., 2006; Dasmahapatra and Medda, 1982; Haux and Norberg, 1985; Khanna and Singh, 1983; Korsgaard, 1990; Korsgaard and Petersen, 1979; Sharpe and MacLatchy, 2007; Vlaming et al., 1977). Many studies have reported rather crude measures of lipid responses, with little to no detailed information regarding altered biochemical classes. Contemporary investigations of biochemical effects of EDC exposure in fish often employ metabolomics, allowing for simultaneous profiling of a broad spectrum of endogenous biomolecules. However, limited information exists regarding long-term effects, because studies are often terminated immediately following exposure with little to no depuration period. For example, Ekman et al. (2009) reported sustained effects on lipid metabolite profiles of male fathead minnows exposed to 17α-ethinylestradiol following an 8-day depuration period, but time points beyond this period were not evaluated.

To assess long-term effects of intermittent EDC exposures, more information is needed regarding the biochemical aspects of long-term effects. The objectives of this study were to: (1) evaluate long-term effects of early life-stage estrogen exposure on endogenous hepatic metabolism, (2) compare the utility of polar and nonpolar metabolite profiling for assessment of long-term effects of exposure, and (3) provide insight to biochemical pathways associated with long-term physiological effects resulting from estrogen exposure in male fish.

Methods

Animals and Rearing Conditions

Wild-type (Hd-rR strain) Japanese medaka embryos were obtained from 6-10 breeding pairs from Aug. 10 to Aug. 17, 2017 at US Geological Survey Columbia Environmental Research Center (CERC, Columbia, MO). Medaka embryos were shipped overnight to the University of Georgia Aquatic Toxicology Lab (Athens, GA, USA), where they were incubated at 28 °C in gently circulating carbon-filtered dechlorinated tap water with added salts to achieve desired hardness (~250 mg $CaCO_3$/L). Upon hatching, larval medaka were transferred to glass 75-L holding aquaria and fed freshly-hatched *Artemia nauplii* twice daily. Approximately 3 weeks post-hatch, pulverized Tetramin® fish flakes (Tetra, Blacksburg, VA) were introduced into the diet, and juveniles were fed a combination of *A. nauplii* and fish flakes for the duration of the study. Upon development of sexually dimorphic coloration (approximately 6 weeks post-hatch), juvenile medaka were sorted based on sexually dimorphic traits to obtain male fish for the exposure. At 8 weeks post-hatch, a total of n = 44 putative male juvenile medaka were randomly distributed among four 40-L glass exposure aquaria recirculating with 30 L gently aerated carbon-filtered dechlorinated tap water, and fish were allowed to acclimate for 3 days

prior to exposure. All fish were handled in accordance with approved University of Georgia Institutional Animal Care and Use protocols (AUP # A2016 06-028-A1).

Estrogen Exposure

Following acclimation, carbon filters were removed from tanks, and juvenile medaka were then exposed to nominal E2 concentrations of either 0 or 100 ng/L in 40-L glass aquaria (n = 2 tanks per treatment) filled with 30-L dechlorinated carbon-filtered tap water for 21 days. A solution of E2 (30 mg/L) dissolved in methanol was used to obtain a nominal concentration of 100 ng/L in two randomly selected aquaria, and the remaining two aquaria were dosed with an equivalent volume of methanol and treated as a solvent control group (0 ng/L E2). To maintain exposure concentrations and to remove waste, 50% of water was siphoned and replaced with freshly dosed water three times/week for the duration of the exposure. For measurement of estrogen concentrations, water samples were collected on day 1 (immediately after initial dosing), days 8 and 15 (immediately before water change), and day 22 (immediately prior to ending the exposure). Grab samples of equal volume from each replicate were composited by treatment group for a total volume of 1.0 L, and solid-phase extraction (SPE) was performed with Oasis HLB cartridges (500 mg; Waters, Milford, MA, USA), which were frozen (-80 °C) until elution and analysis. During exposure, water temperature was maintained using a water bath heated to 27 °C, and photoperiod was 16:8 hrs (light:dark). After 21 days, exposure was ceased and medaka were transferred to a single UV-filtered system recirculating with clean carbon-filtered dechlorinated tap water at ~27 °C, with exposure replicates held separately among four 75-L glass aquaria until dissection.

Analysis of Estrogen Exposure Concentrations

E2 exposure concentrations were analyzed via LC/MS following derivatization with dansyl chloride as described by Ke et al. (2014). Briefly, SPE cartridges were warmed to room temperature and extracts were eluted into 20-mL glass tubes with 5 mL methanol followed by 5 mL of dichloromethane. Extracts were evaporated to ~1 mL under a steady flow of nitrogen and rinsed with clean methanol into amber glass HPLC vials, where they were evaporated under vacuum to dryness. Following derivatization, extracts were injected into a ZORBAX Eclipse XDB column (150 x 1.0 mm, particle size = 3.5 µm, pore size = 80 Å; Agilent, Santa Clara, CA, USA) and analyzed using a Thermo TSQ Quantum Access triple quadrupole mass spectrometer. A prepared standard solution of E2 was serially diluted, derivatized, and analyzed along with water samples, and a standard curve was generated to facilitate calculation of exposure concentrations. One blank (ultrapure de-ionized water) was extracted and analyzed for every time point for quality assurance. Measured E2 concentrations during exposure were 127.9 ± 80.0 ng/L for the '100 ng/L' (nominal concentration) exposure group and 4.7 ± 7.7 ng/L in the control group (likely due to excretion of endogenous E2). The limit of detection for E2 was 0.99 ng/L.

Tissue Dissection

Approximately 30 weeks post-hatch, medaka tissues were dissected over a period of 3 days for metabolomics analysis, determination of genetic sex, and histological processing. Individuals were processed in a randomized fashion and euthanized with an overdose of pH-buffered MS-222 (500 mg/L) prior to dissection. Length (nearest mm) and weight (nearest mg) were measured and condition factor (CF = weight (g) / 100 * Length (cm)^3) for each individual. The entire liver of each fish was removed and placed in a chilled 2-mL centrifuge tube, which was immediately flash frozen and stored at -80 °C until extraction. The caudal fin of each fish

was excised and placed into 95% ethanol for determination of genetic sex. Fish were then decapitated and the trunk of each fish containing the gonadal tissue was placed into a histology cassette and preserved in buffered 10% formalin solution for histological processing.

Gonad Histology

Gonads were evaluated histologically for the presence of abnormalities or sex-reversal. Histological processing was performed by the College of Veterinary Medicine Histology Lab at the University of Georgia. Briefly, fish trunks preserved in formalin were dehydrated and embedded in paraffin wax. Three histological step-sections approximately 6 µm in thickness, each 75 µm apart (spanning a total of ~150 µm in depth) and were taken from the medial region of each fish trunk, placed on microscope slides, and stained with hematoxylin and eosin to facilitate microscopic evaluation. Slides were examined using compound microscopy for phenotypic gonadal sex and gonadal abnormalities. Individuals for which fewer than two complete sections of gonadal tissue were obtained were excluded from further evaluations to avoid variability due to the potential for unknown sex-reversal or gonadal intersex.

Determination of Genetic Sex

Determination of genetic sex of each individual was accomplished via polymerase chain reaction (PCR) to identify the presence/absence of the *DMY* gene in DNA extracted from the ethanol-preserved caudal fin of each individual. Methods for DNA extraction and PCR followed those used by Shinomiya et al. (2004) with *Taq* polymerase (New England Bio-Labs, inc., Ipswich, MA, USA). Briefly, DNA was extracted from caudal fins and PCR was performed with the following primers for *DMY* and *DMRT1*:

PG17.5, CCGGGTGCCCAAGTGCTCCCGCTG

PG17.6, GATCGTCCCTCCACAGAGAAGAGA

Samples were placed in a thermal cycler under the following conditions: 95 °C for 2 min, followed by n = 27 cycles of 95 °C for 15 sec, 58-49 °C (i.e. 58 °C for the first cycle, and decreasing by 0.3 °C for each subsequent cycle to 49 °C by the last cycle), followed by 68 °C for 70 sec and samples were held at 12 °C prior to electrophoresis. PCR products were analyzed via electrophoresis in 1% agarose gel, and the presence or absence of the *DMY* gene was used to determine the genetic sex of test animals. A total of 18 fish (n = 9 from the control group and n = 9 from the E2-exposed group) determined as females (both genotypically and histologically) were inadvertently included in the study due to error in sexing of juveniles based on sexually dimorphic coloration prior to exposure. Control females were subsequently pooled and evaluated as a separate group for reference.

Liver Extraction

Metabolomics extractions and analyses of liver tissues were performed at the US EPA National Exposure Research Laboratory (NERL, Athens, GA, USA). Extraction procedures followed those detailed by Viant et al., (2007). Briefly, frozen liver samples (in addition to n = 5 blanks to verify no cross-contamination during extraction) were randomly distributed prior to extraction, and a tissuelyzer was used to homogenize tissues in a bi-phase solvent mixture of methanol/water (polar phase) and chloroform (non-polar phase). Samples were then centrifuged to separate phases, and polar and nonpolar phases were decanted into separate 2 mL amber glass HPLC vials. Quality control samples were created by pooling remaining extract from all individuals, homogenizing, centrifuging, and decanting to obtain aliquots equivalent in volume for each phase, which were hereafter processed and analyzed simultaneously. Both phases were evaporated under vacuum, and dried extracts were stored at (-80 °C) until analysis. Prior to analysis, extracts were thawed and reconstituted in either 150 µl 30% acetonitrile (nonpolar

phase) or 200 µl 75% acetonitrile (polar phase). Samples were vortexed for 5 min to ensure complete dissolution of extracts and were then transferred to micro-target inserts placed in HPLC vials for analysis.

Metabolomics Analysis

Both polar and nonpolar metabolite profiles were acquired using a Thermo TSQ Quantum Access triple quadrupole MS (+ESI mode), and all data were collected and processed using Excalibur (v. 2.0.7, Thermo Fisher Scientific, Waltham, MA, USA). Separation of 20 µL polar extract injections was achieved using an Accucore HILIC column (150 x 2.1 mm, particle size = 2.6 µm, pore size = 80 Å; Thermo Fisher Scientific) maintained at 40 °C with a gradient elution of solvent A (ultrapure water with 0.1% formic acid) and solvent B (acetonitrile with 0.1% formic acid) with flow rate of 400 µl/min. The initial condition was set to 2% solvent A for 3 min, which was increased to 20% solvent A by 20 min, 30% solvent A by 25 min, and 50% solvent A by 35 min. The column was rinsed with 100% solvent A for 4 min and conditioned with 2% solvent A for 5 min prior to the injection of each sample. Separation of 20 µL nonpolar extract injections was achieved using a Kinetex C18 column (150 x 2.1 mm, particle size = 2.6 µm, pore size = 100 Å; Phenomenex, Torrance, CA, USA) maintained at 40 °C with a gradient elution of solvent A (ultrapure water with 0.1% formic acid) and solvent B (acetonitrile with 0.1% formic acid) with flow rate of 200 µl/min. The initial condition was set to 70% solvent A for 3 min, which was decreased to 2% solvent A by 50 min, where it was maintained until 55 min. The column was rinsed with 100% solvent B for 4 min and conditioned with 70% solvent A for 5 min prior to the injection of each sample. The following mass spectrometry parameters were used for both polar and nonpolar methods: sheath gas, 30; Auxilliary gas, 15; sweep gas, 0; capillary temperature, 300 °C; Spray voltage, 3.5 kV; m/z scan range, 50-800.

Statistical Analysis (Morphometrics)

Normality of morphometric data were assessed using Shapiro-Wilk's test ($\alpha = 0.05$), and equality of variance among treatment groups was assessed using Bartlett's test ($\alpha=0.05$). Though morphometric data were determined to be normally distributed and variances equal among groups, due to the unequal sample sizes of experimental groups, non-parametric statistical analyses were employed to evaluate differences among groups. Kruskal-Wallis one-way analysis of variance was used to evaluate differences in total length, body weight, and condition factor ($\alpha = 0.05$), and a Mann-Whitney U test was used for pair-wise comparisons of males and females ($\alpha = 0.05$).

Data Processing and Statistical Analysis (Metabolomics)

Chromatograms were exported as raw files, converted to mzMXL format, and imported into XCMS Online (xcmsonline.scripps.edu) for data preprocessing and alignment (Tautenhahn et al., 2012). Recommended parameters for processing of single quadrupole HPLC metabolomics data were used for peak detection, retention time correction ('Obiwarp' method), and alignment of both polar and nonpolar data. Processed polar and nonpolar data were exported as csv files and uploaded separately to Metaboanalyst (metaboanalyst.ca) for statistical analysis. Analytes close to the baseline were filtered by mean intensity value (lowest 40%) and metabolite profiles were normalized by the sum intensity value. Analyte relative abundance values were mean-centered and pareto scaled prior to statistical analysis. Partial least squares discriminant analysis (PLS-DA) was conducted to evaluate overall differences among females, control males, and E2-exposed males for both polar and nonpolar datasets. The top 25 variable importance in projection (VIP) scores were identified for relevant components, and the relative directional response of groups was noted for each analyte. A series of t-tests ($\alpha = 0.05$) and fold-change analyses (fold-

change threshold = 2.0) were conducted to evaluate differences between metabolite profiles of control males and E2-exposed males. Putative metabolite annotations were performed for analytes of interest by searching median m/z values used in alignment (assuming protonated molecular ions, i.e. M+H) using the Human Metabolome Database (hmdb.ca) and METLIN (metlin.scripps.edu) with a mass error threshold of ≤ 100 Δppm. Analytes without plausible hits at this threshold were annotated using a mass error threshold of ≤ 300 Δppm and flagged as speculative annotations.

Results

Fish Morphology, Histology and Genetic Sex

Significant differences in body weight ($p = 0.33$), total length ($p = 0.71$), or condition factor ($p = 0.36$) were not observed between females and males (Fig. 2.1). Differences between male exposure groups in body weight ($p = 0.17$), total length ($p = 0.51$), and condition factor ($p = 0.25$) were also insignificant. Histological evaluation revealed sexually mature gonadal tissue in all fish. A total of n = 18 individuals (nine from each treatment) were found to have ovarian tissue and no testicular tissue in the area examined and were determined as females histologically. Ovarian tissues from females contained oocytes in various stages of development, including pre-vitellogenic, post-vitellogenic, and atretic oocytes. Testes of all male fish contained spermatogenic tissues in various stages of development, from spermatogonia to mature sperm. Evidence of intersex was not detected among any individuals. However, E2-exposed males had an increased prevalence of abnormal connective tissues (91%) compared to control males (0%), with regions of the testis composed almost entirely of fibrotic tissues (Fig. 2.2). Analyses of genetic sex confirmed that the n = 18 individuals with ovarian tissues included in the

study were XX females, despite efforts to obtain a male-only cohort prior to exposure. Genetic sex determined by PCR was in agreement with histological sex in all cases, confirming that sex reversal did not result from exposure to E2.

Metabolomics

Analysis of liver metabolite profiles of Japanese medaka detected a total of 690 spectral features in the polar phase and 1068 spectral features detected in the nonpolar phase. Results of statistical analyses of both polar and nonpolar liver metabolite profiles showed strong sex-differences between males and females. Both polar (Fig. 2.2A) and nonpolar profiles (Fig. 2.2B) showed clear separation of males from females along PLS-DA Component 1, but differences between control and E2-exposed males were less pronounced. Polar metabolite profiles of control and exposed males were practically indistinguishable by PLS-DA due to near-complete overlap between both male groups; therefore, results of this analysis were not investigated further. However, nonpolar profiles showed moderate separation of control and exposed males along Component 1, and E2-exposed males on average trended toward females, and 95% confidence ellipses of female and E2-exposed males overlapped slightly. Pairwise analyses found a total of n = 19 nonpolar spectral features differing significantly ($p < 0.05$, FC > 2.0) between control and exposed males, and only n = 5 polar features differed. Significantly differing analytes were generally higher in exposed males, with the exception of two nonpolar analytes and one polar analyte.

Putative nonpolar metabolites important for separation in PLS-DA analysis, as indicated by the top 25 VIP scores, included mostly high molecular weight glycerophospholipids, ranging from m/z 703 to 834 and included phosphatidic acids (PAs), phosphatidylcholines (PCs), phosphatidylserines (PSs), and phosphatidylinositols (PIs; Table 2.1). In addition, three peptides,

each composed of four amino acid residues, were also important for separation along Component 1. Peptide 1 was composed entirely of nonpolar amino acid residues alanine, cysteine, phenylalanine, and tryptophan, and Peptide 2 contained two acidic residues (aspartic and glutamic acids) as well as two uncharged polar residues (threonine and tyrosine). Peptide 3 was composed of two cysteine residues, one basic residue (lysine), and one uncharged polar amino acid (tyrosine). Annotations of spectral features differing significantly between control and exposed males in pairwise comparisons included n = 6 peptides and amino acid analogs, as well as n = 3 glycerophospholipids, and n = 10 undetermined analytes (Table 2.2). Peptides were generally reduced in exposed males and were composed primarily of nonpolar amino acid residues (including methionine, alanine, and tryptophan). Glycerophospholipids, including two phosphatidyl inositols and one phosphatidylinositol phosphate were also significantly higher in exposed males. Putative metabolite annotations for polar analytes included an amino acid analog (O-acetylserine), one phosphorylated carbohydrate (undetermined hexose-phosphate), a pyrimidine derivative (thiamine pyrophosphate), and two undetermined analytes. Annotation of many metabolites of interest was not achievable due to a lack of plausible database search hits.

Discussion

In this study, we found evidence that estrogen exposure in juvenile male medaka may lead to prolonged effects on the nonpolar liver metabolome, and changes observed in the liver metabolome could reflect organizational or activation-associated effects of estrogen exposure. Long-term metabolic effects are likely to influence hepatic physiology, as lipids and other nonpolar metabolites play crucial and diverse physiological roles in fish and other organisms. Alterations to the liver metabolome could indicate or mediate adverse effects in the liver and

related organ systems that persist throughout the life-cycle of male fish. In this study, estrogen exposure was also associated with fibrosis in the testis. We hypothesized that intersex would be observed in exposed males, and although intersex was not observed upon termination of the study, it is possible that testicular oocytes resulting from estrogen exposure were sloughed or regressed by the time of termination of the experiment. Thus, fibrosis observed in the testes of exposed males may represent morphological remnants of intersex. Little is known about the regression of testicular oocytes, but it could contribute to changes in the liver metabolome via transport and catabolism of oocyte components. Also, genomic alterations and dysregulation of hormone signaling cascades could have prolonged influence on hepatic metabolism. In the present study, PIs and PIPs were significantly higher in E2-exposed males, which have important regulatory functions in cells and often define specialized membrane domains used for membrane transport and protein-binding (Alberts et al., 2015). These results suggest that changes in nonpolar liver metabolites resulting from estrogen exposure may indicate or mediate long-term changes in physiology.

In this study, the nonpolar liver metabolome of adult male medaka exhibited prolonged effects of early life-stage estrogen exposure, in contrast with the polar liver metabolome, in which very few long-term effects were observed. Metabolite profiling may have utility for ecosystem monitoring, such as assessing recovery from EDC exposure following remediation efforts. In a study of caged adult male fathead minnows exposed to wastewater effluent containing EDCs, Davis et al., (2013) reported rapid recovery of the polar liver metabolome, which suggests that fish may quickly return to a normal physiological state following depuration. However, results of the present study suggest that effects on nonpolar metabolite profiles may persist for extended periods of time; whereas, long-term effects may not be reflected by the

relatively transient polar metabolome. Therefore, monitoring of nonpolar metabolite profiles may offer considerable advantage for long-term assessment of ecosystem recovery. Nonpolar metabolites may be a source of sustained biomarkers of EDC exposure and could have diagnostic utility to complement physiological endpoints indicative of fish health. To better assess the utility of metabolomics for ecosystem monitoring, more information is needed regarding temporal aspects of metabolome recovery following exposure to EDCs. For example, there could be differences in metabolome recovery of longer-lived fish species with different life-histories. Also, the liver was chosen as the source of metabolite profiles in the present study due to knowledge of extensive hepatic metabolic processes, but similar effects may also persist in other tissues and biofluids. In addition, long-term metabolic effects may also result from exposure to other classes of EDCs.

Due to limitations of mass spectroscopy methods employed in this study, the specificity as well as degree of certainty pertaining to annotations assigned to features of interest is relatively low. Ideally, high-resolution MS methods capable of detecting accurate mass or tandem MS/MS methods should be used in order to reliably annotate structural details of metabolites, particularly with regard to medium to large molecules. The present study provides insight to inform future studies investigating long-term metabolomic effects of estrogen exposure in fish, but important information regarding physiological function could be elucidated by detailed structural information, including the identity of fatty acyl components and the amino acid sequence of peptides.

Overall, results of this study suggest that nonpolar liver metabolite profiles may be useful for monitoring long-term effects of EDC exposure in fish. In contrast with polar metabolite profiles, which are typically favored for the diversity of metabolites and relative ease of

annotation, lipids and other nonpolar metabolites may have greater utility for development of long-term biomarkers of EDC exposure. In addition, changes in the nonpolar metabolome could provide insight into biochemical mechanisms associated with long-term physiological endpoints relevant to adverse outcomes.

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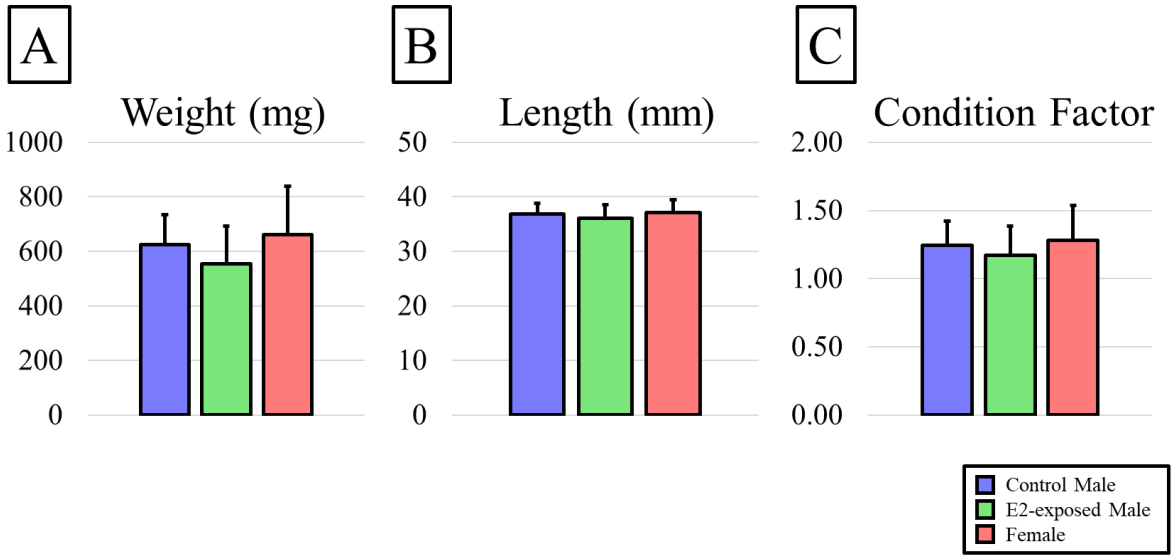


Figure 2.1. Mean ± SD morphometric measurements of control male (blue), E2-exposed male (green), and female (red) Japanese medaka.

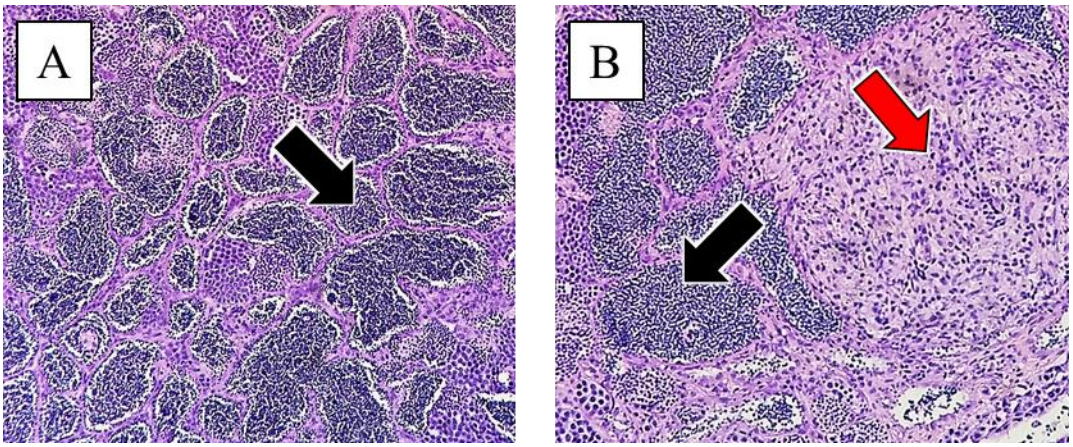


Figure 2.2. Representative histological sections of adult testicular tissues from control male Japanese medaka (A), and male medaka exposed to 127.9 ng/L 17β-estradiol for 3 weeks as juveniles (B) following a 4-month depuration period. Black arrows indicate mature sperm, and the red arrow indicates abnormal fibrotic tissue. H&E stain, 400x

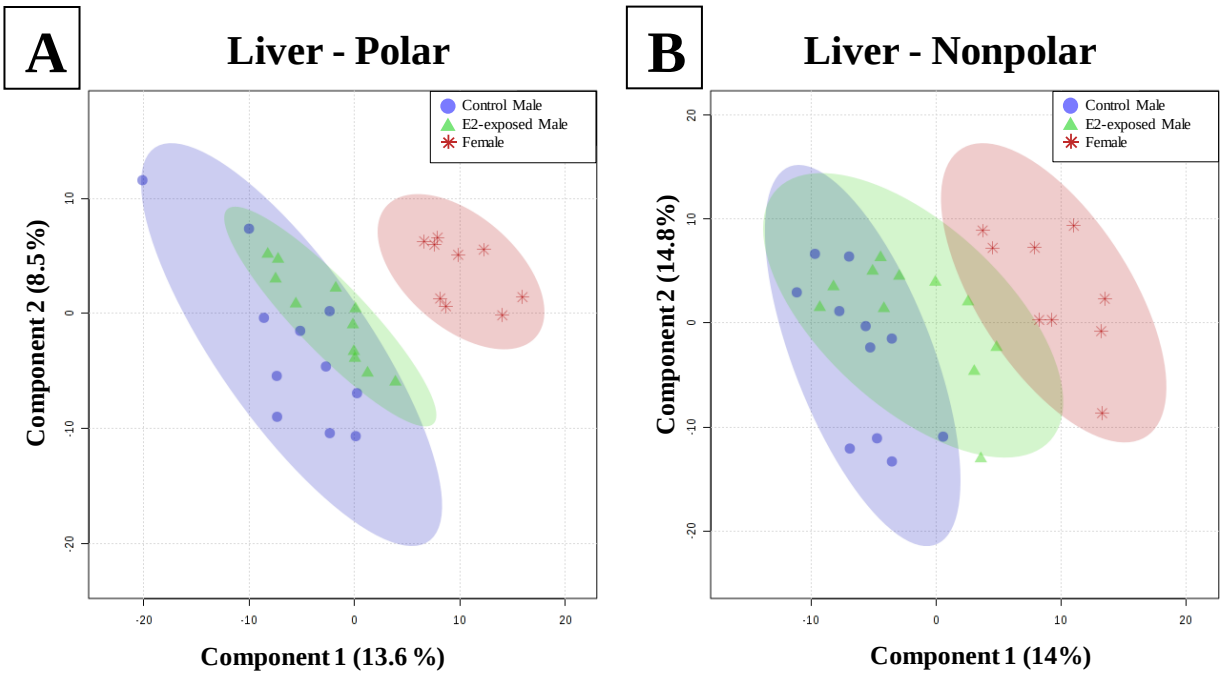


Figure 2.3. PLS-DA scores plots representing differences in polar (A) and nonpolar (B) liver metabolite profiles of control male (blue), E2-exposed male (green), and female (red) Japanese medaka. E2 exposures were conducted for 3 weeks as juveniles, followed by a 4-mo. depuration period. Whereas near-complete recovery of polar metabolite profiles in apparent, there appear to be long-term effects of feminization on nonpolar metabolite profiles.

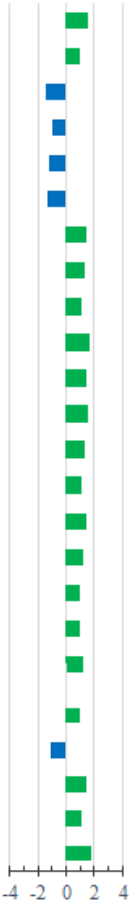
Table 2.1. Putative chemical class and annotations of nonpolar liver metabolite profile features representing relative differences among control female, control male, and male Japanese medaka exposed to 17β-estradiol (E2, 127.9 ng/L) as juveniles, followed by a 4-mo. depuration period. Putative metabolite features shown were important for separation along PLS-DA Component 1, as determined by the top 25 variable importance in projection (VIP) scores. Retention times and m/z values represent the median value used for alignment. For lipids, only the lipid class and mass are annotated. Columns on the right denote relatively elevated (↑), medial (•), and reduced (↓) response among groups, and elevated responses are highlighted by group for ease of interpretation.

Chemical Class	Putative Metabolite Annotation	m/z	RT (min)	VIP Score	Control Male	E2-exposed Male	Female
Amino acids, peptides, and analogs	Ala Cys Phe Trp*	526.21	22.86	4.38	↑	•	↓
	Asp Glu Thr Tyr*	527.20	22.85	2.47	↓	•	↑
	Cys Cys Phe Lys*	500.19	20.36	2.28	↑	•	↓
Glycero-Phospholipids	Phosphatidylserine (834)	834.52	57.56	4.76	↓	•	↑
	Phosphatidylserine (786)	786.52	47.27	4.54	•	↑	↓
	Phosphatidic acid (703)	703.51	49.60	4.06	↓	•	↑
	Phosphatidylserine (734)	734.49	40.50	4.00	↑	•	↓
	Phosphatidylserine (804)	804.48	49.20	3.89	↓	•	↑
	Phosphatidylethanolamine (786)	786.49	48.04	3.53	↓	•	↑
	Phosphatidylserine (802)	802.44	46.77	3.17	↓	•	↑
	Phosphatidylserine (778)	778.46	48.60	3.10	•	↑	↓
	Phosphatidylserine (768)	768.48	57.15	3.00	↓	•	↑
	Phosphatidylserine (704)	704.47	49.60	2.94	↑	•	↓
	Phosphatidylinositol (835)	835.49	57.52	2.84	↓	•	↑
	Monoglycosyl diacylglycerol (761)	761.49	45.43	2.75	•	↑	↓
	Phosphatidylethanolamine (732)	732.47	54.36	2.66	↓	•	↑
	Glycerophosphoglycerol (787)	787.53	47.17	2.50	•	↑	↓
	Phosphatidylinositol (805)	805.47	49.20	2.49	↑	•	↓
	Phosphatidylserine (758)	758.50	55.07	2.49	•	↑	↓
	Phosphatidylserine (818)	818.51	55.45	2.33	↑	•	↓
	Phosphatidylinositol (779)	779.45	48.60	2.30	↓	•	↑
	Phosphatidylinositol (827)	827.43	45.67	2.25	↑	•	↓
Purine derivatives	8-oxoguanine	166.03	4.35	4.94	•	↓	↑
Unknown	NA	826.44	45.68	3.14	↑	•	↓
	NA	898.49	44.45	2.66	↑	•	↓

*amino acid sequence unknown

Table 2.2. Putative chemical class and annotations values for nonpolar liver metabolite profile features differing significantly ($p < 0.05$, fold-change [FC] > 2.0) between control and 17β-estradiol- (E2, 127.9 ng/L) exposed male Japanese medaka. Exposures were conducted for 3 weeks as juveniles, followed by a 4-mo. depuration period. Features are ordered by lowest p-value within each chemical class. For large lipid molecules, only the lipid class and mass are annotated. On the right, log2 Fold change values are displayed for comparison (green, higher in E2-exposed group; blue, higher in control group).

	Chemical Class	Putative Metabolite Annotation	m/z	RT (min)	*p*-value	FC	log2(FC)
Nonpolar	*Amino acids, peptides, and anologs*	Linoleoyl glycine	338.29	46.50	0.002	2.89	
		Met Trp Trp Trp*	708.32	26.74	0.007	2.03	
		Ala Ala Glu Ile*	403.22	33.11	0.028	-2.76	
		His Lys Met Met*	546.26	24.55	0.033	-2.01	
		Phe Ile Ile Trp*	578.33	36.16	0.033	-2.34	
		Asp Pro Arg Tyr*	550.26	30.35	0.035	-2.57	
	Glycero-phospholipids	Phosphatidylinositol phosphate (915)	915.44	42.21	0.012	2.62	
		Phosphatidylinositol (801)	801.44	44.15	0.031	2.52	
		Phosphatidylinositol (903)	903.44	42.69	0.035	2.15	
	Unknown	Unknown	798.44	45.39	0.006	3.20	
		Unknown	914.46	42.21	0.008	2.72	
		Unknown	884.45	42.69	0.009	2.80	
		Unknown	902.47	42.71	0.013	2.51	
		Unknown	873.45	42.89	0.016	2.20	
		Unknown	634.30	34.50	0.019	2.70	
		Unknown	864.46	45.30	0.019	2.34	
		Unknown	872.46	42.88	0.022	2.04	
		Unknown	839.42	44.62	0.034	2.00	
		Unknown	800.44	44.15	0.039	2.35	
Polar	*Amino acid analogs*	O-Acetylserine	381.07	1.43	0.033	2.03	
	Carbohydrates	Hexose phosphate	245.04	2.26	0.009	-2.08	
	Pyrimidine derivatives	Thiamine pyrophosphate	425.09	1.49	0.005	2.72	
	Unknown	Unknown	123.97	18.51	0.015	2.05	
		Unknown	348.03	36.83	0.043	3.29	



*amino acid sequence unknown

CHAPTER 3

EXPLORING BIOTIC AND ABIOTIC FACTORS ASSOCIATED WITH THE OCCURRENCE OF TESTICULAR OOCYTES IN FISHES FROM AN AGRICULTURAL WATERSHED[2]

[2] Matthew L. Urich, Jennifer C. Brennan, W. Matthew Henderson, Brian J. Irwin, Donald E. Tillitt, Robert B. Bringolf. To be submitted to *Science of the Total Environment*

Abstract

Incidences of intersex in freshwater fish reported in the Southeastern United States and throughout much of the rest of the world have caused concern for fish population levels due to associated adverse reproductive effects. Though intersex is often associated with exposure to endocrine disrupting chemicals from point sources of anthropogenic pollution (e.g. municipal wastewater effluents) or agricultural land use, causes of intersex among wild fish remain unclear, particularly in areas absent obvious sources of pollution. Evidence contradicting popular theories involving chemical exposure has raised questions regarding the involvement of alternative causative factors such as elevated surface water temperature, or nitrate exposure. However, studies utilizing integrated approaches and considering multiple factors of various categories simultaneously to understand intersex among wild fish are lacking. We compared the results of an intersex survey of fishes throughout the Upper Coosa River System to measurements of several variables of interest, including fish morphometrics, surface water chemistry, land use, and seasonal sediment estrogen concentrations and estrogenicity. A series of statistical models was generated to identify factors associated with intersex in this region, and the most plausible models were selected using AICc. Intersex was most common among black basses and other centrarchids, and factors important for highly plausible models included maximum surface water temperature, sediment estrogen concentrations, localized agricultural land use, and fish size. These results suggest that intersex may result due to a combination of diverse environmental and individual factors. Factors identified may be used to inform management practices to control incidences of intersex in the Coosa River Basin and similar systems.

Introduction

Despite concern for adverse population-level effects associated with widespread reports of intersex in freshwater fish, causes remain poorly understood. Intersex among wild fish is often found in association with endocrine disrupting chemicals (EDCs) found in industrial and municipal effluents (Bjerregaard et al., 2006; Blazer et al., 2007, 2012; Hinck et al., 2008; Pollock et al., 2010; Vajda et al., 2008), but many incidences have been reported in the absence of any major point-sources of pollution. In fact, some incidences of intersex appear to be more closely related to non-point source pollution, specifically agricultural land use, than wastewater effluent (Blazer et al., 2014). Agricultural runoff can release a considerable amount of estrogenic EDCs known for feminizing effects in fish (Ciparis et al., 2012; Jenkins et al., 2009; Lange et al., 2002; Lee et al., 2014; Soto et al., 2004; Yonkos et al., 2010). Exposure to estrogenic compounds has been repeatedly shown to induce intersex and other abnormalities in laboratory studies (see Leet et al. [2011]). However, extrapolating laboratory results to wild fish populations is often problematic, and intersex can occur at high levels even in relatively pristine areas (Iwanowicz et al., 2016). Environmental factors such as elevated nitrate levels and water temperature (Bringolf et al., 2015), as well as biotic factors such as fish size (Blazer et al., 2007) have also been suggested as potential factors, yet comprehensive analyses accounting for a broad scope of biotic and abiotic factors are lacking.

Especially concerning are the adverse reproductive effects associated with intersex and associated potential for population-level effects. Studies of intersex in common roach (*Rutilus rutilus*) showed reduced sperm production, sperm motility, and fertilization success compared to normal males from the same populations, with severity of intersex correlated with a decrease in viable offspring (Jobling et al., 2002). Harris et al. (2011) reported a 76% reduction in

reproductive success among intersex males in the common roach compared to normal males. Relevant information is limited or unknown for many wild species in which intersex is common, but some studies suggest a potential for adverse population-level effects. For example, intersex fathead minnows were found among a population that experienced reproductive failure following long-term estrogen exposure (Kidd et al., 2007). In this case, whether intersex was a causative factor in the resulting population crash, or perhaps only a benign side-effect of estrogen exposure is unknown. More information is needed to assess ecological risk associated with intersex, and given its widespread occurrence in the environment, understanding causative factors is critical to identifying areas or populations of concern and recognizing the scope of potential ecological harm.

The Upper Coosa River (UCR) Basin in northwestern Georgia and southern Tennessee (USA) is among the most bio-diverse systems in the world, but within the past few decades, dramatic population declines have caused concern. Seine surveys conducted from 1996 to 2012 in the Conasauga River, one of three major tributaries of the UCR suggested a dramatic decline in the abundance of several rare fish species (Golder Associates, Inc., 2010; Hagler and Freeman, 2012). Causes of the declines are largely unknown, as areas experiencing the greatest decline are generally upstream of major municipal or industrial sources of waste, and analyses of current use pesticides, PAHs (Sharpe and Nichols, 2007), and glyphosate (Lasier, 2012) have not indicated levels of concern. However, potentially harmful levels of estrogens have been measured in Conasauga River sediments and surface waters (Lasier et al., 2016), and Jacobs (2013) detected intersex among 10 of 13 (76.9%) male sunfish (family Centrarchidae) collected from Conasauga River tributaries, potentially implicating estrogens in the feminization of fish in the region. Aside from this brief survey, information regarding intersex in this region is lacking, and current

evidence is insufficient to understand causative factors and potential implications with observed population declines. Understanding the extent of intersex throughout the region could be useful to identify causative factors of intersex of concern, and may also help to explain possible implications of intersex in population declines within the UCR and in comparable regions.

To better understand the relationships between intersex and selected biotic and abiotic factors in the UCR, we conducted a survey of intersex throughout the region and produced predictive models using a broad range of variables. Variable categories including fish morphometrics, water quality, land use, seasonal sediment estrogenic activity, and sediment estrogen concentrations were investigated for relationships with intersex at sites across a gradient of forested, agricultural, and urban development within the Upper Coosa River Basin. The primary objectives of this study were to: (1) determine the prevalence, severity, and geographic and taxonomic distribution of intersex among large-bodied fishes of the Upper Coosa River Basin, and (2) identify relationships of variables associated with and/or predictive of the occurrence of intersex in the region.

Materials and Methods

Study Area

A total of eight sites within the three major river basins of the UCR (Conasauga River, n=3; Coosawattee River, n=3; and Etowah River, n=2) were selected to reflect varying degrees of agricultural and municipal land use. Specific sampling locations (Fig. 3.1) were selected based on points of access and were meant to reflect a broad range of land use practices. Upstream sites (Upper Conasauga, Upper Coosawattee 1 and 2, and Upper Etowah) located in the headwaters of the UCR were selected due to relatively limited development and agricultural land use. The

Upper Conasauga site is located in Jacks River just upstream of its confluence with the Conasauga River in an area protected by the US Forest Service, and land cover in that watershed is predominantly forest. The Middle Conasauga site was selected due to its proximity to intensive agricultural land use and represents 3 sample sites that were subsequently pooled because of lack of variability among the sites and their close proximity (< 12 river miles) to one another. Watershed delineation for Middle Conasauga was conducted using coordinates for the most downstream site. Downstream sites (Lower Conasauga, Lower Coosawattee, and Lower Etowah) were selected in each major UCR tributary to reflect relatively high levels of human development and/or agriculture.

Fish Sampling

Fish were collected during the spring from 2015-2017 during the pre-spawn period using a variety of sampling techniques including boat electrofishing, hoop netting, and angling. Due to the presence of protected species, electrofishing is not permitted in the Upper Conasauga River. Therefore, fish at those sites were sampled without the use of electricity. Sexually mature male fish of non-protected species from the following families were targeted for collection at each site: Centrarchidae (sunfishes), Cyprinidae (minnows), Ictaluridae (catfishes), Catostomidae (suckers). Whenever possible, immature, female, or reproductively spent individuals were released, and protected species were always immediately released. Water chemistry (pH, DO, conductivity, and temperature) was measured at each site during sampling to characterize conditions. Upon capture, the fish were euthanized with an overdose (1500 mg/L) of pH-buffered tricaine mesylate (MS-222) solution. Total length (nearest mm) and weight (nearest 0.1 g) were measured for each fish to calculate Fulton's condition factor (CF = [total weight (g) / total length (mm) ^{3}] * 100,000; Ricker, 1987). Gonads were excised and weighed to the nearest

0.01 g to facilitate the calculation of gonadosomatic index (GSI = [gonad weight (g) / total weight (g)] * 100). Testes were then encased in pre-labeled plastic histology cassettes and preserved (≥ 96 hrs.) in plastic containers with neutral-buffered 10% formalin solution and stored at room temperature prior to histological processing. All fish were handled in accordance with approved University of Georgia Institutional Animal Care and Use protocols (AUP # A2016 06-028-A1).

Gonad Histology

Testes fixed in formalin from each male fish were prepared for histopathological evaluation and examined under a compound microscope for the presence of TO. Tissue processing and slide preparation was performed by the Diagnostic Laboratory at the University of Georgia College of Veterinary Medicine (Athens, GA, USA). Briefly, preserved testes were dehydrated in alcohol, embedded in paraffin wax, and because of the small size of most fish, a single 4-µm, medial, longitudinal section was taken from each gonad to maximize visible surface area (Jacobs, 2013; Kellock et al., 2014). For testes too large for a single cassette, multiple cassettes were used, and a single longitudinal section was taken from each cassette. Sections were mounted on a glass microscope slide and stained with hematoxylin and eosin. Slides were examined under a compound light microscope at the Aquatic Toxicology Laboratory at the University of Georgia (Athens, GA). If present, TO were enumerated for each fish. Intersex was identified by the presence of at least one oocyte in the testicular tissue.

Watershed and Land Use Delineation

To evaluate relationships between land use and intersex within the UCR, watershed land use characteristics were determined for each fish collection site with QGIS (v. 3, http://qgis.osgeo.org) using the following publicly available databases: National Land Cover

Database 2011 (https://www.mrlc.gov), National Hydrologic Dataset (https://www.usgs.gov), and National Elevation Dataset (10-meter resolution, https://catalog.data.gov). For each site, geographic coordinates were used to delineate two watershed areas: (1) the total watershed area drained by each sample site (illustrated in Fig. 3.1), and (2) watershed area within a 5-km radius upstream of each sample site. Land use characteristics for the Middle Conasauga site were determined by coordinates of the farthest downstream of the pooled sites. For each watershed area, land use and land cover percentages were generated. For simplicity, similar land use categories were pooled for all sites as follows: Total developed land use was calculated by grouping all developed categories ('developed-open,' 'developed-low,' developed-medium,' and 'developed-high'). Similarly, total agriculture was calculated by grouping 'pasture and hay' and 'cultivated crops' categories, total forested land area by grouping 'deciduous-,' 'evergreen-,' and 'mixed-forest' categories, and total wetland area by grouping 'woody wetlands' and 'emergent wetlands' categories.

Environmental Sample Collection and Water Chemistry

From August 19, 2015 to August 18, 2016, surface water samples were systematically collected from all sites included in the intersex fish survey (with the exception of Lower Etowah, which was subsequently added as a fish sampling site) for characterization of water chemistry. Surface water samples were collected on the first Wednesday of each month, in addition to 9 samples from each site opportunistically collected during or immediately following rain events predicted to affect the entire study area. A total of 21 samples was collected at each site with the exception of Lower Conasauga, where only 20 samples were collected due to hazardous conditions on one occasion. Approximately 4 L of surface water were collected from at least 2 M from the riverbank at a minimum depth of 10 cm in 4-L amber glass jars with a wooden pole or a

rope at sites where a bridge is present. With every set of samples, a field blank (deionized water - 18 MΩ specific resistance) was taken to the field and handled as an additional surface water sample to assess contamination from field conditions during sampling. Immediately following collection, a sub-sample was transferred into an opaque 250-mL HDPE jar for water quality and nutrient measurements. Water temperature and conductivity were measured on-site with a conductivity meter (Orion model 1214000, Thermo Fisher Scientific Inc., Waltham, MA), and pH was measured in the laboratory following the filtration step outlined below (Orion® ion-selective meter #720A with Triode electrode, Thermo Fisher Scientific Inc., Waltham, MA). Both samples were kept on ice during transport and prior to analysis.

In the laboratory (< 24 h after sampling Con5), cold sub-samples were vacuum filtered into a 500-mL HDPE vacuum flask with a pre-rinsed 0.45 µm polyethersulfone membrane (Sterlitech, Kent, WA) contained in a 90-mm Buchner funnel (Cole Parmer, Vernon Hills, IL). Twenty milliliters of filtered sample were then transferred to a 30-mL polyethylene cup and allowed to warm to room temperature to facilitate nitrate measurements. Nitrate concentrations (as mg NO3-N/L) were determined using an Orion® ion-selective meter (#720A) with a Nitrate Ionplus® Sure-Flow® electrode (Orion #9707BNWP). The low-level calibration technique supplied by the manufacturer was employed because concentrations below 1.4 mg NO3-N/L were expected. For these analyses, plastic labware was employed in all stages of sample handling and analysis after the initial sample collection. Nitrate concentrations in blank samples were less than 0.1 mg NO3-N/L.

Sediment samples were collected concomitantly with surface water samples on eight sampling trips (4 samples during each spring and summer) from areas with fine, depositional particles with a stainless-steel garden trowel to remove the top 2 cm of sediment. On each

collection trip, sediments from each site were composited and homogenized in a stainless-steel bowl before being transferred to clean 500 mL amber glass jars. Collection equipment was rinsed thoroughly with methanol between sites. A single discrete water sample was collected in clean 4-L amber glass jars at a depth of 1 m from each site during each collection trip. All environmental samples were kept on ice and protected from light during transport to the lab, where they were stored at 4 °C prior to extractions, which were conducted within 24 hrs of collection. A separate aliquot of sediment was physically characterized by determining moisture content, particle-size distribution (Miller and Miller, 1987), and loss-on-ignition (Davies, 1974) to estimate total organic material (TOM).

Sediment Extraction

Methods adapted from Wang et al. (2012) were used to extract sediments for measurement of estrogens and estrogenicity. Briefly, ~30 g wet sediment was extracted in a 50-mL glass centrifuge tube using three aliquots of 10 mL ethyl acetate. Centrifuge tubes were vortexed for 1 min, ultra-sonicated for 15 min, and centrifuged at 1500 xg for 10 min after each aliquot of ethyl acetate was added. The supernatant from each round of extractions was decanted and combined into a 50-mL glass tube and a steady flow of N2 gas at room temperature was used to evaporated to near dryness. Extract volumes were continually monitored until they reached ~1 mL and were transferred to amber glass autosampler vials. Drying tubes were rinsed thoroughly by vortexing additional ethyl acetate, which was added to the autosampler vials evaporated to dryness under vacuum before being stored at -80 °C until further analysis. One blank (30 mL MilliQ water, pH 3) was extracted with every 10 samples for quality assurance, and a duplicate sediment sample was spiked with an estrogen standard mixture to determine % recovery. All solvents used were HPLC grade.

Estrogen Analyses

Sediment E1, E2, E3, and EE2 concentrations were measured via LC/MS following derivatization using dansyl chloride (Ke et al., 2014). Briefly, extracts were reconstituted in 200 µL 10% acetonitrile and 100 µL each of 0.2 M sodium bicarbonate in water and 1mg/mL in acetone was added to the vials. Samples were capped, vortexed for 1 min, and heated at 60 °C for 20 min. Samples were brought to room temperature and transferred to micro-target inserts. A prepared standard mixture containing E1, E2, E3, and EE2 was serially diluted, derivatized, and analyzed along with field samples, and a standard curve was generated to facilitate concentration calculations for each estrogen. Estrogens used to generate standard curves and derivatizing agents were obtained from Millipore Sigma (St. Louis, MO, USA). Spike recoveries were 97.6 % for E1, 100.9 % for E2, 56.6 % for E3, and 81.5 % for EE2. Values for estrogen concentrations were not corrected for recovery prior to modeling.

CALUX assay

A chemical activated luciferase gene expression (CALUX) assay previously described by Brennan and Tillitt (2018) was used to determine estrogenicity of sediment extracts. CALUX experiments were performed at the Columbia Environmental Research Center, U.S. Geological Survey in Columbia, MO (USA). Briefly, VM7Luc4E2 cells were exposed in a 386-well plate to 1.25 mg sediment equivalents (wet weight) of sediment extract resuspended in methanol and the luminescence of cell lysates was measured for each well. Measured luminescence values were background subtracted and normalized to the amount of protein in each well, derived from the simultaneously conducted fluorescamine assay. E2 standard concentration bioluminescence measurements fit a sigmoidal dose-response curve, which were transformed and used to generate a standard curve. The average of two replicate experiments was taken for each extract, and

estrogenicity of sediment samples were expressed as mean percent of maximum luciferase expression (induced by 1 nM E2, EC100).

Statistical Analysis

Statistical analyses were conducted in R (http://www.r-project.org/, v. 3.4.2) with significance based on $\alpha \leq 0.05$. All analyses of water chemistry and sediment estrogenicity data were conducted using mean values of three pooled sites for the Middle Conasauga site. Nitrate, estrogen, and estrogenicity values less than the limit of detection (LOD) were replaced by LOD/ $\sqrt{2}$ for statistical comparisons. To better reflect the bioavailability of sediment-bound contaminants, sediment estrogenicity, estrogen concentrations, and contaminant concentrations were corrected for % moisture content to reflect dry weight concentrations ([ng/g sediment (dry wt.)] = [ng/g sediment (wet weight)] * [1- % moisture]) and normalized to TOM ([ng/g sediment (dry wt.)] / TOM [g])

Morphometric and intersex data were analyzed using non-parametric statistical methods due to differences in sample sizes among sites. A Kruskal-Wallis test for significance was used to evaluate differences among sites in mean testicular oocyte counts among sunfish and black bass species. Mann-Whitney U tests were performed to evaluate differences in total length, HSI, and GSI between normal males and intersex males.

Modeling and Model Selection

To evaluate relationships with occurrence of intersex and morphometric and environmental variables, a series of binomial generalized linear models (GLMs) was performed in R with the glm function in the stats package. Fish collected from Lower Etowah were excluded from models because abiotic variables were not measured at this site. Continuous explanatory variables were mean-centered and scaled prior to modeling, and correlations

(Pearson R) were calculated between all scaled continuous variables prior to modeling. Variables that were highly correlated ($R > 0.7$, or < -0.7) were not included in the same model to avoid multicollinearity. To explore associations of explanatory variables with TO among individuals of taxa known to exhibit relatively high levels of intersex, two datasets were used to evaluate all models: (1) including only sunfish (n = 374), and (2) including only black bass (genus *Micropterus*, n = 119). Abiotic variables for each collection site, including mean and maximum water chemistry measurements throughout the year sampled, land use/cover, sediment estrogenicity and environmental contaminant measurements, as well as individual morphometric and taxonomic factors for each fish were considered for inclusion in models. A total of 30 variables (in addition to species) were selected based on documented or suspected environmental relevance to intersex and fish health (Table 3.1). A set of 99 unique models was generated for each taxonomic data subset, with species included as a fixed-effect variable in all cases. Each of the 30 selected variables appeared a total of n = 6 times throughout each model set, including one model for each variable in which species was the only other variable included.

To select the most informative models, Akaike's information criteria corrected for small-sample bias (AICc) was determined for each model (Akaike, 1973; Burnham and Anderson, 2002), and model selection criteria were slightly modified from those used by Grieshaber et al. (2018). Briefly, models were ranked from lowest to highest AICc, and ΔAICc was calculated for each model. Akaike weight (w_i) calculated for each model was used to determine the relative likelihood of each model, with the highest w_i indicating the most plausible model (Burnham and Anderson, 2002). To evaluate relative strength of models within the candidate set, models were ranked, and those with w_i within 10% of the top-ranked model were included in the confidence set. Further, 'highly plausible' models were identified with $\Delta AICc < 2$ and were used to generate

predictions of intersex that were compared with observations. To determine the relative importance of variables explaining intersex occurrence, weights of models in which a variable appeared were summed (Σw_i) across a 95% confidence set (cumulative $w_i \geq 0.95$). Variables were then ranked according to Σw_i, with the highest rank indicating the most important variables for explaining intersex.

Results

Intersex Survey

In total, 486 sexually mature male fish were evaluated for the presence of testicular oocytes. A total of 26 different species were surveyed including individuals from seven families: Lepisosteidae (n=3), Sciaenidae (n=4), Cyprinidae (n=5), Ictaluridae (n=6), Percidae (n=8), Catostomidae (n=73), and Centrarchidae (n=388). Mean total length (± SD) by family was 762 ± 80 mm for Lepisosteidae, 347 ± 46 mm for Sciaenidae, 219 ± 27 mm for Cyprinidae, 373 ± 105 mm for Ictaluridae, 227 ± 60 mm for Percidae, 395 ± 105 mm for Catostomidae, and 216 ± 74 mm for Centrarchidae. Mean body weight (± SD) by family was 989.5 ± 307.5 g for Lepisosteidae, 606.6 ± 309.9 g for Sciaenidae, 119.6 ± 40.7 g for Cyprinidae, 601.8 ± 522.8 g for Ictaluridae, 167.0 ± 148.5 g for Percidae, 993.9 ± 760.3 g for Catostomidae, and 208.2 ± 230.5 g for Centrarchidae. A total of 101 (20.8%) individuals sampled had TO within the area of testes examined, with TO counts ranging widely from n=1 to over 1000 per individual. Mean TO count (±SD) among all taxa sampled was 6.7 ± 64.8 per individual, and mean TO count among centrarchids was 13.8 ± 101.5. Oocytes ranged in diameter from approximately 15-50 µm and were previtellogenic in all cases.

TOs were detected in centrarchids and catostomids but were not detected in other families sampled. Overall intersex prevalence in centrarchids (25.0%) was far higher than catostomids (2.7%), in which only 2 of 73 individuals sampled had detectable TO; intersex was not detected in lepisosteids, cyprinids, sciaenids, ictalurids, or percids. Within the family Centrarchidae, the overall prevalence of intersex was highly variable among species, with some species as high as 64.4% (coosa bass, *Micropterus coosae*, n = 45), and others 0.0% (green sunfish, *L. cyanellus*, n = 1; warmouth, *L. gulosus*, n = 4; spotted sunfish, *Lepomis punctatus*, n = 1). Overall intersex prevalence within the genus *Micropterus* was 47.9%. Three of the four centrarchid species in which overall intersex prevalence exceeded 20% (coosa bass; largemouth bass; spotted bass, *M. punctulatus*) belong to the genus *Micropterus* (black bass species), and the other to the genus *Lepomis* (redear sunfish, *L. microlophus*)..

Intersex was detected at every site sampled, with overall prevalence ranging from 14.3% of individuals at Upper Etowah to 40.8% at Upper Coosawattee 1. Intersex prevalence among sunfishes and black basses varied geographically, but no clear directional trend was evident between upper and lower sites, and prevalence among the three river basins sampled was similar (Fig. 3.2A). Within the centrarchid family, prevalence ranged from 15.1% at Middle Conasauga to 43.5% at Upper Coosawattee 1, and prevalence among black basses exhibited the greatest variability among taxa surveyed, ranging from 25.0% at Middle Conasauga to 81.8% at Upper Coosawattee 1.

Water Chemistry

Mean water chemistry variables varied substantially among sites, particularly within the Conasauga River (Table 3.2). For all sites except for Lower Etowah, at which water chemistry data were not collected, mean surface water temperature ranged from 16.6 ± 5.9 °C (Upper

Conasauga) to 19.5 ± 6.1 °C (Lower Conasauga). Mean conductivity ranged from 13.6 ± 1.8 µS/cm at Upper Conasauga to 158.9 ± 41.1 µS/cm at Lower Conasauga. Mean pH ranged from 6.8 ± 0.2 at Upper Conasauga to 8.0 ± 0.2 at Lower Conasauga. Mean NO3-N concentrations ranged from 0.008 ± 0.02 mg/L at Upper Conasauga to 0.53 ± 0.21 mg/L at Lower Conasauga. Mean phosphorus concentrations ranged from 15.9 ± 5.9 µg/L at Upper Etowah to 49.5 ± 70.5 µg/L at Lower Conasauga.

Watershed Land Use

Total watershed area varied among sampling locations, ranging from 104 km^2 (Upper Conasauga) to 2853 km^2 (Lower Etowah), naturally increasing at downstream sites (Table 3.3). Total watershed land use/cover characteristics also varied among sites, with up to 99.5% forest and very little anthropogenic influence at some sites (Upper Conasauga). Anthropogenic land use generally increased at downstream sites, with some sites as high as 24.9% developed (Lower Etowah) or 17.9% agricultural land (Lower Conasauga; Fig. 3.2B). With two sites (Lower Etowah and Lower Coosawattee) located below reservoirs, the percentage of open water also showed some variation among sampling locations, ranging from 0.1% (Upper Etowah) to 2.17% (Lower Etowah) within the study area.

Sediment Estrogen Analyses

Normalized sediment estrogen concentrations assessed via LC/MS varied substantially among sites in both spring and summer (Fig. 3.2C). E2 was detectable at two sites in the spring and two sites in the summer; the highest level (2582 ng/kg) of E2 was measured in the spring sediment of Upper Conasauga. EE2 was detected at three sites in the spring and two sites in the summer; the highest EE2 concentration was measured in spring sediment at Middle Conasauga (165.8 ng/kg). E1 was detectable in n = 1 sediment sample in the spring (Middle Conasauga;

84.1 ng/kg) and one sediment sample in the summer (Middle Conasauga; 2.0 ng/kg). E3 was not detected in spring or summer samples.

CALUX Assay

Relative sediment estrogenicity, assessed by normalized percent induction of CALUX assay relative to the EC100 (Fig. 3.2C) indicated several sediment samples induced estrogenic responses substantially higher than the baseline response. The highest levels of estrogenicity in the spring were measured in sediments at Upper Coosawattee 1 (20% EC100), and the highest level of summer sediment estrogenicity was measured at Lower Conasauga (41% EC100). Sediments collected at sites with relatively high levels of intersex incidence were generally estrogenic, and estrogenicity was generally higher in sediments in which estrogens were detected via LC/MS (Fig. 3.2).

Intersex Model Selection

Results of model selection indicate associations of several measured explanatory variables with the incidence of intersex for sunfish and for black bass species (Table 3.4). For sunfish, six models were within 10% of the highest w_i of 0.2662, with only three models having $\Delta AICc < 2$. Similarly, for black bass, 11 models were within 10% of the most plausible model ($w_i = 0.1991$), and only 4 highly plausible models had $\Delta AICc < 2$. A total of 23 models were included in the 95% confidence set for sunfish models, and 35 models were included in the black bass 95% confidence set. Although some variables performed relatively well when modeled individually, top-ranked models frequently included combinations of multiple variables from different categories, including fish morphometrics, surface water chemistry, sediment estrogenicity and estrogen concentrations, and land use variables.

Highly feasible models were moderately accurate in predictions of intersex incidence (Fig. 3.3). The relative importance of explanatory variables, as assessed by Σw_i for models within the 95% confidence set including each variable (Table 3.4), demonstrated a relatively high likelihood that particular variables are associated with intersex fish in the UCR. Variables of relatively high importance were generally consistent for both sunfish and black bass, with maximum surface water temperature and fish weight both appearing within the top three ranked variables for both groups. Variables of relatively high importance for explaining intersex in sunfish and in black bass (Table 3.5) included body weight (Wt), maximum (MxTemp) and average surface water temperature (AvTemp), summer sediment E1 concentration (E1Su), spring sediment estrogenicity (EstrSp), percentage of proximal (within 5 km upstream) agricultural land use (Ag5), and maximum surface water nitrate concentration (MxNO3).

Discussion

In this study, as in previous studies, members of the family Centrarchidae, and particularly black bass species, had relatively high intersex prevalence and TO counts compared to other fish taxa. Although the primary taxonomic group targeted in this study was the family Centrarchidae, and relatively few individuals from other families were sampled, the vast majority of intersex individuals in this study were centrarchids, and intersex prevalence among this group (25%) was far higher than any other family. The only family other than Centrarchidae with detected TO was Catostomidae, in which in 2 of 73 individuals (2.7%) had TO in the area examined. The prevalence of intersex among centrarchid species was highly variable. The three centrarchid species surveyed in which TO was not detected all had low sample sizes ($n \leq 4$ individuals per species), so perhaps all centrarchid species in this region exhibit some intersex.

Of the four centrarchid species with an intersex prevalence of greater than 20% (coosa bass, largemouth bass, redear sunfish, and spotted bass), three belong to the genus *Micropterus* (black bass species, overall intersex prevalence = 47.9%). Previous surveys of this genus conducted in the United States reported similar results. For example, Hinck et al. (2008) reported 42%, Bringolf et al. (2015) reported 48%, and Grieshaber (2018) reported 40% intersex prevalence among black bass. Results of the current study support previous suggestions that incidences of TO may be much more likely to occur among centrarchids, and certain centrarchid species including members of the genus *Micropterus*. A higher intersex prevalence may suggest an increased sensitivity to EDCs or other causative factors of intersex induction, but may also support suggestions of a natural background prevalence of intersex among certain fish taxa, including centrarchid species.

Model selection identified a limited set of highly plausible models for both sunfish and black bass (Table 3.4). Variables of relative importance for models explaining intersex suggested the involvement of biotic factors as well as abiotic factors previously suspected to alter endocrine function. Body weight was positively associated with intersex in highly plausible models and was the highest ranked variable of importance for both sunfishes and black bass, suggesting a higher frequency of intersex as fish grow larger. The higher probability of intersex predicted as body weight increases (Fig. 3.4A) may potentially be explained by higher body burdens of EDCs and/or longer histories of chemical exposure in larger fish. However, the nature of relationships between TO and fish size/age remains poorly understood. Age has been previously suggested as a potential factor in intersex occurrence in black basses (Hinck et al., 2009), but little is known regarding age distributions of intersex fish. Maximum surface water nitrate concentration was positively associated with intersex for both sunfishes and black bass, and was among the 10

highest ranking variables in both cases. Surface water nitrate is often sourced from anthropogenic activity and usually co-occurs with suspected EDCs, but nitrate itself is also suspected as an endocrine disruptor (Guillette and Edwards, 2005; Edwards and Hamlin, 2018; Poulsen et al., 2018), so levels of nitrate may influence the incidence of intersex in fish, as suggested by Bringolf et al. (2015). Surface water temperature held very high importance in top-ranking models for sunfishes and black bass, which implies that exposure to higher environmental temperatures may positively influence incidence of intersex. Average marginal predicted probabilities for intersex among black bass suggest an increased probability of intersex with higher maximum surface water temperatures (Fig. 3.4B). Evidence suggests that water temperature can affect sex steroid production in the testes of tilapia (*Oreochromis mossambicus*; Kime and Hyder, 1983), common carp (*Cyprinus carpio*; Kime and Manning, 1986), and rainbow trout (*Oncorhynchus mykiss*; Manning and Kime, 1985). Further, elevated rearing temperatures have been associated with reduced aromatase mRNA and estradiol levels in Japanese flounder (*Paralichthys olivaceus*; Kitano et al., 1999), as well as altered sex determination, gonad differentiation, and skewed sex ratios in several species (Conover and Kynard, 1981; Nomura et al., 1998; Römer and Beisenherz, 1996; Strussmann et al., 1996; Struussmann et al., 1996). The association of lower impoundment surface area with intersex in largemouth bass reported by Bringolf et al. (2015) may support the involvement of temperature, because fish inhabiting smaller lentic bodies of water typically experience higher water temperatures in warm climates. This study contributes to evidence suggesting that rising surface water temperatures may influence the levels of intersex in fish and also contributes to a growing body of evidence suggesting that climate change may exacerbate the effects of EDCs in fish (Brown et al., 2015; Keller et al., 2015). Climate change and chemical stressors are among the

biggest threats to the environment, and these results support previous findings that a combination of exposure to chemical contaminants and elevated surface water temperatures may pose an increasing risk to global fish health and population sustainability.

Intersex may play a role in documented fish population declines within the UCR and elsewhere, but this study did not investigate intersex among imperiled species; therefore, direct inferences to populations of concern cannot be made. However, intersex observed among non-imperiled species may be used to speculate the relative likelihood of intersex incidence among coinhabiting fish populations. In the present study, incidences of intersex observed in the Conasauga River, where a number of sensitive species occur, were mostly limited to centrarchid species and were not considerably higher than those observed elsewhere in the UCR. Therefore, if similar trends exist among species of concern, intersex does not likely substantially contribute to regional population declines observed in the Conasauga. However, differences in intersex susceptibility among species not surveyed in this study are unknown, and the potential threat posed by intersex and other feminizing effects of estrogens cannot be ruled out at this time. The primary goal of this study was to better understand relationships between intersex and variables of interest within the UCR, but similar relationships may exist in comparable river systems. The UCR is fairly unique because of its vast biodiversity and extensive use of particular agricultural practices (i.e., widespread application of poultry litter as fertilizer) in certain regions. However, many of the species investigated in this study (e.g. largemouth bass) are found throughout much of North America, and others (e.g. coosa bass) are closely related to species of particular interest for intersex research (i.e., smallmouth bass). Hence, similar relationships are likely to exist in comparable river systems, particularly those lacking major municipal and industrial effluents, and with extensive agricultural land use within the watershed.

In the present study, some results were unexpected and appeared to contradict presumed hypotheses suggested by previous findings. For example, upstream sites were primarily included for reference, and levels of contaminants and intersex were generally expected to be lower than those detected at downstream sites, reflecting a gradient of anthropogenic land use. Interestingly, there was no clear evidence indicating higher levels of intersex or sediment contaminants at downstream sites. In fact, despite lower levels of anthropogenic land use, some of the highest levels of sediment estrogens and estrogenicity, as well as intersex, were found at upstream sites. These results may suggest the existence of unknown sources of contamination, such as septic system leachate or runoff containing herbicides used for forest management. Alternatively, intersex susceptibility may vary among populations inhabiting different reaches of the river. Also interesting was a negative relationship between intersex and variables hypothesized to positively influence intersex (i.e. sediment estrogens, estrogenicity, and agricultural land use) among top-ranked models. Although total watershed agricultural land use did not appear in candidate model sets, localized agricultural land use (Ag5) had a negative effect on intersex in models, suggesting perhaps an indirect effect of local agriculture such as the use of riparian buffers.

Due to inherent error associated with histological detection of TO, at least some individuals may have had TO outside the area examined, thereby introducing a chance of false negative determination of intersex. Furthermore, although sampling locations were geographically distant, movement of fish among sites could have skewed results. Whereas centrarchid species are not generally thought to move large distances within riverine habitats (Gatz and Adams, 1994), other taxa (e.g. catostomids) regularly migrate within river systems, particularly during spring months when fish surveys for the present study were conducted.

Models produced in this study indicate a significant association of several measured variables with intersex incidence in fishes of the UCR, and top-ranked models are generally predictive of the intersex condition (Fig. 3.3). However, much variability remains unexplained, and the nature of associations between intersex and predictor variables largely unknown. Directional relationships (positive or negative) with intersex were generally consistent among models for variables of high importance, but some variables (e.g. sediment E1 concentration and spring sediment estrogenicity) displayed ambiguity among models, with a positive effect in certain models and a negative effect in others. These contradictions contribute to uncertainty in interpreting relative variable importance and may suggest the existence of false positives or unknown interactions among explanatory variables of interest. Validation of suggested relationships among variables by additional sampling and inclusion of additional variables and interaction terms in models may aid interpretation of contradictory model results.

In this study, models incorporating multiple sources of biotic and abiotic factors are the most informative for explaining intersex variability. Inclusion of easily discernible factors such as body weight and surface water temperature were highly important for model plausibility. Despite considerable evidence suggesting the influence of morphometric and physical environmental factors on intersex in fish, such factors are rarely incorporated into comprehensive investigations of potential causes, often in favor of exclusively investigating the roles of xenobiotic exposure and/or anthropogenic land use. Chemical pollutants almost certainly play a role in many incidences of intersex, but extraneous variability observed among and within populations may be partially explained by the inclusion of a broad scope of biotic and abiotic factors. The ecological risk associated with intersex will not likely be realized without first understanding potentially complex interactions among multiple biotic and abiotic factors that

likely contribute to its incidence. This study found that multiple biotic and abiotic factors from various categories may be associated with intersex in freshwater fish. Fish weight and surface water temperature appear to be particularly important factors to explain the occurrence of intersex among sunfish and centrarchids. Natural resource management practices to increase shade and reduce stream temperature, such as the use of riparian buffers, may be important for the control of intersex in freshwater fish.

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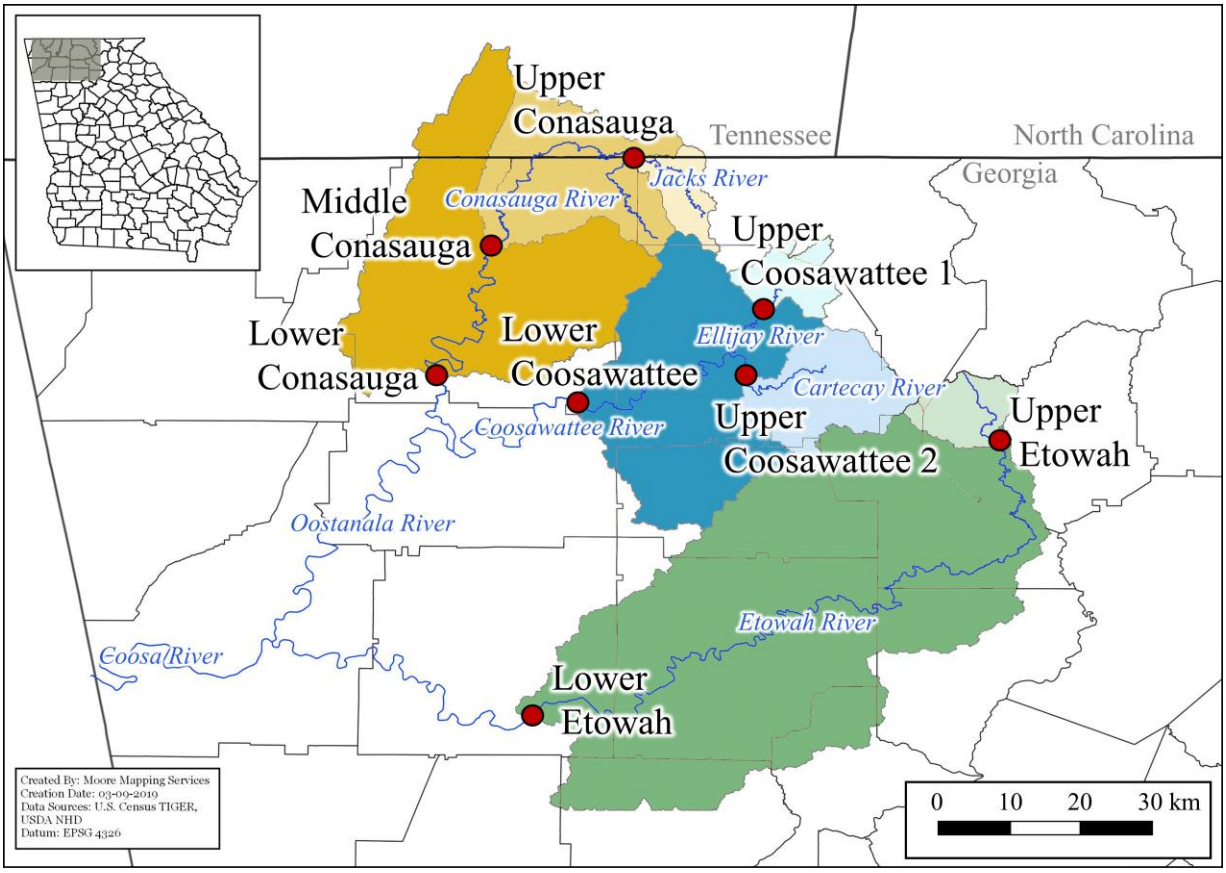

Figure 3.1. Fish sampling locations in the Upper Coosa River Basin and their respective watershed areas. Fish collected from Spring 2015 to 2017 were evaluated histologically for the occurrence of testicular oocytes.

Table 3.1. Categories, abbreviations, and explanations of hypothesized explanatory variables selected for inclusion in models explaining incidence of intersex among fish sampled from the Upper Coosa River System.

Category	Abbreviation	Variable Explanation
Biotic Data	Sp	Fish species (fixed effect)
	TL	Fish total length
	Wt	Fish body weight
	CF	Fish condition factor
	GSI	Fish gonadosomatic index
Land Use	WA	Total watershed area at fish sampling site
	OW	% open water area within total watershed at fish sampling site
	OW5	% open water area within 5 km radius of fish sampling site
	Dev	% developed area within total watershed at fish sampling site
	Dev5	% developed area within 5 km radius of fish sampling site
	For	% forested area within total watershed at fish sampling site
	For5	% forested area within 5 km radius of fish sampling site
	Wtlnd	% wetland area within total watershed at fish sampling site
	Wtlnd5	% wetland area within 5 km radius of fish sampling site
	Ag	% wetland area within total watershed at fish sampling site
	Ag5	% agricultural area within 5 km radius of fish sampling site

continues

Table 3.1 (continued)

Category	Abbreviation	Variable Explanation
Water Quality	AvTemp	Average surface water temp measured at fish sampling site during water quality monitoring
	MxTemp	Maximum surface water temp measured at fish sampling site during water quality monitoring
	AvpH	Average surface water pH measured at fish sampling site during water quality monitoring
	MxCond	Maximum surface water conductivity measured at fish sampling site during water quality monitoring
	AvNO3	Average surface water NO3-N conc.measured at fish sampling site during water quality monitoring
	MxNO3	Maximum surface water NO3-N conc.measured at fish sampling site during water quality monitoring
	AvP	Average surface water phosphorus conc. measured at fish sampling site during water quality monitoring
Sediment Estrogens and Estrogenicity	E1Sp	Spring sediment E1 conc. measured at fish sampling site
	E1Su	Summer sediment E1 conc. measured at fish sampling site
	E2Sp	Spring sediment E1 conc. measured at fish sampling site
	E2Su	Summer sediment E2 conc. measured at fish sampling site
	EE2Sp	Spring sediment EE2 conc. measured at fish sampling site
	EE2Su	Summer sediment EE2 conc. measured at fish sampling site
	EstrSp	Spring sediment estrogenicity measured at fish sampling site
	EstrSu	Summer sediment estrogenicity measured at fish sampling site

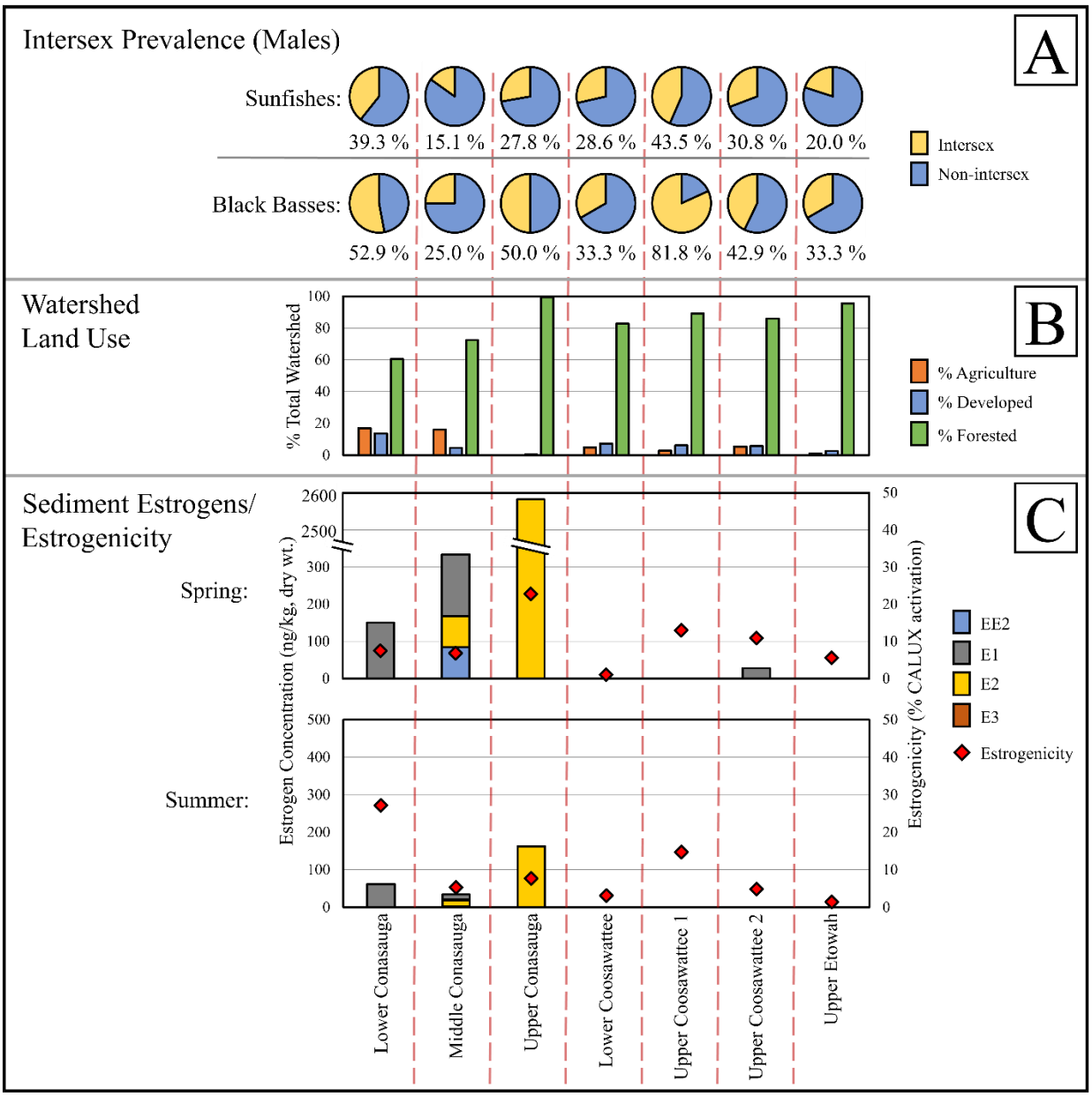

Figure 3.2. Prevalence of intersex observed among sunfishes and black bass species at each sampling location in the Upper Coosa River system (A), compared with respective agricultural, developed, and forested land use percentages within the watershed of each sampling location in the Upper Coosa River system (B), and estrogen concentrations (left y-axis) and relative estrogenicity (right y-axis) of Upper Coosa River sediments collected in spring or summer (C). Values shown are normalized to sediment dry weight total organic material. Estrogenicity represents % activation of estrogen-responsive CALUX assay compared to the EC100.

Fish were collected in spring 2015-2017 during the pre-spawn period.

Table 3.2. Mean water chemistry variables (±SD) measured at fish sampling locations on a monthly basis, in addition to major precipitation events, in the Upper Coosa River System from Aug 2015 to Aug 2016.

Sampling Location	Temperature (°C)	Conductivity (µS/cm)	pH	Hardness	Alkalinity	NO3-N (mg/L)	Total N (mg N/L)	Phosphorus (µg/L)
Lower Conasauga	19.5 ± 6.1	158.9 ± 41.1	8.0 ± 0.2	57 ± 15	69 ± 18	0.53 ± 0.21	0.768 ± 0.209	49.5 ± 70.5
Middle Conasauga	18.5 ± 6.0	86.8 ± 22.1	7.8 ± 0.2	32 ± 8	37 ± 9	0.32 ± 0.10	0.618 ± 0.147	40.9 ± 58.9
Upper Conasauga	16.6 ± 5.9	13.6 ± 1.8	6.8 ± 0.2	5 ± 1	5 ± 1	0.008 ± 0.02	0.338 ± 0.082	16.3 ± 6.3
Lower Coosawattee	18.5 ± 4.8	39.2 ± 6.4	7.3 ± 0.1	12 ± 3	13 ± 2	0.29 ± 0.09	0.597 ± 0.085	32.7 ± 42.8
Upper Coosawattee 1	18.1 ± 5.2	24.5 ± 2.6	7.2 ± 0.2	8 ± 1	9 ± 2	0.17 ± 0.05	0.472 ± 0.100	18.6 ± 16.9
Upper Coosawattee 2	17.5 ± 5.3	24.8 ± 2.8	7.1 ± 0.1	8 ± 1	9 ± 2	0.44 ± 0.08	0.742 ± 0.115	30.9 ± 38.5
Upper Etowah	16.6 ± 4.8	14.2 ± 1.4	7.0 ± 0.1	6 ± 2	7 ± 1	0.09 ± 0.03	0.355 ± 0.117	15.9 ± 5.9

Table 3.3. Watershed area and % land use/cover for total watershed area and buffered watershed area (within 5 km radius) for fish and environmental sampling locations.

	Land Use/Cover in Total Watershed Area								
Sampling Location	Total Area (km2)	Open Water	Total Developed	Total Agriculture	Total Forest	Total Wetland	Barren	Shrub/ Scrub	Grassland/ Herbaceous
Lower Conasauga	1781.0	0.4%	13.7%	16.9%	60.5%	0.9%	0.2%	4.7%	2.7%
Middle Conasauga	608.0	0.2%	4.5%	16.1%	72.4%	0.4%	0.1%	4.0%	2.2%
Upper Conasauga	104.0	0.0%	0.4%	0.0%	99.5%	0.0%	0.0%	0.1%	0.0%
Lower Coosawattee	1371.0	1.2%	7.3%	4.9%	82.7%	0.2%	0.1%	1.8%	1.8%
Upper Coosawattee 1	120.0	0.2%	6.1%	2.7%	89.0%	0.2%	0.0%	0.8%	0.9%
Upper Coosawattee 2	313.0	0.1%	5.7%	5.4%	86.0%	0.2%	0.0%	1.1%	1.5%
Upper Etowah	136.0	0.1%	2.5%	0.8%	95.6%	0.1%	0.0%	0.3%	0.7%
	Land Use/Cover in Buffered (5km) Watershed Area								
Sampling Location	Total Area (km2)	Open Water	Total Developed	Total Agriculture	Total Forest	Total Wetland	Barren	Shrub/ Scrub	Grassland/ Herbaceous
Lower Conasauga	61.0	2.7%	11.2%	11.2%	59.7%	2.8%	0.4%	7.5%	4.7%
Middle Conasauga	31.1	1.7%	8.8%	32.6%	45.4%	2.0%	0.1%	5.1%	4.2%
Upper Conasauga	18.4	0.0%	0.0%	0.0%	100.0%	0.0%	0.0%	0.0%	0.0%
Lower Coosawattee	43.3	10.3%	6.8%	7.5%	70.3%	0.9%	0.0%	1.9%	2.2%
Upper Coosawattee 1	34.5	0.7%	7.9%	6.9%	81.4%	0.6%	0.0%	1.5%	0.9%
Upper Coosawattee 2	30.3	0.0%	10.5%	9.6%	74.9%	0.0%	0.4%	2.5%	2.0%
Upper Etowah	37.9	0.1%	3.2%	2.2%	92.6%	0.1%	0.0%	0.6%	1.3%

Table 3.4. Candidate models explaining incidence of intersex among sunfish (family Centrarchidae) and black bass spp. (genus Micropterus) sampled throughout the Upper Coosa River System. Models are ranked in order of descending AICc weight (Wi), indicating the most plausible models. Shading indicates 'highly plausible' models within 2 AICc units of the top-ranked model. (↑) indicates a positive relationship with intersex incidence, and (↓) indicates a negative relationship.

Taxon	Model Parameters	Rank	K	AICc	ΔAICc	Wi
Sunfish	Sp + Wt (↑) + MxTemp (↑) + Ag5 (↓)	1	16	263.06	0.00	0.2569
	Sp + Wt (↑) + MxTemp (↑) + MxNO3 (↑) + EstrSp (↓)	2	17	263.27	0.21	0.2313
	Sp + Wt (↑) + MxTemp (↑) + E1Su (↓)	3	16	263.47	0.41	0.2093
	Sp + TL (↑) + AvTemp (↑) + E1Su (↓)	4	16	266.43	3.37	0.0476
	Sp + Wt (↑) + AvTemp (↑) + E1Su (↓)	5	16	266.57	3.51	0.0444
	Sp + TL (↑) + WA (↑) + Wtlnd5 (↑)	6	16	267.57	4.51	0.0269
Black Bass	Sp + Wt (↑) + MxTemp (↑) + Ag5 (↓)	1	6	140.84	0.00	0.1841
	Sp + Wt (↑) + MxTemp (↑) + E1Su (↓)	2	6	141.78	0.94	0.1150
	Sp + Wt (↑) + AvTemp (↑) + E1Su (↓)	3	6	142.01	1.17	0.1026
	Sp + Wt (↑) + MxTemp (↑) + MxNO3 (↑) + EstrSp (↓)	4	7	142.67	1.83	0.0737
	Sp + TL (↑) + AvTemp (↑) + E1Su (↓)	5	6	143.25	2.41	0.0552
	Sp + TL (↑) + WA (↑) + Wtlnd5 (↑)	6	6	144.11	3.27	0.0359
	Sp + EstrSp (↑) + AvTemp (↑)	7	5	144.15	3.31	0.0352
	Sp + EstrSu (↑) + MxTemp (↑)	8	5	144.22	3.38	0.0340
	Sp + EstrSu (↑)	9	4	144.43	3.59	0.0306
	Sp + Wtlnd5 (↑)	10	4	144.63	3.79	0.0277
	Sp + AvTemp (↑)	11	4	144.66	3.82	0.0273

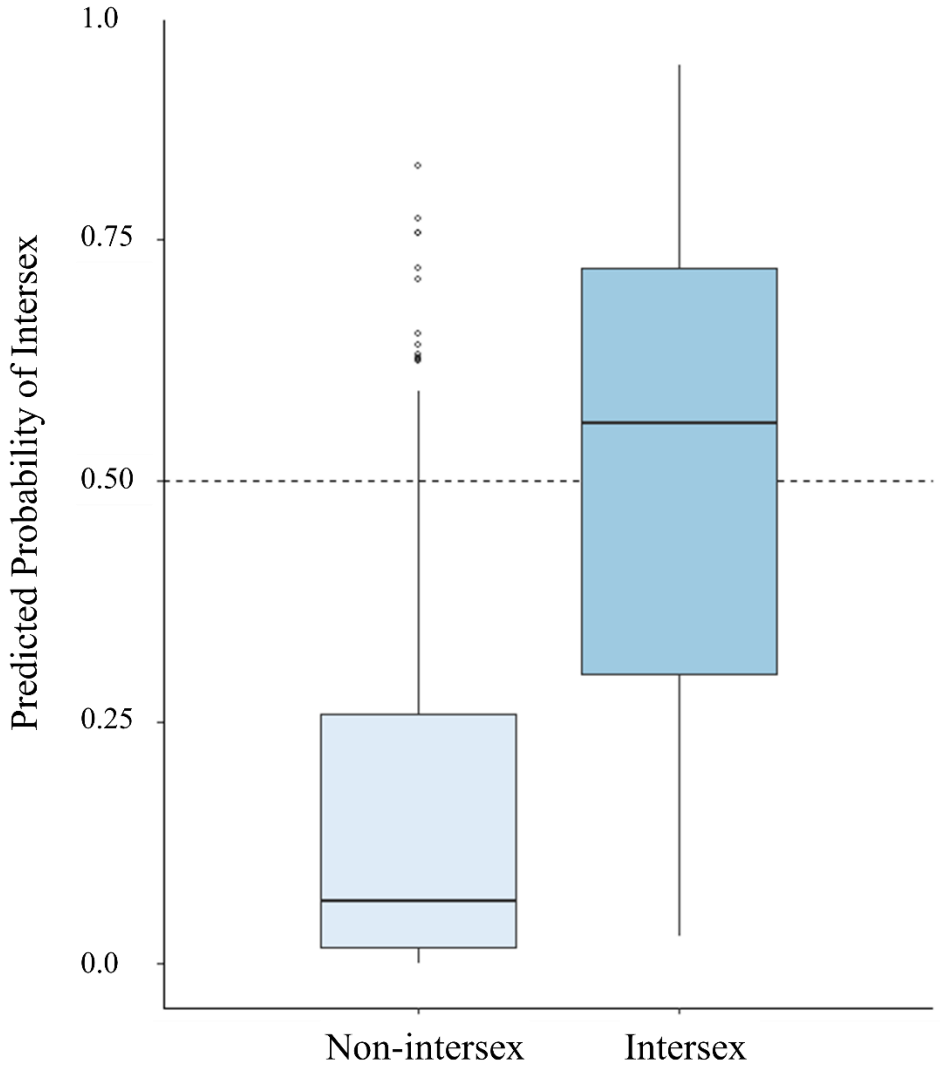

Figure 3.3. Box and whisker plot of centrarchid intersex observations in the Upper Coosa River System determined via histology (x-axis) vs. generalized linear model predictions of intersex probability (y-axis). Predictor variables for this model are fish species and total length, total watershed area, and watershed % wetland area within a 5km radius upstream of sampling location.

Table 3.5. Ranking of variable importance in 95% confidence model sets explaining incidence of intersex among fish taxa sampled from the Upper Coosa River System, as determined by the sum of AICc weights of models containing each variable. Variables shaded in grey did not appear in the 95% confidence set.

Sunfish			Black Bass		
Variable	Rank	ΣAICc Weight	Variable	Rank	ΣAICc Weight
Wt	1	0.7490	Wt	1	0.4816
MxTemp	2	0.7357	MxTemp	2	0.4387
E1Su	3	0.3013	E1Su	3	0.2728
Ag5	4	0.2569	AvTemp	4	0.2450
EstrSp	5	0.2428	Ag5	5	0.1841
MxNO3	6	0.2384	EstrSp	6	0.1349
AvTemp	7	0.1158	EstrSu	7	0.1313
TL	8	0.0910	Wtlnd5	8	0.1176
Wtlnd5	9	0.0537	TL	9	0.1033
EstrSu	10	0.0271	MxNO3	10	0.0799
WA	11	0.0269	WA	11	0.0359
AvNO3	12	0.0128	Wtlnd	12	0.0358
Dev	13*	0.0125	Ag	13	0.0237
MxCond	13*	0.0125	For	14	0.0214
Ag	15	0.0122	AvNO3	15	0.0184
For5	16	0.0099	E2Su	16	0.0148
AvP	17	0.0047	GSI	17	0.0146
Wtlnd	18	0.0045	CF	18	0.0132
EE2Su	19	0.0040	Dev	19	0.0123
E2Su	20	0.0038	MxCond	20	0.0116
GSI	21*	0.0000	AvP	21	0.0101
For	21*	0.0000	Dev5	22	0.0053
Dev5	21*	0.0000	AvpH	23	0.0051
E2Sp	21*	0.0000	E1Sp	24	0.0050
AvpH	21*	0.0000	E2Sp	25	0.0045
E1Sp	21*	0.0000	EE2Sp	26*	0.0044
EE2Sp	21*	0.0000	EE2Su	26*	0.0044
CF	21*	0.0000	OW	28*	0.0000
OW	21*	0.0000	OW5	28*	0.0000
OW5	21*	0.0000	For5	28*	0.0000

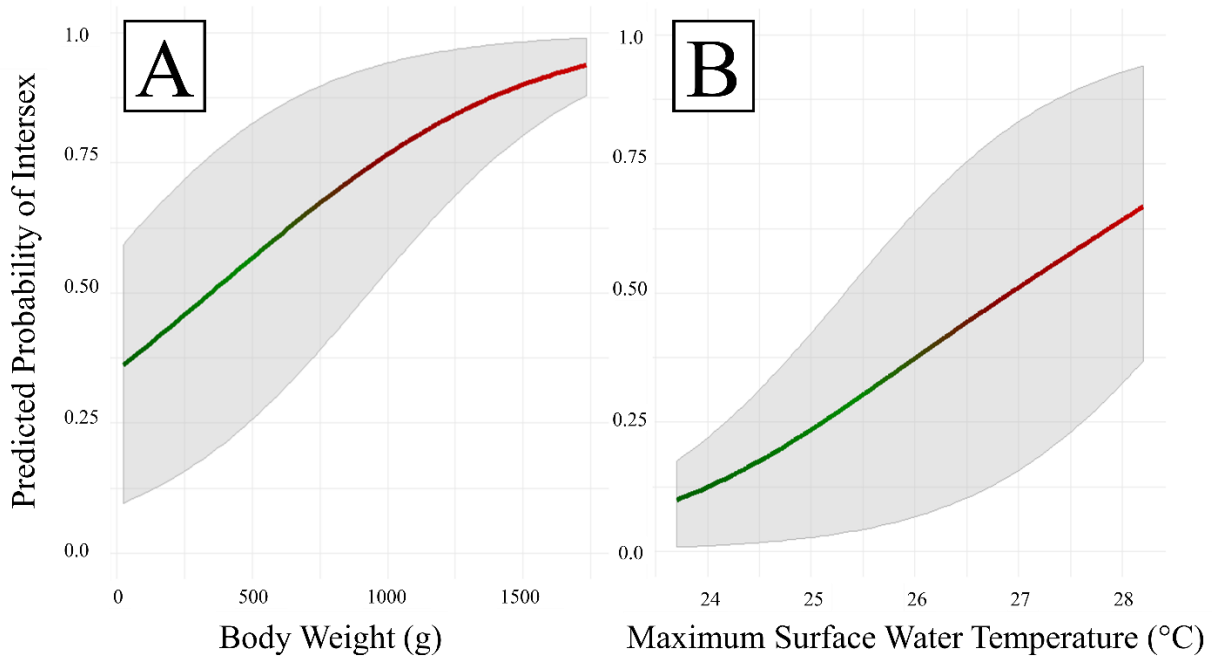

Figure 3.4. Average marginal predicted probabilities and interquartile range for predictions (shaded area) of intersex incidence for individual adult black bass in the Upper Coosa River System (Georgia, USA) over a range of body weights (A) and maximum surface water temperatures (B). For a given value, 50% of black bass individuals are predicted to fall within the shaded area.

CHAPTER 4

ASSOCIATION OF LIVER AND BLOOD PLASMA METABOLITES WITH TESTICULAR OOCYTES IN WILD LARGEMOUTH BASS[3]

[3] Matthew L. Urich, David M. Skelton, W. Matthew Henderson, Robert B. Bringolf. To be submitted to *Environmental Science and Technology*

Abstract

Numerous reports regarding the incidence of testicular oocytes (TO) among freshwater fish has frequently been linked to reduced sperm fertility and motility, yet the potential for population-level effects and associated biochemical changes are still poorly understood. Current methods to positively identify TO in fish are tissue invasive and lethal. Further, in order to estimate of TO prevalence among wild populations, large numbers of adult males would need to be sacrificed. Thus, the identification of non-invasive metabolic indicators through use of untargeted LC/MS-based metabolomics in intersex fish may aid in identification of key cellular pathways and lead to the development of useful biomarkers in intersex fish. We investigated metabolic profiles of purported male (individuals identified as male based on the presence of testes, prior to histological evaluation) largemouth bass with TO counts ranging from 0 – 164 (per longitudinal histological section) from an impoundment in the Piedmont region of Georgia (U.S.). The levels of several liver and blood plasma analytes were highly significantly ($p<0.01$) correlated with the number of oocytes detected in the testis. Analytes involved in metabolic pathways such as the urea cycle lend insight to the mechanisms associated with intersex induction in these fish. Development of targeted analytical methods to detect biomarkers of TO, particularly in non-lethal tissues/fluids, would greatly enhance efforts to obtain sufficient sample sizes and allow for repeated sampling of individuals. In addition, elucidation of associated pathways could inform adverse outcome pathways and ecological risk assessments by increasing our understanding of biochemical mechanisms, spatial and temporal trends, causative factors and adverse effects associated with intersex on an individual and population level.

Introduction

Despite concerns regarding the potential ecological effects of increased incidence of intersex among freshwater fish, current knowledge is often insufficient to identify causes or assess associated risks. Reports of testicular oocytes (TO, a form of gonadal intersex in which developing egg cells occur amongst testicular tissue) are globally distributed (Bahamonde et al., 2013) and highly prevalent (up to 100%) among males from some populations. TO occurs in a broad range of taxa including reptiles (Guillette et al., 1994) and amphibians (Hayes et al., 2002), but is particularly prevalent among fish species of the families Cyprinidae, Catostomidae, and Centrarchidae, among others (Bahamonde et al., 2013). Black bass species, including smallmouth bass (*Micropterus dolomieu*) and largemouth bass (LMB, *Micropterus salmoides*) are among the most commonly reported species with intersex (Abdel-moneim et al., 2015). Black bass exhibit a broad range (0-100% of males) of TO prevalence among sites, yet the significance and implications of locally high levels of TO among certain sites, species, and/or populations are mostly unknown.

Though evidence is scarce, the potential for reduced reproductive fitness among individuals and populations with high levels of TO raises concern for widespread ecological implications. In a study of the wild common roach (*Rutilus rutilus*) sampled from polluted streams in the United Kingdom, individuals with severe TO had reduced sperm motility and fertility compared with conspecifics lacking TO all together (Jobling et al., 2002). Links between TO and adverse population-level effects are further supported by the detection of TO in a population of fathead minnows (*Pimephales promelas*) that declined in numbers following exposure to a synthetic estrogen (Kidd et al., 2007). The potential for TO-mediated adverse

outcomes highlights the need to understand causes and mechanisms of induction of TO, as well as the potential manifestation of population-level effects.

Exposure to complex chemical mixtures and other factors such as temperature, population density, and genetics likely play a role in the induction of TO. Still, extensive sampling efforts are necessary to draw meaningful conclusions regarding risk assessments, development of adverse outcome pathways, and fisheries management. Variability in prevalence and severity of TO among wild populations is often attributed to endocrine disrupting chemicals (EDCs) exposure, particularly during sensitive early life stages. Chemical exposure, however, does not appear to be directly implicated in all incidences of TO, as in reported cases at reference sites (Iwanowicz et al., 2016) and within laboratory control populations (Grim et al., 2007). When EDC exposure is suspected in the induction of TO in the environment, measured contaminants levels often fail to fully explain variability in observed TO levels (Kolpin et al., 2013). To better understand causative factors and ecological implications of TO, relationships with geo-spatial, temporal, ecological, and taxonomic factors must be established.

Efforts to determine the extent and mechanisms of ecological risk associated with TO are severely limited by lethal histological methods of TO detection. Histological evaluation of large numbers of sexually mature male fish is required to make meaningful statistical comparisons, but lethal sampling is not prudent or practical for populations at risk of decline. Further, histological methods disallow for repeated sampling of individuals, hindering the investigation of temporal aspects of TO development. Thus, the development of non-lethal methods of TO identification is necessary to broaden the scope of investigations into causes and effects of TO. Recently, efforts have been made to develop non-lethal laparoscopy-based methods, (MacLeod et al., 2017) but

histological evaluation of gonadal tissues remains the only widely-accepted method for identification of TO in fish.

Identification of molecular markers of TO, particularly from non-lethally collected biological samples, would greatly expand the diagnostic ability of scientists to evaluate TO among fish species of interest. Molecular endpoints have been found in association with TO in fish, but most still require invasive or lethal sampling, and none display specificity or reproducibility required to reliably indicate TO. Biomarkers associated with feminization in male fish due to estrogen exposure, including reduced gonadosomatic index (GSI) and plasma vitellogenin (Vtg, an egg yolk protein) levels, are only loosely associated with TO and lack specificity to differentiate between intersex individuals and cohabiting estrogen-exposed male fish with no TO (Bahamonde et al., 2015). Induction of the gene ovarian structure protein-1 (OSP-1), is strongly associated with TO in Japanese medaka (*Oryzias latipes*; Abdel-Moneim et al., 2015), but analysis of OSP-1 relies on a species-specific sequence for detection. Transcript and protein markers such as OSP-1 offer great potential for the investigation of TO, but their utility is currently limited to organisms with well-characterized genomes. Without effective biomarkers for TO among wild fish, the associated risk cannot be widely and accurately assessed, and the potential ecological threat posed by TO will remain a significant concern for regulators, fisheries managers, and researchers among others.

Metabolomics is a practical option for development of non-lethal methods to detect molecular biomarkers of TO in wild fish. Biological matrices, including some that can be collected non-lethally, contain hundreds to thousands of endogenous biochemical metabolites relevant to the physiological status of organisms. For example, metabolomics has been used to study the effects of EDCs on fish. In a laboratory study, urinary metabolite levels of fathead

minnows were found to display markedly different profiles when exposed to EDCs individually as well as in mixtures (Collette et al., 2010). Davis et al. (2013) demonstrated the utility of field-based exposure monitoring using metabolite profiles of caged fathead minnows exposed *in situ* to EDCs in the environment. This study demonstrated the ability to detect sex-specific responses to EDC exposure in metabolite profiles. Sex-specific responses were also reported in the skin mucus of fathead minnows exposed to Bisphenol-A (Ekman et al., 2015), a compound previously shown to induce TO in fish (Kang et al., 2002). These data suggest that minimally invasive techniques may be used to characterize the feminization of male fish, even in transient biofluids. Similar techniques may be used to examine biological matrices of wild fish for biomarkers of TO. In the present study, we evaluated the utility of LC/MS-based metabolomics for the identification of metabolite biomarkers associated with TO in liver and blood plasma from wild male LMB with varying TO counts. The objectives of this study were to: 1) identify candidate analytes for development of biomarkers of TO among wild LMB, and 2) elucidate biochemical mechanisms associated with the incidence of TO.

Materials and Methods

Site Selection and Fish Collection

Adult LMB were collected by boat electrofishing from a single creek-fed impoundment (~7 ac.) in Wilkes Co., Georgia, USA during April, 2014. This site was selected based on a previous report of TO among male LMB with a prevalence of 72% (Kellock et al., 2014). Fish targeted for this experiment ranged between 250 and 350 mm total length to insure fish were sexually mature and of similar size. All fish outside this range were released immediately. Relative weight (Wr) for each fish was calculated according to methods developed by Blackwell

et al. (2000) for LMB. pH, DO, and temperature were measured before and after sampling (Hach HQ40D, USA) and ranged from 6.07-6.96, 9.5-10.1 mg/L, and 17.8-20.1 °C, respectively. All fish were handled in accordance with approved University of Georgia Institutional Animal Care and Use protocols (AUP # A2016 06-028-A1).

Tissue Collection

Upon collection, a numbered zip-tie was placed around the mandible of each fish. Fish (no more than 5 at a time) were held in an aerated on-boat holding tank for no longer than 15 min. In the order in which they were collected, each fish was euthanized with an overdose of buffered tricaine methanesulfonate (MS-222, 500 mg/L). Fish were weighed (nearest 0.01g) and measured for total length (TL, nearest mm), and immediately processed for blood and liver samples. Blood was collected ventrally from the caudal vessels in heparinized syringes and expelled into heparinized 2 mL centrifuge tubes. Blood samples were kept on ice, transferred to the aquatic lab, then centrifuged (10 min. at 2000rpm, 4 °C). The plasma was carefully collected and stored at -80 °C until analysis. Approximately 100-150 mg of liver tissue was collected from the posterior tip of the lateral-most lobe of the liver in each fish and immediately flash frozen in liquid nitrogen and stored at -80 °C to prevent degradation of metabolites. Dissection tools and surfaces were rinsed with methanol, then de-ionized water and wiped thoroughly between each dissection to prevent cross-contamination. During processing, the whole liver and whole gonad were weighed (nearest 0.01 g) to facilitate calculation of HSI and GSI. Testicular tissue was collected from each male fish. A small piece (~5% of total tissue) was immediately flash frozen in liquid nitrogen and stored at -80 °C for metabolomics analysis. The rest of the tissue was preserved in 10% formalin solution for histopathological processing and evaluation.

Gonad Histology

Testicular tissue fixed in 10% formalin was prepared for histopathological evaluation. Briefly, testis from each fish was embedded in paraffin wax, and a single 4 μm longitudinal section from the central region was obtained using a microtome. Each section was mounted on a glass microscope slide and stained with hematoxylin and eosin and examined under a compound light microscope. Testicular oocytes were identified and enumerated to determine the total number of oocytes detected per individual (TO count). Individuals with one or more TO within the area examined were identified as intersex, and individuals without oocytes within the area examined were identified as male. An intersex severity index (SI) developed by Blazer et al., (2007), as modified by Kellock et al. (2014) was assigned to each intersex fish to assess distribution of intersex severity within the individuals sampled. Briefly, SI of 1 is a testis section with only one TO, SI of 2 is a section with multiple focally distributed TO, SI of 3 is multiple TO within a cluster, and SI of 4 is defined as multiple clusters of TO.

Tissue Extractions

Extractions of liver and blood plasma were performed by adapting established methods for FHM tissue analysis (Ekman et al., 2012). Briefly, a bi-phase extraction was performed using a methanol/water solution (polar fraction) and chloroform (non-polar fraction) in a 96-well format. After phases were separated and solvents were evaporated from the extracts, plates containing both polar and non-polar fractions were stored at (-80 °C) until analysis.

LC/MS Analysis and Data Processing

High-resolution untargeted metabolomics was conducted on the polar phase of each tissue extract (full scan mode) in both positive (+ESI) and negative (-ESI) ionization modes. Analyses were conducted using an Accela 1250 UHPLC with an Open Accela Autosampler

(Thermo Fisher Scientific) paired with a Q-Exactive mass spectrometer (Thermo Fisher Scientific) with a heated electrospray ionization source (HESI-II). LC separation of a 2 µL sample injection was performed using a Hypersil aQ C18 (100 mm x 2.1 x 1.9; Thermo Fisher Scientific) maintained at 50 °C with a gradient elution of solvent A (ultrapure water with 0.1% formic acid) and solvent B (acetonitrile with 0.1% formic acid) with flow rate of 600 µl/min. The initial condition was set to 100% solvent A, decreased to 80% A at 4 min, 40% A at 5.5 min, and 5% at 8.0 min, where it was maintained until 9.0 min. The column was rinsed with 100% solvent B for 4 min and conditioned with 100% solvent A for 5 min prior to the injection of each sample. The following mass spectrometry parameters were used: probe heater temperature, 390 °C; sheath gas, 60; Auxilliary gas, 15; sweep gas, 2; capillary temperature, 320 °C; S-lens RF level, 35%; Spray voltage, 3 kV (positive mode), 2.8 kV (negative mode); m/z scan range, 65-850; mass resolution, 70,000; automatic gain control target, 1×10^6; max injection time, 100 ms. Analyte peaks from each ionization mode were detected and integrated using the small molecule component extraction detection algorithm (SIEVE v2.1, Thermo Fisher Scientific), and relative abundance was determined by measuring area under the curve for each analyte peak and normalizing all analyte values per individual to 1.

Statistical Analysis

Student's 2-tailed t-tests ($\alpha = 0.05$) were used to evaluate mean morphometric differences between male and female fish, and one-way ANOVA ($\alpha = 0.05$) was used to evaluate mean morphometric differences among male, female, and intersex classes. Metabolomics data were pareto scaled prior to partial least squares discriminant analysis (PLS-DA, SIMCA-13.0, Umetrics Inc.). Visual separation and Q2 values were used to evaluate the utility of metabolite profiles for prediction of sexual phenotype (male, female, or intersex). Pearson's correlation

coefficient (R) was calculated between the relative abundance of each analyte and the number of oocytes detected for each fish (R, V. 3.3.1), and only analytes with highly significant ($p \leq 0.01$) correlations were selected for targeted annotation and interpretation of results. Analytes of interest were putatively annotated by searching the accurate mass in public databases (METLIN, Scripps Institute; Human Metabolome Database, hmdb.ca) using a mass error tolerance ≤ 4 ppm. Pathway analysis was conducted using putative metabolite IDs in MetaboAnalyst 4.0.(Chong et al., 2018) Significantly altered pathways ($p \leq 0.05$) were identified to aid in interpretation of results.

Results

Fish Collection

Total length of all phenotypic male LMB (n = 39) collected from the impoundment ranged from 277 – 350 mm (304 ± 15 mm), and females (n = 8) ranged from and 287-340 mm (271 ± 16 mm). Wr ranged from 0.579 – 0.939 (0.742 ± 0.068) for phenotypic males and from 0.679 – 0.909 (0.735 ± 0.073) for females. Neither length ($p = 0.39$) nor Wr ($p = 0.78$) differed significantly between macroscopic male and female fish (Student's two-tailed t-test, $\alpha = 0.05$). When considering three sexual phenotypes based on histological identification of TO among phenotypic males (i.e. male, female, or intersex), one-way ANOVA paired with Tukey's post-hoc test ($\alpha = 0.05$) revealed no differences in mean total length ($p = 0.35$) nor mean Wr ($p = 0.73$) among groups. As may be expected during the pre-spawn period, mean GSI differed significantly between females and intersex ($p < 0.01$), and female and male ($p < 0.01$) groups, but did not differ between male and intersex fish ($p = 0.90$). Mean HSI differed significantly only between female and intersex groups ($p < 0.01$).

Gonad Histology

Of the 39 phenotypic male LMB collected, 11 had no identifiable oocytes (TO count = 0) within the testicular tissue examined, and 28 individuals had TO counts ranging from 1-164. Oocytes were pre-vitellogenic in all cases and ranged from approximately 20 to 50 μm in diameter. Distribution of intersex SI reflected a broad range severity, with at least 5 individuals from each SI 1-4 (Fig. 4.1).

Metabolomics

Analysis of liver samples detected 470 analytes in positive mode and 279 analytes in negative mode. In blood plasma, 368 analytes were detected in positive mode, and 202 analytes were detected in negative mode. Multivariate model analysis revealed consistency between tissues sampled, with PLS-DA analyses from liver and blood plasma showing moderately clean separation in both tissues between females and other phenotypes (Fig. 4.2). Males with TO, however, did not distinctly separate from males without TO in the blood plasma analysis (Fig 4.2A), and even less separation was observed using liver data (Fig.4.2B). Despite a lack of separation of the intersex phenotype in multivariate models, univariate correlation analyses yielded a total of 28 analytes having highly significant ($p < 0.01$) correlations with TO count, including 2 from the liver and 26 from blood plasma (Table 4.1). Of these, 16 were putatively annotated (mass error tolerance ≤ 4 ppm), and represent a broad range of chemical classes, including amino acids, carbohydrates, and lipids (Table 4.1). Metabolites putatively identified play diverse and varied roles in biological processes, including the urea cycle, citric acid cycle, glutathione metabolism, and glycolysis.

Discussion

In this study, we explored use of LC/MS-based metabolomics and found it may be a useful approach for identifying biomarkers of TO in teleost fish. Although multivariate models were poorly predictive, univariate analysis revealed relationships between TO count and the levels of endogenous putative metabolites measured in this study, and altered biochemical pathways associated with TO were putatively identified.

The relative abundance of uremic toxins such as guanidinosuccinic acid and citrulline in blood plasma suggests that metabolism and/or excretion of nitrogenous wastes may be disrupted in fish with higher TO counts, and that elevated levels of endogenous urea cycle intermediates may play a role in the incidence of TO among LMB. A considerable number of putative metabolites positively correlated with TO count are urea cycle constituents and are considered uremic toxins. Data on the effects of these compounds in fish physiology are limited or non-existent, but research in other species suggests that uremic toxins are directly and/or indirectly related to cellular pathways that regulate levels of steroid and peptide hormones, as well as normal function, maintenance, development, and differentiation of testicular tissue. For example, uremic (elevated levels of uremic toxins) human male patients with terminal renal failure have higher serum levels of estradiol and luteinizing hormone (LH), and lower levels of anti-mullerian hormone (AMH; Eckersten et al., 2015). Estradiol and LH play critical roles in gonadal development and maintenance, and estrogens including estradiol and some EDCs are well documented for their feminizing effects on male fish. AMH is an important regulator of testicular function and contributes to the differential regulation of gonadotropins in rats (Garrel et al., 2016). Interestingly, AMH displays male-biased expression patterns in developing fish of several species (Baron and Guiguen, 2003; von Hofsten et al., 2005; Miura et al., 2002;

Rodríguez-Marí et al., 2005; Yoshinaga et al., 2004), and is thus suspected for its involvement in testicular differentiation in fish (Schulz et al., 2007). This information suggests linkages between elevated levels of urea cycle constituents and the incidence of TO in LMB, possibly via modulation of sex-specific hormone levels.

Interestingly, particular uremic metabolites identified in the present study are suspected for involvement in nitric oxide (NO) biosynthesis pathways in vertebrates. NO is a potent signaling molecule that decreases androgen levels by inhibiting several steroidogenic enzymes such as steroidogenic acute regulatory protein (StAR), cytochrome P450-sidechain cleavage enzyme (SCC), and 3β-hydroxysteroid dehydrogenase (3βHSD), (Del Punta et al., 1996; Panesar and Chan, 2000) and can affect the development, motility, and viability of sperm (Del Punta et al., 1996; Kostić et al., 1998; Panesar and Chan, 2000; Ratnasooriya and Dharmasiri, 2001; Rosselli et al., 1995). Both guanidinosuccinic acid (GSA) and citrulline, urea cycle constituents positively correlated with TO count in the present work, have each been noted for their involvement in NO synthesis and signaling. GSA is considered a stable NO mimic which activates NO pathways and NO-mediated effects *in vivo* and *in vitro* (Noris and Remuzzi, 1999). Furthermore, the synthesis of GSA from argininesuccinate has been suggested to stimulate a positive feedback loop of NO synthesis, further potentiating activation of NO pathways in the presence of GSA (Aoyagi et al., 1999). Citrulline is also closely related to NO biosynthesis and NO-mediated pathways. Citrulline is formed as a product in the synthesis of NO from arginine, a reaction catalyzed by nitric oxide synthase (NOS). Therefore, elevated levels of citrulline in fish with higher TO counts may be indicative of increased NOS activity and subsequently elevated NO concentrations. Citrulline is not only a product of NO synthesis, but can also act as a substrate for NO production, and evidence suggests that citrulline may even be superior to

arginine in terms of induction of NO synthesis (El-Hattab et al., 2012). Modulation of NO pathways leading to altered steroidogenesis, could have significant effects on gonadal development in fish, particularly during early developmental stages in which bi-potential gametocytes or germ cells are present. Positive correlations between TO count and the levels of urea cycle constituents in the present study suggest that TO may be associated with uremic toxin-mediated disruption of endogenous NO pathways, potentially resulting in modulation of steroid hormone levels in gonadal tissue. Elevated levels of urea cycle intermediates, such as GSA and citrulline suggest that urea levels may also be elevated. In humans, GSA is thought to be synthesized from arginine when enzymes that facilitate urea production are inhibited by excessive build-up of urea (Noris and Remuzzi, 1999). In the present study, we are unable to confirm elevated urea levels in fish with TO, as it was not within the mass ranges scanned. Future research investigating biochemical mechanisms associated with the incidence and induction of TO could be informed by exploring hypotheses related to urea cycle, NO signaling, and interactions between these pathways and their constituents.

In the present study, we sought to minimize extraneous variability in order to isolate responses associated with TO. Therefore, we investigated metabolite profiles of individuals from one population and one species, encompassing a narrow size (and presumably age) distribution, collected from a single site, during a brief time period. Biomarkers of effect were targeted with this approach, and environmental concentrations of potentially causative compounds were not measured. Although quantitative information on chemical exposure would be useful for investigating potential causes of TO induction, the sampled fish were confined to the impoundment and likely experienced a similar history of chemical exposure during their lifetime. Actively minimizing variability may be useful in the initial stages of biomarker development, but

to develop a robust procedure that can be applied under different circumstances, variability must be addressed and accounted for. Future research should further examine the robustness of these relationships among chemical, spatial, temporal, taxonomic, and genetic factors, and investigate their utility in statistical models that could be used for predictive purposes. Advancement of these techniques could allow for the development of targeted analytical screening procedures that use non-lethal sampling methods to estimate the prevalence and severity of TO among wild fish populations. Such developments could significantly advance the pace of TO research, allow for repeated sampling of individuals, and better our ability to assess risk associated with TO across fish taxa, particularly among imperiled species.

Though the chemical exposure history of the fish sampled in this study is largely unknown, underlying mechanisms associated with TO may be similar among different exposure scenarios. Whereas compounds from a variety of chemical families and sources induce TO in fish (and some incidences are suggested to arise spontaneously), the apical biochemical pathways leading directly to or from TO induction may be very similar in all or most cases of TO, regardless of chemical exposure (or lack thereof). Modulations of these pathways and their constituents represent potential biomarkers of effect (as opposed to biomarkers of exposure). As such, rather than indication of chemical exposure, these differences represent the net effects of interactions between exogenous and endogenous factors resulting in or leading from the incidence or severity of TO.

This study found that liver and blood plasma are potential sources for metabolic biomarkers of TO in wild fish, but analysis of other biological matrices may yield additional information. The liver was chosen as a candidate tissue in this study due to its high metabolic activity, whereas blood plasma can be sampled non-lethally and acts as an intermediary bodily

compartment among several organ systems. Blood chemistry is useful for determining organism-level effects and mechanisms, but many endogenous and exogenous factors may contribute to extraneous variability within this tissue. A better understanding of TO-associated mechanisms may be gained by examining the levels of metabolites directly from the affected tissue. In terms of utility for elucidating apical mechanisms, biomarkers of effect, and informing adverse outcome pathways, levels of metabolites within the testes are possibly even more informative. Furthermore, analysis of other non-lethal biological matrices such as skin mucus may also produce insightful data and further minimize invasiveness of sampling procedures. Urinary metabolites may also be informative, given the abundance of nitrogenous waste compounds, such as those highlighted in this study, typically excreted in the urine. Future research investigating biomarkers and mechanisms of TO incidence and induction could be well-informed by analysis of metabolite levels in additional biological matrices, such as testicular tissue, blood plasma, urine, and skin mucus.

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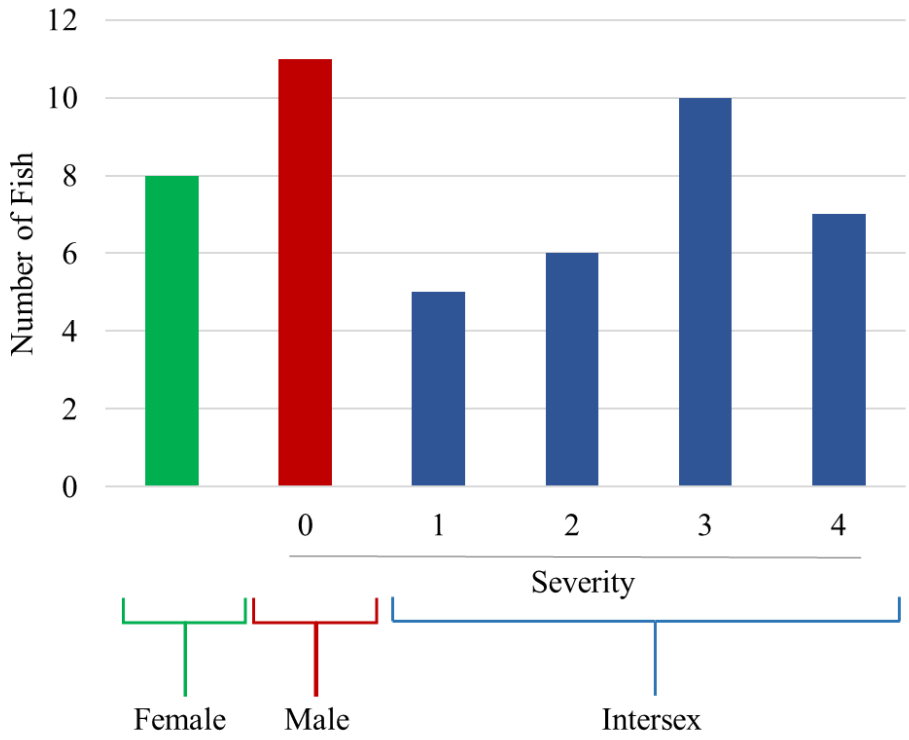

Figure 4.1. Distribution of gonadal phenotypes of wild LMB sampled from an impoundment in Wilkes Co., GA (USA), along with intersex severity index for male (n = 0 TO detected), and intersex (at least 1 TO detected) fish.

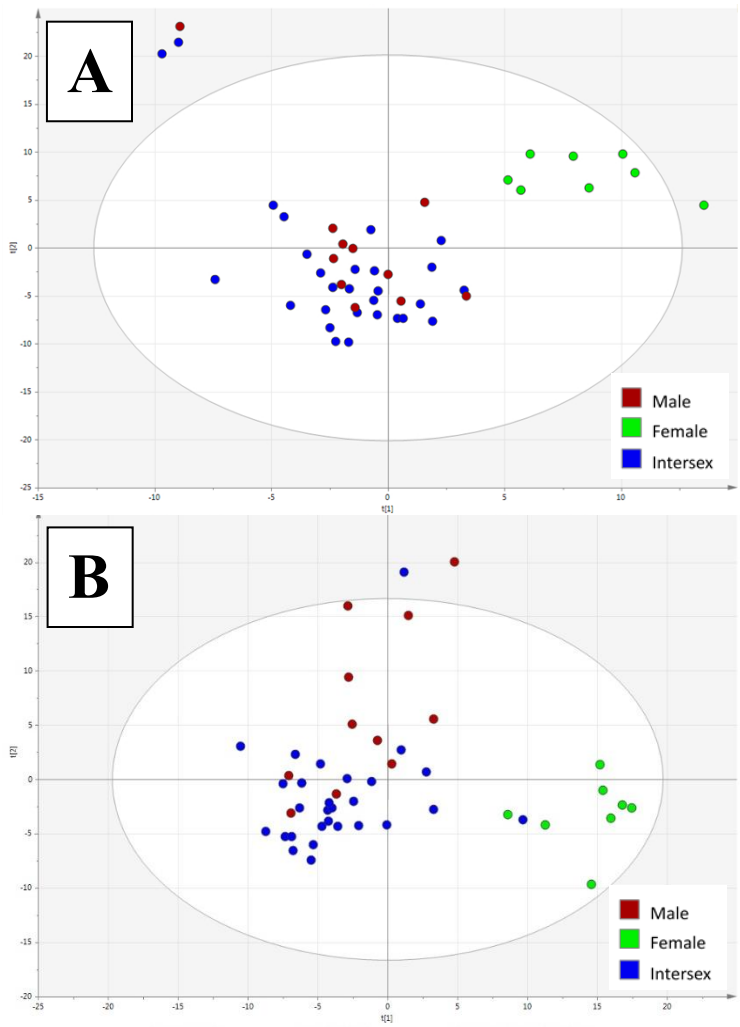

Figure 4.2. PLS-DA scores plots representing differences in the relative abundance of metabolites detected in positive ionization mode in blood plasma (A) and liver (B) of female (green) and male largemouth bass with TO (intersex, blue), and without TO (male, red).

Table 4.1. Putative metabolite annotations of analytes significantly ($p < 0.01$) correlated with the number of testicular oocytes detected in individual largemouth bass collected from an impoundment in Wilkes Co., GA (USA).ESI (+/-) indicates the ionization mode in which the analyte was detected. P = blood plasma, L = liver.

Chemical Class	Putative Annotation	m/z	Mass Error (Δppm)	ESI (+/-)	Tissue	Pearson R (~TO)
Alkyl Sulfates	Ethyl Hydrogen Sulfate	124.9915	3	-	P	0.582
Amino Acids, Peptides and Analogs	Citrulline; Argininic Acid	174.0886	3	-	P	0.608
	Serine	104.0355	3	-	P	0.597
	Guanidinosuccinic acid	176.0658	3	+	P	0.562
	Pyrroline	128.0354	2	-	P	0.531
	Proline	116.0707	3	+	P	0.490
	Tyrosine	180.0669	2	-	P	0.475
	Threonine	118.0510	0	-	P	0.453
	Serine	106.0498	1	+	P	0.445
	Glycine	76.0393	4	+	P	0.413
Carbohydrates	Erythrose	119.0350	0	-	P	0.447
Carboxylic Acids	Butenedioic acid	115.0038	2	-	P	0.466
Lipids and Lipid-like Molecules	Phosphatidyl choline*	806.5685	1	+	P	0.428
	Propionylcarnitine	218.1386	1	+	P	0.419
Non-metal	Phosphoric acid	98.9841	1	+	P	0.412
Pyrimidine	Cytidine	244.0927	0	+	P	0.446
Unknown	-	157.9899	N/A	+	P	0.620
	-	82.0138	N/A	+	P	0.513
	-	296.0214	N/A	-	L	0.479
	-	90.9767	N/A	+	P	0.476
	-	100.0756	N/A	+	P	0.475
	-	146.0299	N/A	+	P	0.457
	-	128.0194	N/A	+	P	0.455
	-	80.0344	N/A	+	P	0.439
	-	232.0914	N/A	+	P	0.436
	-	83.0215	N/A	+	P	0.419
	-	229.0327	N/A	-	L	0.414
	-	127.0866	N/A	+	P	0.410

* Unknown isomer

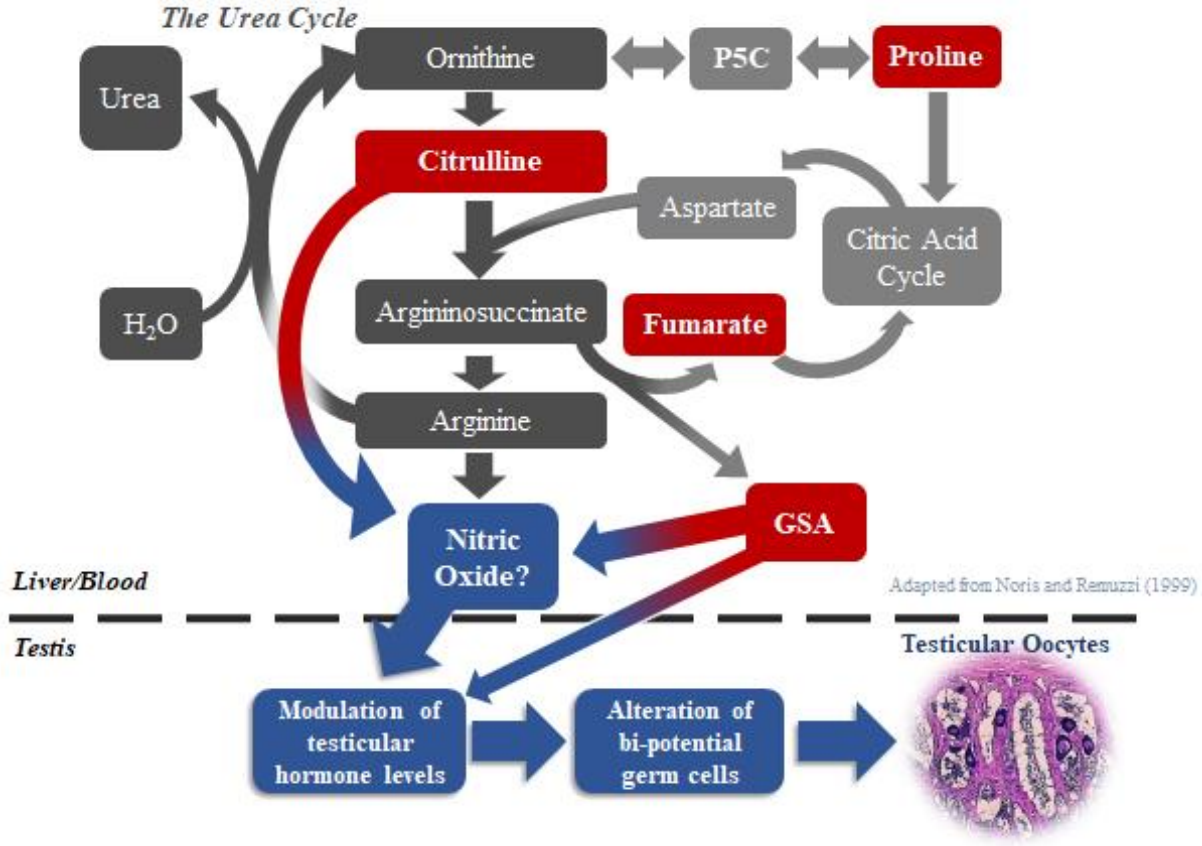

Figure 4.3. Proposed mechanism of TO induction in largemouth bass, as related to the ornithine-urea cycle and associated pathways. Putative metabolites shown in red were significantly ($p <$ 0.01) correlated with the number of TO detected in each individual. Metabolites shown in grey were not identified/measured in our analysis. Items shown in blue depict the hypothesized pathways of NO induction and subsequent induction of TO. GSA, guanidinosuccinic acid; P5C, pyrroline-5-carboxylic acid. Biochemical pathways adapted from Norris and Remuzzi (1999).

CHAPTER 5

EVALUATION OF GONAD METABOLITES AND BLOOD PLASMA BIOCHEMICAL PROFILES OF INTERSEX LARGEMOUTH BASS[4]

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Abstract

Testicular oocytes (TO) is a gonadal intersex condition frequently observed among freshwater fish and could hold substantial implications for biodiversity and sustainability of fisheries worldwide. Placing TO within the toxicological context of adverse outcome pathways (AOPs) is an important priority for ecotoxicologists due to its widespread occurrence and existing knowledge of associations with harmful chemical exposure and adverse effects in fish. However, key event relationships between EDC exposure, incidence of TO, and adverse outcomes have yet to be fully elucidated – in part, due to unexplained geographic and taxonomic variability, but also due to complexities associated with linking toxicological and physiological factors to associated population-level effects. An understanding of the physiological status of reproductive systems in intersex fish is a major component needed to assess ecological risk associated with adverse reproductive effects. In the present study, alterations to gonad metabolite profiles associated with TO were determined using GC-MS-based metabolomics to investigate physiological states associated with intersex in largemouth bass collected from an impoundment in Georgia (USA). In addition, clinical blood biochemical screens were conducted to evaluate markers of fish health. Results suggest that physiological changes associated with energy expenditure, gonad lipid and protein metabolism, and thermal and/or hypoxic stress is related to the occurrence of TO. These results provide novel insight to explain cellular pathways underlying the intersex condition in male largemouth bass (LMB, *Micropterus salmoides*) and to identify candidate analytes for biomarker discovery. Overall, these results highlight the need to understand relationships between effects of chemical exposure and nonchemical factors related to climate change.

Introduction

Testicular oocytes (TO) is the most commonly observed form of gonadal intersex among freshwater fish. Characterized by the presence of immature egg cells within testicular tissue, TO has been reported in a wide diversity of gonochoristic (fixed-sex) species and occurs frequently among particular taxa. In freshwater teleost fishes, TO is largely considered to be an abnormal feminization, but some have speculated that it may occur, at least in part, as a natural phenomenon (Bahamonde et al., 2013). After decades of research investigating potential causes of intersex in fish, TO can clearly be induced by exposure to endocrine disrupting chemicals (EDCs) under certain conditions. Field studies and surveys of wild fish that evaluated potential environmental causes of TO have identified associations between incidences of TO in wild fish exposed to chemicals resulting from anthropogenic land use. As a result, geographic variation in TO prevalence is frequently attributed to various sources of anthropogenic contaminants. However, involvement of other factors is suspected because in many cases, results are inconclusive or inconsistent (see Bahamonde et al. [2013] for a review). Ultimately, TO among wild freshwater fish appears to be widely circumstantial, depending on a variety of known and unknown interdependent factors. To gain a better understanding of causative factors of TO, characterizing physiological effects associated with the condition is necessary.

Laboratory experiments have contributed much to our understanding of TO induction in fish, but inconsistencies exist even in highly controlled environments, and the environmental relevance of such findings remains unclear. Chemical exposure is clearly capable of inducing TO in some species under certain circumstances, but the degree to which these findings can be extrapolated to explain TO observed in wild fish is uncertain. Laboratory studies have used well-characterized laboratory fish species to investigate and demonstrate TO induction via exposure to

suspected causal compounds. Indeed, several compounds have been found to induce various degrees of intersex including TO in laboratory species such as Japanese medaka (*Oryzias latipes*) and zebrafish (*Danio rerio*; Abdel-moneim et al., 2015). The incidence and magnitude of these responses is dependent on multiple factors, including the timing and duration of exposure, chemical concentration and potency, and whether fish are exposed to chemicals individually or within a mixture of compounds that may either agonize or antagonize particular biochemical pathways. Even among controlled laboratory experiments, a great deal of variability exists in exposure scenarios and responses of exposed fish. For example, some studies demonstrate induction of TO at environmentally relevant chemical concentrations (low ng/L; Depiereux et al., 2014, Metcalfe et al., 2001), and others report TO only at relatively high concentrations exceeding those commonly expected to occur in the environment (100 ng/L or more; Hirakawa et al., 2012). Furthermore, TO is sometimes observed among laboratory control and reference groups (Grim et al., 2007), substantiating hypotheses involving non-chemical factors. Even in circumstances in which a high degree of certainty can be placed on environmentally relevant causative factors, the taxonomic distance of model fish species like Japanese medaka and wild species of interest such as LMB obscures the environmental relevance of such findings. Considering the large amount of taxonomic variability exhibited by fish with TO, one would reasonably suspect that substantial differences exist among wild fish taxa in terms of sensitivity, mechanisms of induction, and potential consequences.

Of major concern is evidence for adverse reproductive effects associated with TO, which could have the potential to elicit population-level effects. Studies of intersex in common roach (*Rutilus rutilus*) have shown reduced sperm production and motility, as well as reduced fertilization success compared to normal males from the same populations, with severity of

intersex correlated with a decrease in viable offspring (Jobling et al., 2002). Harris et al. (2011) reported a 76% reduction in reproductive success among intersex male common roach compared to normal males. Fuzzen et al. (2015) reported reduced fertilization success in wild rainbow darters (*Etheostoma caeruleum*) with severe intersex. Relevant information is limited or unknown for many wild species in which intersex is common, but some studies suggest that TO may have adverse population-level consequences. For example, TO was observed in a fathead minnow (*Pimephales promelas*) population that experienced reproductive failure following long-term estrogen exposure (Kidd et al., 2007). In this case, TO is directly confounded with chemical exposure, and whether TO contributed to the resulting population crash or was perhaps only a benign side-effect of estrogen exposure in uncertain. The reproductive physiology associated with intersex is too poorly understood to reliably infer population-level effects, but the evidence merits further investigation. More information regarding physiological effects associated with intersex is needed to assess ecological risk of TO and to understand its role in adverse outcomes.

An important aspect of any potentially harmful biological condition or disease state relates to the mechanisms associated with its incidence. Identifying biochemical changes within the affected tissue may yield information relevant to molecular initiating events, as well as associated adverse physiological states. Analytical techniques applied in the field of metabolomics have provided important information about physiological conditions associated with diseased states, and are commonly used to study complex diseases such as cancers. Measuring levels of endogenous metabolites, collectively referred to as the metabolome, can yield important information regarding pathogenesis and related physiological effects of a condition. In a toxicological setting, metabolomics is used to uncover mechanisms of toxicity to better understand how effects of chemical exposure are produced on a biochemical level.

Investigation of the molecular physiology within an intersex gonad could yield important information relevant to the induction of testicular oocytes and provide insight into biochemical mechanisms of intersex induction resulting from chemical exposure.

Whether TO is considered a pathological condition, a direct effect of chemical exposure, or a natural phenomenon, metabolomics offers a unique opportunity to better understand the physiological state of wild intersex fish on a molecular level. To date, most research investigating intersex on a sub-cellular level has been limited to gene expression investigations of laboratory-reared species such as the fathead minnow (*Pimephales* promelas; Feswick et al., 2016). Gene expression assays conducted on wild intersex fish have also provided valuable information about differential gene expression in intersex fish (Bahamonde et al., 2015). However, these techniques are limited to select wild fish species for which relevant RNA primers exist, and the relevance of results to unrelated species of interest is unknown. Metabolomics offers a considerable advantage for studying intersex in that techniques do not require a sequenced genome and thus could be used to develop methods capable of interspecies comparisons. In the case of intersex, preserving most of the testicular tissue for histological confirmation of the condition is necessary. Using metabolomics, valuable information could be gained by analyzing metabolite profiles of very small (~50 mg) gonadal tissue samples, while still providing for adequate tissue for gonad histology. Biochemical activity within the intersex testis may be indicative of physiological and reproductive endpoints critical to assessing ecological risk. More information regarding the relationship of TO with potential adverse effects is needed to anchor the condition into structured ecotoxicological frameworks such as ecological risk assessments and adverse outcome pathways (AOPs).

In the present study, we evaluated the gonad metabolite profiles of wild intersex LMB to reveal information relevant to molecular initiating events of TO and associated adverse physiological effects within the gonad. In addition, blood plasma biochemical profiling was employed to better understand organismal responses associated with TO and to evaluate its utility for classification of individuals with different levels of TO. Methods used in this study could be used to investigate intersex and other gonadal abnormalities in various species and to compare incidences of intersex occurring under different circumstances to better explain variability observed in the field and in laboratory studies.

Methods

Fish Collection

Adult LMB were collected by boat electrofishing from a single creek-fed impoundment (~20 ac.) in Wilkes Co., Georgia, USA during March 2015. This site was selected based on a previous report of high prevalence (80%) of TO among male LMB (Kellock et al., 2014). Fish targeted for the current experiment ranged from 200 to 375 mm total length and fish outside this range were released immediately. Although intersex male fish were the primary subject of this study, females were also collected for reference. Upon collection, a numbered zip-tie was placed around the mandible of each fish. Fish (no more than 5 at a time) were held alive in an aerated on-boat holding tank for ≤ 15 min prior to processing in the order in which they were collected. Each fish was euthanized with an overdose of neutral-buffered tricaine methanesulfonate solution (MS-222, 200 mg/L). Body weight (nearest 0.1g) and total length (TL, nearest mm) were recorded for each fish, and relative weight (Wr) was calculated according to the Blackwell et al., (2000) method for LMB. Water quality variables (pH, DO, and temperature) measured

during sampling (Hach HQ40D, USA) were 7.11, 9.97 mg/L, and 15.2 °C, respectively. All fish were handled in accordance with approved University of Georgia Institutional Animal Care and Use protocols (AUP # A2016 06-028-A1).

Tissue Collection

After recording morphometrics, blood and gonadal tissue samples from each fish were immediately dissected in the field. Blood was collected ventrally from the caudal vessels in heparinized syringes and expelled into heparinized 2 mL centrifuge tubes. Blood samples were kept on ice, transported to the University of Georgia Aquatic Toxicology Laboratory (Athens, Georgia, USA) for centrifugation (10 min. at 1500 xg, 4 °C), and two equal aliquots of plasma from each sample were transferred into separate tubes and frozen prior to biochemical profiling and vitellogenin analysis (vitellogenin analysis reported by MacLeod et al. [2017]). Gonads from each male fish were weighed (nearest 0.001g) to facilitate calculation of gonadosomatic index (GSI = [gonad weight (g) ÷ body weight (g)] ×100). Approximately 50 mg of gonadal tissue was excised from the left gonadal lobe of each fish for metabolomics. Gonad metabolomics samples were taken from a well-vascularized, medial region near the hilum, where TO might be expected to occur. Gonad metabolomics samples were placed in a chilled 2-mL cryogenic tube and immediately flash frozen in liquid nitrogen to prevent degradation of metabolites. Dissection tools and surfaces were rinsed thoroughly with methanol, then de-ionized water, and dried between each fish to prevent cross-contamination. The remaining testicular tissue from all male fish was preserved in 10% neutral-buffered formalin solution for histopathological processing and evaluation. In the laboratory, metabolomics samples were stored at -80 °C until extraction.

Gonad Histology

Testicular tissues preserved in formalin (≥96 h in 10% neutral-buffered formalin) were prepared for histology and evaluated for the presence of TO at the Aquatic Toxicology Laboratory at the University of Maryland (College Park, MD, USA) with the modified methods outlined by MacLeod et al. (2017). Briefly, preserved testes were dehydrated in alcohol, embedded in paraffin wax, sectioned at 6-mm, mounted on glass slides, and stained with hematoxylin and eosin (Presnell et al., 1997). For each male fish, three longitudinal step-sections were cut from the right lobe of the testis, and 5 transverse sections were cut from the left lobe, avoiding areas biopsied for use in another study. Slides were examined by light microscopy (Olympus BH2) for TO. Oocytes were enumerated for each slide, and the total number of TO detected per individual (TO Count) was calculated as the sum of oocytes detected in both testis lobes. For statistical comparisons, male fish were placed into one of two groups based on TO Count to determine differences between individuals with varying degrees of intersex: (1) individuals with TO Count ≤ 20 were identified as 'low TO,' and (2) individuals with TO Count > 20 were identified as 'high TO.'

Blood Plasma Biochemical Profiling

Blood plasma samples were thawed and kept on ice prior to biochemical profiling. Samples were evaluated on a Hitachi® 912 Blood Chemistry Analyzer (Roche Diagnostics, Indianapolis, IN, USA) at the Clinical Pathology Laboratory at the University of Georgia College of Veterinary Medicine for the following 13 bio-chemical variables: alanine aminotransferase (ALT) activity, aspartate aminotransferase (AST) activity, alkaline phosphatase (ALP) activity, bicarbonate, calcium, phosphorous, magnesium, total protein, sodium, potassium, chloride, amylase, and glucose.

Gonad Extraction/Derivatization

Metabolomics extractions and analyses were performed at the US EPA National Exposure Research Laboratory (NERL, Athens, GA, USA). Extraction procedures followed those detailed by (Viant, 2007). Briefly, frozen gonad samples were randomized prior to extraction and homogenized with a tissuelyzer in a bi-phase solvent mixture of methanol/water (polar phase) and chloroform (non-polar phase). Samples were then centrifuged to separate phases, and final volumes of 500 µL (polar phase) and 210 µL (nonpolar phase) were decanted separately into amber glass GC vials. The polar phase used in the current study was evaporated under vacuum, and dried extracts were stored at (-80 °C) until derivatization. After thawing, extracts were derivatized with 100 µL methoxyamine hydrochloride solution (20 mg/mL in pyridine) and placed in a 60 °C oven for 2.5 hr. Samples were vortexed for 1 min at 30 min intervals during incubation. After cooling, 100 µL BSTFA (*N*,*O*-bistrifluoroacetamide) with 10% TCMS (methyltrichlorosilane) was added to each sample. Samples were then placed in the oven again for 1.5 hr and vortexed at 30 min intervals. Derivatized samples were then cooled to room temperature and transferred to micro-target inserts for GC/MS analysis.

GC-MS Metabolomics

Gonad extracts were analyzed on an Agilent® 7890 gas chromatograph (Agilent Technologies, CA, USA) linked to an Agilent 5975c mass spectrometer, and all data were collected and then processed using Chemstation®. Separation of metabolite derivatives was achieved with an Rxi-5Sil MS (30 m, 0.25 µm thickness, and 0.25 mm ID; Restek, PA, USA). All injections (2 µL) were made in the splitless mode. The injector temperature was set to 250 °C, the transfer line temperature was held constant at 280 °C, the source temperature was set to 200 °C, and the trap and detector were set for 350 µA and 300 V, respectively. The carrier gas

was helium and maintained at a constant pressure of 65.20 kPa. The initial oven temperature (60 °C) was held for 2 min and then increased at 6 °C/min to 280 °C with a hold time of 3 min. Instrument blanks were run at the beginning and intermittently throughout the sequence to verify no instrument carry over, and 13 extraction method blanks were randomly included to verify no cross contamination. Mass spectra were acquired over a mass range of 25 to 800 *m*/*z*. Chromatograms were exported as netcdf files and imported into MetAlign (v. 041012) for data preprocessing and alignment. Distributor recommended parameters for fast scan analysis was used (Lommen, 2009). Following alignment, Excel was used to filter and truncate the data as described in Niu et al. (2014).

Statistics (Morphometrics)

Differences in mean morphometrics (total length, body weight, Wr, and GSI) were assessed using non-parametric procedures due to unequal sample sizes among sexual phenotype groups. Morphometric statistical analyses were conducted in R (http://www.r-project.org/, v. 3.4.2), and significant difference was assessed at $\alpha=0.05$ for all tests. Differences in total length, weight and Wr among all three groups (female, low TO, and high TO) were assessed using a Kruskal-Wallis test. Mann-Whitney U tests were performed to evaluate differences in mean total length, body weight, Wr, and GSI for all pair-wise comparisons (i.e., female vs. low TO, female vs. high TO, and low TO vs. high TO).

Statistics (Blood plasma biochemistry)

Statistical analyses of blood plasma biochemistry data were performed in R. To evaluate mean differences among all three sexual phenotypes, a Kruskal-Wallis test was conducted for each blood plasma chemistry variable measured, and false-discovery rate (FDR) adjustments were applied to *p*-values (FDR-adj. *p*-value) to reduce the possibility of type I error.

Additionally, receiver operating characteristic (ROC) curves were generated using the Biomarker Analysis module in Metaboanalyst 4.0 (Chong et al., 2018) to identify plasma chemistry variables associated with higher TO Count and to evaluate diagnostic accuracy for classifying male fish into high TO and low TO groups. A generalized logarithm transformation was performed prior to analysis, and pairwise ratios of blood chemistry variables were computed for inclusion in ROC analysis. A t-test was used to compare mean values for each variable and variable ratio between high and low TO groups. Significance was assessed at $\alpha = 0.05$, and area under the ROC curve (AUROC) was used to assess the relative usefulness of variables for discrimination of high and low TO groups, with an AUC value of 1.0 representing a test with perfect diagnostic accuracy (i.e. no false positives or false negatives).

Statistics (Metabolomics)

Gonad metabolomics data were analyzed statistically with Metaboanalyst 4.0. Prior to analysis, analyte relative abundance values were normalized by sum for each observation, mean-centered, and pareto scaled. Partial least squares discriminant analysis (PLS-DA) was used to assess overall differences among groups by evaluating spatial separation in a scores plot, and Q2 values were used to evaluate the utility of metabolite profiles for prediction of sexual phenotype. The top 25 variable importance in projection (VIP) scores were identified for relevant components and the relative directional response of gonad phenotype groups was noted for each analyte. A series of t-tests ($\alpha = 0.05$) and fold-change analyses (fold-change threshold = 2.0) were conducted to evaluate differences between metabolite profiles of low TO and high TO groups. Metabolite annotations were performed for analytes of interest by comparing mass spectra to the NIST Spectral Library (v. 11) and a match quality threshold of ≥ 0.65.

Results

Fish Collection and Gonad Histology

Thirty-eight adult LMB (16 males; 22 females) were collected during the sampling. Neither total length ($p = 0.52$), body weight ($p = 0.47$), nor Wr ($p = 0.11$) varied by sex. Histological evaluation of preserved testicular tissues revealed an intersex prevalence of 95.5% among males, with TO detected in all males except for one individual. TO Count ranged from 0 to 276 per individual, with a total mean (±SD) of 37.7 ± 59.9 oocytes per individual. For individuals with TO Count ≤ 20 ('low TO' group, n=13, mean TO Count ± SD = 10.0 ± 7.1) total length ranged from 261 to 325 mm, Wr ranged from 0.764 to 0.960, and GSI ranged from 0.177 to 0.606. For individuals with TO Count > 20 ('high TO' group, n=9, mean TO Count ± SD = 77.8 ± 79.3) total length ranged from 278 to 326 mm, Wr ranged from 0.760 to 0.957, and GSI ranged from 0.370 to 0.786. Mean total length ($p = 0.76$), body weight ($p = 1.0$), Wr ($p = 0.09$), and GSI ($p = 0.13$) were not significantly different between low TO and high TO groups.

Blood Plasma Biochemical Profiles

Blood chemistry profiles were variabile among individuals, but significant differences among sexual phenotypes were few (Table 5.1). Mean plasma calcium concentration, significantly higher in females (FDR-adj $p < 0.001$), was the only blood chemistry analyte differing significantly among groups (Table 5.1). Plasma magnesium concentration was also generally higher in females but was marginally insignificant (FDR-adj $p = 0.051$; Table 5.1). Evaluation of ROC curves identified the most useful variables for classification of high TO and low TO males. Although the ratio of plasma ALP/AST was the only significant difference observed ($p = 0.041$) and also had the highest AUROC (0.778, Fig. 5.1), the optimal cutoff for

the ratio of ALP/phosphorus discriminated equally well (AUROC = 0.778), but differences between groups were marginally insignificant (p = 0.056, not shown).

Metabolomics

Following processing and alignment, a total of 3597 spectral features were identified between retention times 6.5 and 35.0 min (Example chromatogram in Fig. 5.2). PLS-DA scores were used to aid in visualization of overall differences and in gonad metabolite profiles from females, low TO, and high TO male groups (Fig. 5.3). Results revealed clear separation of females from males, mainly along the first component. As expected, differences between high and low TO groups were relatively subtle. Separation between high and low TO groups in this model was poor with relatively similar gonad metabolite profiles between male groups. Interestingly, profiles of high TO males on average trended toward females along the first component. The top 25 variable importance in projection (VIP) scores for Component 1 indicated the relative importance of spectral features of interest for separation along the first component, as well as the directional response (elevated [↑], medial [•], or reduced [↓]) of each sexual phenotype for a given spectral feature (Table 5.2). Putative annotations included organic alcohols, amino acids, carbohydrates, carboxylic acids, and phosphate. Glycerol (VIP = 4.45) responses were elevated in females, medial in high TO males, and reduced in low TO males, as were amino acid analogs dimethyl glycine (VIP = 3.94) and aminobutyric acid (VIP = 4.33). Glutamine (VIP = 4.03) responses were elevated in high TO males, medial in low TO males, and reduced in females. However, most amino acids and analogs, including those with the highest VIP scores were reduced in females, medial in high TO males, and elevated in low TO males. These included pyroglutamic acid (VIP = 8.58), glycine (VIP = 5.12), alanine (VIP = 4.49), aminobutyric acid (VIP = 4.33), glutamine (VIP = 4.03), and dimethyl glycine (VIP = 3.94). The

carbohydrates methyl glucopyranoside (VIP = 5.27) and an undetermined disaccharide (i.e., disaccharide 1; VIP = 4.79) were elevated in females, medial in low TO males, and reduced in high TO males. Responses of carboxylic acids were mixed, with succinic acid (VIP = 4.00) reduced in females and elevated in high TO males, and responses of two different spectral features of lactic acid (VIP = 3.82 – 5.22) mixed among groups. Of note, five spectral features putatively annotated as phosphate (VIP scores ranging from 4.02 to 18.22) held great importance for separation along Component 1, but directional responses varied among the five features. However, levels of phosphate, including the feature most important for separation along Component 1 (VIP = 18.22), were generally elevated in low TO males, medial in females, and reduced in high TO males. In addition, 7 spectral features for which annotations could not be assigned were also important for separation along PLS-DA Component 1.

T-tests and accompanying fold change of testicular analytes indicated 45 spectral features with mean values varying significantly ($p < 0.05$ and FC > 2) between low TO and high TO males (Table 5.3). Putative annotations represented similar chemical classes to those important for PLS-DA separation of all groups, and four putative metabolites (glutamine, phosphate, lactic acid, and an undetermined nucleoside phosphate [i.e., "AXP"]) were found in common between both analyses (Table 5.3). Polyols (myoinositol, inositol phosphate, and myoinositol phosphate) were elevated in high TO males compared to low TO males (Table 5.3). Amino acids and analogs (glutamine, creatinine, amino isobutyric acid, valine, aspartic acid, glutamic acid, and pyroglutamic acid) were mostly elevated in high TO males, except for glutamine and creatinine which had the opposite trend (Table 5.3). Carbohydrates (undetermined monosaccharide phosphates 1, 2, 3, and 4, methyl-N-acetyl-glucosamide, and ribose 5-phosphate), carboxylic and carbamic acids (malonic acid, carbamic acid, and lactic acid), and hydroxy acids (malic acid)

were generally elevated in high TO males, with the exception of monosaccharide phosphate 1, which was reduced in high TO males (Table 5.3). Nucleosides and derivatives were also generally elevated in high TO males, with exception of one undetermined nucleoside phosphate (AXP 4; Table 5.3). Phosphate and phosphate esters (O-phosphoethanolamine) exhibited the some of the highest fold-change values of any chemical class. Members of this class generally were elevated in high TO males, with considerable exception of three phosphate features that were greatly reduced (up to 2000-fold) in high TO males (Table 5.3). In addition, two features without reliable annotations were elevated in high TO males.

Discussion

In this study, we detected and putatively annotated features of interest in intersex gonad metabolite profiles and evaluated differences in blood plasma biochemical profiles associated with increased number of TO in largemouth bass. To our knowledge, this is the first study to demonstrate the utility of metabolomics to identify biochemical differences in gonadal tissues of wild intersex fish. We putatively annotated several testicular analytes that are indicative of altered biochemical pathways in intersex testes and identified plasma biochemicals that indicate the physiological state associated with intersex fish. These results provide novel insight to elucidate cellular pathways underlying the intersex condition in male LMB and to identify candidate analytes for biomarker discovery.

Although differences in blood plasma biochemistry data between high TO and low TO groups of male fish were not significant (FDR-adj $p > 0.05$), results of ROC analyses suggest that ratios of plasma chemistry variables may be useful for discrimination between high and low TO males. The ratio of ALP/AST yielded a mean sensitivity (true positive rate) of 0.8 and 1-

specificity (false positive rate) of 0.9, for an AUROC = 0.778 (Fig. 5.1A), indicating moderate accuracy in discriminating between high and low TO individuals (an AUROC of 1.0 with an ROC curve passing through the top left corner of the graph indicates 100% accuracy). This finding suggests that an elevated presence of TO in male LMB is associated with a decrease in plasma ALP and a corresponding increase in plasma AST activity. AST can be indicative of increased amino acid catabolism and energy expenditure in fish exposed to higher temperatures (Savoie et al., 2008) and is an established marker of hepatic ischemia-reperfusion injury, a form of liver damage related to hypoxia (Camargo et al., 1997). In rat cells exposed to hypoxic conditions, ALP showed a significant decrease in activity (Utting et al., 2006). In addition, Atlantic cod (*Gadus morhua*) ALP activity reportedly decreases with increasing temperature (Ásgeirsson et al., 1995). These results suggest that the ratio of plasma ALP/AST in intersex LMB may be indicative of physiological adversities and could serve as a candidate for biomarker discovery. The discovery of blood plasma biomarkers for intersex would be a significant finding, considering current methods for determination of intersex are lethal, but small amounts of blood could be collected non-lethally. Repeating this work with larger sample sizes of LMB in other populations would be useful to validate these findings, and analysis of nonpolar metabolite profiles may yield additional information. In addition, interspecific comparisons may shed light on taxonomic differences in mechanisms of intersex induction, susceptibility, and related effects.

Metabolomics analysis revealed features of gonad metabolite profiles associated with sexual phenotype and the intersex condition. Separation of sexual phenotype groups in the PLS-DA model was achieved primarily between females from male groups (Fig. 5.3). However, on average high TO males appeared to trend closer to females than did low TO males, suggesting that subtle similarities in gonad metabolomes of females and high TO males may be indicative of

increased presence of oocytes within the gonads. To identify these commonalities associated with oocytes, analyte directional response patterns (Table 5.2) increasing or decreasing in an order consistent with the level of oocytes in sexual phenotype groups (i.e. a medial [•] response of high TO individuals) serves to highlight putative metabolites of interest. Glycerol, which was elevated in females and reduced in low TO males is indicative of lipid metabolism and has been shown to be released by adipocytes following exposure to estrogen (Palin et al., 2003). Of note, the putative metabolites glycine, alanine, and pyroglutamic acid were lowest in females and highest in low TO males. These are important components of the fish egg proteins vitellogenin (Reading et al., 2009) and vitelline (Darie et al., 2004). A negative relationship between the relative abundance of these analytes with the number of oocytes in the gonads may reflect increased utilization of free amino acids for egg protein synthesis. Previous studies have reported increased levels of alanine in fish and fish cells exposed to estrogenic EDCs (Davis et al., 2016; Ekman et al., 2008; Teng et al., 2013). Hence, the negative relationship between the level of TO and levels of testicular alanine observed in the present study suggest that intersex is related to pathways other than those activated by the estrogen receptor.

Pairwise analyses of low TO and high TO males suggest that features of the gonad metabolome associated with increased presence of oocytes in the testes (Table 5.3) may reflect differences in energy metabolism, protein synthesis, and phospholipid production between groups. Levels of amino acids were generally elevated in the gonads of high TO males (with the exception of glutamine and creatinine, which were reduced), which likely reflects differences in protein and peptide biosynthesis. Glutamine, glutamic acid, aspartic acid, and pyroglutamic acid were all significantly different between high TO and low TO groups. These amino acids are primary substituents of gonadins, a class of peptides with demonstrated ability to alter steroid

hormone levels in male rats (Ruiz-Alcaraz and del Rio-Garcia, 2005). Altered levels of these amino acids in intersex LMB could be related to differences in gonadin metabolism. Generally, increased levels of phosphorylated carbohydrates in testes of high TO males may indicate differences in glycogenolysis or pentose phosphate pathways. Levels of ribose-5 phosphate, elevated in testes of high TO males, is a product of the pentose phosphate pathway (PPP). PPP plays an important role for oocyte development in other species, including protection of spermatogenic tissue from oxidative stress (Williams and Ford, 2004). In addition, PPP also plays an important role in lipogenesis for oocyte development. For example, female mice exposed to an androgen experienced reduced oocyte lipid content through inhibition of PPP (Jimenez et al., 2013). Thus, activation of PPP in intersex fish could be associated with oxidative stress and/or the production of lipids for oocyte development. Spectral features representing phosphate were not all in agreement, so false annotation of some peaks might have occurred due to errors in alignment and/or unresolved analytes. However, two features putatively annotated as phosphate were highly reduced in high TO males and showed the largest magnitude of change (131.7- and 2072.3-fold) of any feature measured. Phosphorylated sugars and alcohols, nucleoside phosphates, and O-phosphoethanolamine were elevated in high TO males, which may suggest increased incorporation of phosphates for energy storage and synthesis or modification of more complex biomolecules such as nucleic acids and lipids.

Overall, results of this study suggest that higher levels of TO in LMB seem to be associated with physiological stress. Chemical contaminants were not measured in this study, so exposure to unknown chemicals may have played a role in the occurrence of intersex. However, this study site is not known to have a history of chemical contamination, and toxicological factors are not suspected as a cause of intersex in this case. In small, un-aerated impoundments

of the Southeastern United States such as the one sampled in the current study, elevated temperatures and hypoxic conditions are fairly common during warmer periods, which can contribute to physiological stress (Esch and Hazen, 1980), particularly when combined with stresses associated with spawning. In a survey of LMB from impoundments of Georgia (USA), including the one sampled in the present study, Bringolf et al. (2015) found intersex to be closely associated with decreased impoundment surface area, suggesting that higher temperatures and/or lower levels of dissolved oxygen typical of smaller impoundments may play a role. Glycogenolysis and glycolysis can be induced by a variety of physiological stressors in fish, including thermal and hypoxic stress (Mukundan et al., 1986). For example, environmental hypoxia induces glycogenolysis (Wright et al., 1989), as well as glycolysis pathways, transgenerational testicular abnormalities, and epigenetic effects in fish (Wang et al., 2016). Therefore, similar mechanisms may be involved with the occurrence of intersex in fish. Signs of elevated glycogenolytic and glycolytic activity associated with intersex in the present study, such as elevated levels of testicular sugar-phosphates, lactic acid, and other carboxylic acids, in addition to reduced blood plasma ALP/AST ratio may indicate the involvement of hypoxic and/or thermal stress. Because all fish were collected from the same impoundment, differences observed within the sampled population could be attributed to individual-level variability in sensitivity to thermal and/or hypoxic stress or perhaps variation in habitat selection within different thermal strata of the impoundment.

The potential involvement of temperature (and/or the associated relationship with dissolved oxygen) in the induction of TO would not be surprising, considering evidence that temperature may alter sex steroid production in the gonads (Kime and Hyder, 1983; Kime and Manning, 1986; Manning and Kime, 1985) as well as sex determination and differentiation

(Conover and Kynard, 1981; Nomura et al., 1998; Römer and Beisenherz, 1996; Strussmann et al., 1996; Struussmann et al., 1996) in various species. LMB are extremely adaptable to different environments and exhibit a relatively wide range of thermal tolerance (Mulhollem et al., 2015). However, variability is likely to exist among individuals and populations, which may in part be related to intra- and inter-population variability in intersex reported among LMB. Furthermore, interspecies differences in thermal tolerance may help to explain relatively high levels of intersex reported in closely related species (e.g. smallmouth bass, *M. dolomieu*). Centrarchids, which predominate reports of intersex in North America, nest in shallow areas with slow waterflow during in the spring/summer (Bain et al., 1988; Clark et al., 2008), predisposing them to higher temperatures, particularly within small, confined bodies of water. If higher temperatures are related to the induction of intersex, this nesting behavior may partially explain why TO is so common in centrarchids.

In this study, we identified blood plasma biochemistry variables related to fish health, as well as features of the gonad metabolome associated with increased presence of TO in male LMB. Based on these results, the occurrence of intersex seems to be associated with differences in energy expenditure, gonad lipid and protein metabolism, and thermal/hypoxic stress. This information may aid in explaining the role of intersex in AOPs by revealing adverse physiological states associated with TO in LMB. Only one population of a single species was examined in the present study, but similar relationships may exist in other incidences of intersex. Methods used in the present study could be applied to various species and populations of interest to investigate causative factors and relationships with measures of fish health. For example, we did not investigate EDC exposure in the current study, but valuable information could be learned by applying metabolomics analysis of gonadal tissues of fish with various exposure histories to

link biochemical indicators of apical effects with the occurrence of TO. Indications of thermal and hypoxic stress in individuals with elevated TO counts highlight the need to understand the relationships between intersex, elevated temperatures, hypoxia, and measures of fish health and reproduction. Whether temperature and/or hypoxia are directly or indirectly connected with the incidence intersex in the present work is unknown, but understanding the role of temperature in relation to intersex and other suspected factors could aid in explaining extraneous variability observed in the environment. If elevated water temperature is directly involved in the induction of intersex conditions in fish, it could exacerbate existing feminizing effects of EDC exposure, but could also support suspicions that intersex may occur independently of chemical exposure. Moreover, direct involvement of water temperature and/or hypoxia in the occurrence of intersex would hold implications related to rising global temperatures due to climate change. This work contributes to a growing body of evidence suggesting that global climate change may exacerbate effects of EDC exposure (Brown et al., 2015; Hooper et al., 2013; Keller et al., 2015), which could adversely affect the health of global fish populations.

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Table 5.1. Mean (± SD) blood plasma biochemical profiles of female, low TO (≤20 testicular oocytes), and high TO (>20 testicular oocytes) male largemouth bass collected from an impoundment in Georgia (USA). ALP = alkaline phosphatase activity; ALT = alanine aminotransferase activity; AST = aspartate aminotransferase.

	Electrolytes						
Group	Sodium (g/L)	Chloride (g/L)	Potassium (mg/L)	Bicarbonate (mg/L)	Calcium (mg/L)	Phosphorus (mg/L)	Magnesium (mg/L)
Female	3.74 ± 0.10	3.60 ± 0.16	86.5 ± 38.4	530.1 ± 96.4	165.7** ± 28.3	117.1 ± 17.6	35.3* ± 3.5
Male - High TO	3.72 ± 0.06	3.71 ± 0.18	83.0 ± 37.9	474.6 ± 117.3	114.1 ± 11.6	112.2 ± 13.7	30.9 ± 1.6
Male - Low TO	3.76 ± 0.08	3.68 ± 0.24	79.4 ± 32.6	450.6 ± 120.9	118.8 ± 9.8	105.6 ± 19.7	32.1 ± 4.1

	Macromolecules			Enzyme Activities		
Group	Glucose (g/L)	Cholesterol (g/L)	Total Protein (mg/L)	ALP (U/L)	ALT (U/L)	AST (U/L)
Female	1.28 ± 0.55	6.20 ± 2.04	34.06 ± 3.13	22.81 ± 9.25	8.25 ± 6.91	77.25 ± 51.42
Male - High TO	1.31 ± 0.28	6.11 ± 1.10	32.11 ± 3.06	19.67 ± 7.32	5.89 ± 5.60	83.44 ± 47.17
Male - Low TO	1.37 ± 0.39	5.90 ± 1.19	33.08 ± 3.59	26.92 ± 10.22	5.54 ± 4.75	72.62 ± 62.19

*marginally insigificant (FDR-adj p = 0.051)

** highly significant (FDR-adj p < 0.01)

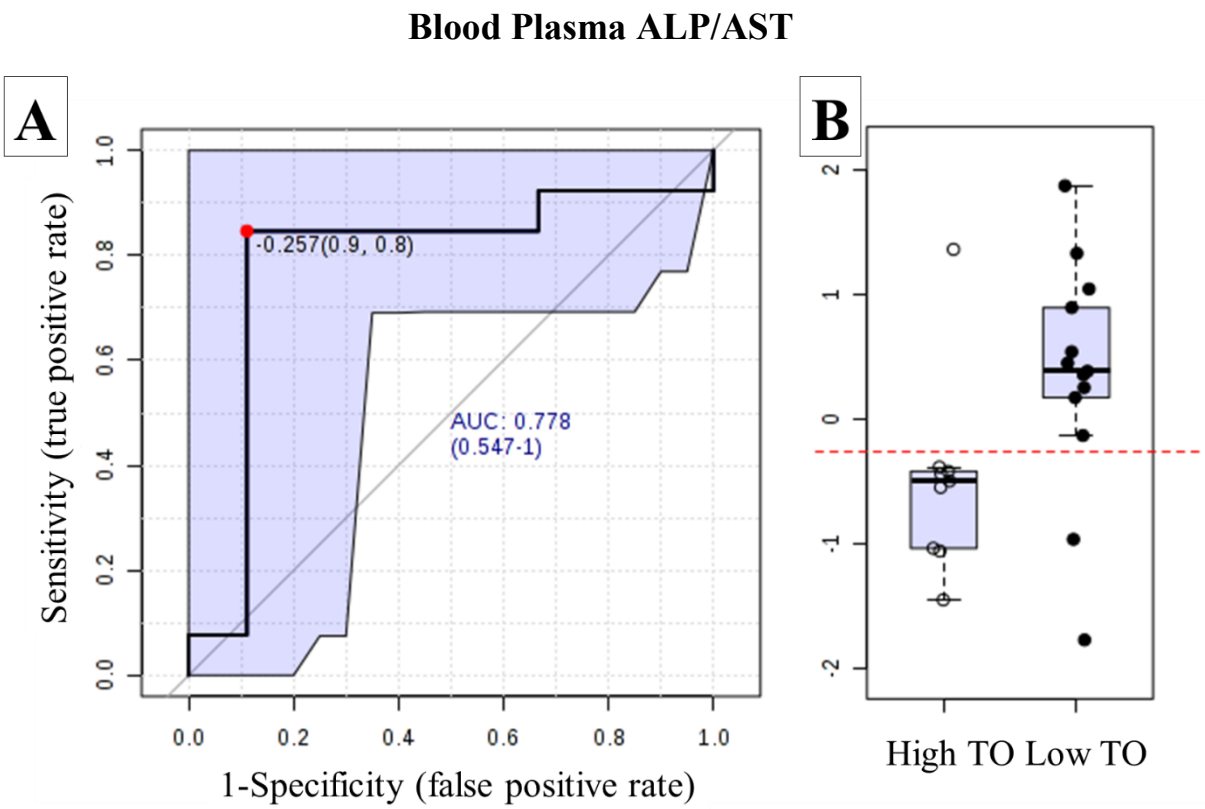
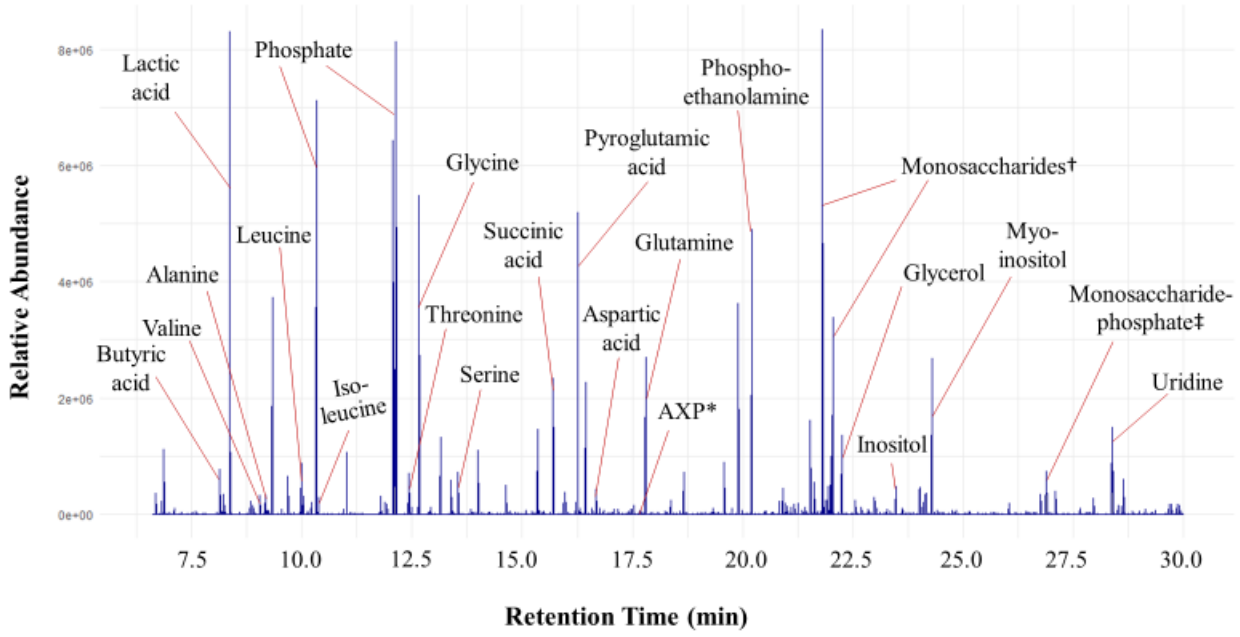


Figure 5.1. Receiver operating characteristic (ROC) curve and 95% confidence band for the ratio of alkaline phosphatase (ALP) and aspartate phosphatase (AST) activity (generalized log transformed) in blood plasma of male largemouth bass with high TO (> 20 testicular oocytes) and low TO (≤ 20 testicular oocytes; A). e.g., Curves passing through the upper left corner have 100% diagnostic accuracy. Boxplot representing differences between high and low TO groups in plasma ALP/AST ratio (B). The red dot on the ROC curve, as well as the red dotted line in the corresponding boxplot represents the optimal cutoff value.



*undetermined nucleoside phosphate
†undetermined monosaccharides
‡undetermined monosaccharide-phosphate

Figure 5.2. Example gonad metabolomics total ion chromatogram from largemouth bass collected from impoundment in Northeast Georgia (USA) aligned and processed, along with select analyte annotations.

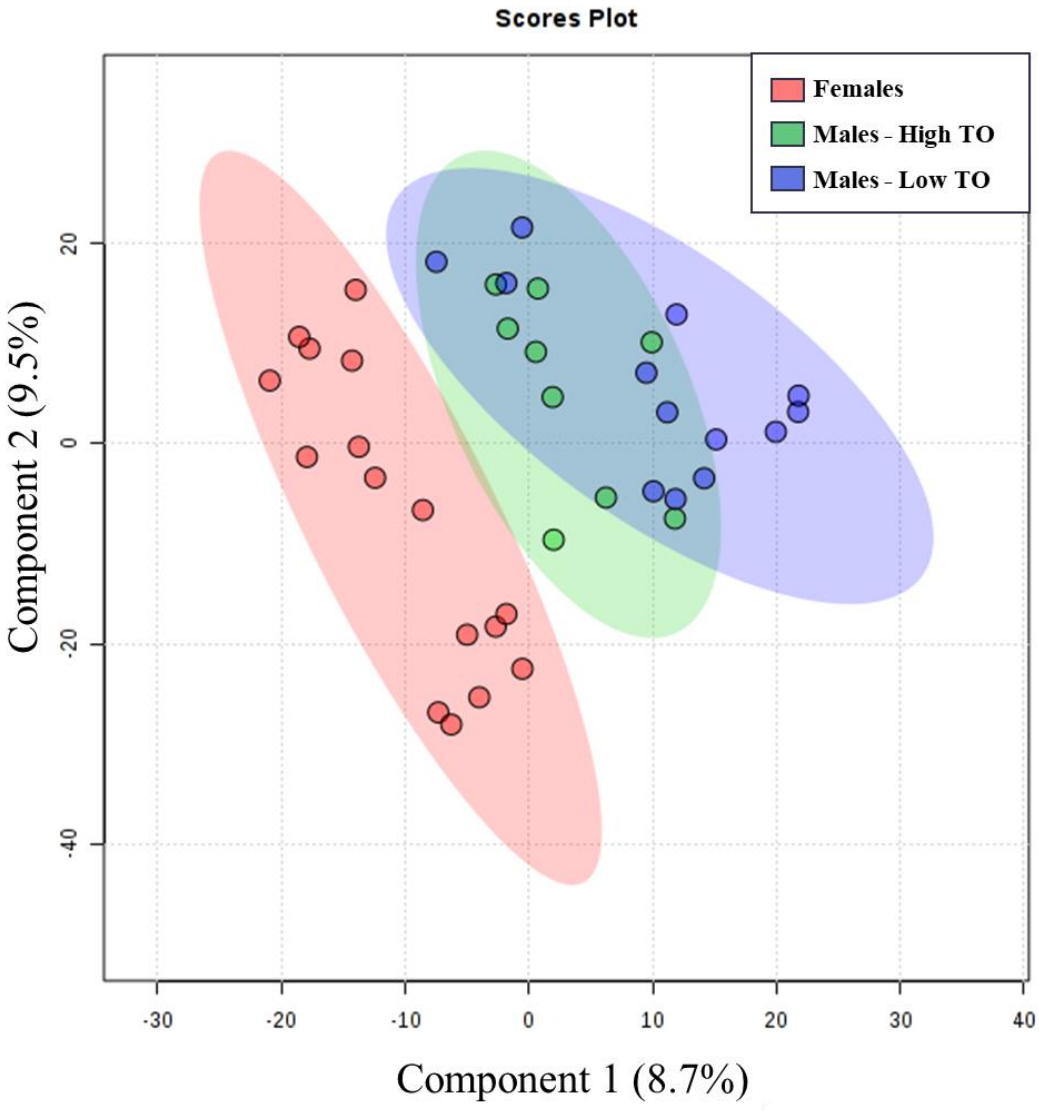


Figure 5.3. PLS-DA scores plot representing differences in individual gonad metabolite profiles of female (red) and male largemouth bass with varying levels of testicular oocytes ('Low TO' - males with low testicular oocyte count [≤ 20, blue]; 'High TO'- males with high testicular oocyte count [> 20, green]) from a Georgia (USA) impoundment. Each point represents an individual fish, and distance between points represents overall differences in gonad metabolome.

Table 5.2. Chemical class, putative annotation, and retention time for the top 25 PLS-DA variable importance in projection (VIP) scores for spectral features of Component 1, representing relative differences in gonad metabolite profiles of largemouth bass from a Georgia impoundment (Low TO, testicular oocyte count ≤ 20; High TO, testicular oocyte count >20). Columns on the right denote relatively elevated (↑), reduced (↓), and medial (•) responses among sexual phenotypes. Duplicate annotations are spectral bins representing the same putative metabolite.

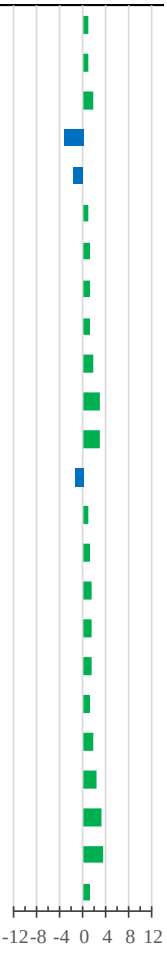
Chemical Class	Annotation	m/z	RT (min)	VIP Score	Female	Male - High TO	Male - Low TO
Alcohols	Glycerol	73	22.25	4.45	↑	•	↓
Amino acids and analogs	Pyroglutamic acid	156	16.26	8.58	↓	•	↑
	Glycine	174	12.60	5.12	↓	•	↑
	Alanine	116	9.11	4.49	↓	•	↑
	Aminobutyric acid	147	7.89	4.33	↑	•	↓
	Glutamine	246	17.80	4.03	↓	↑	•
	Dimethyl glycine	73	7.32	3.94	↑	•	↓
Carbohydrates and carbohydrate conjugates	Methyl glucopyranoside	241	10.17	5.27	↑	↓	•
	Disaccharide 1*	204	21.54	4.79	↑	↓	•
Carboxylic acids	Lactic acid	147	7.96	5.22	•	↓	↑
	Succinic acid	73	15.70	4.00	↓	↑	•
	Lactic acid	73	8.13	3.82	↓	↑	•
Nucleoside Phosphates	AXP 1**	255	14.21	4.33	↓	•	↑
Phosphates	Phosphate	299	11.99	18.22	•	↓	↑
	Phosphate	299	12.18	8.12	↑	↓	•
	Phosphate	73	12.01	6.22	•	↓	↑
	Phosphate	300	11.97	5.07	•	↓	↑
	Phosphate	301	12.19	4.02	•	↑	↓
Unknown	Unknown 1	77	6.88	6.51	↑	•	↓
	Unknown 2	73	6.82	5.63	↑	↓	•
	Unknown 3	221	7.35	4.53	↑	•	↓
	Unknown 4	73	6.68	4.46	↑	•	↓
	Unknown 5	73	6.80	3.96	↑	•	↓
	Unknown 6	258	9.77	3.86	↑	•	↓
	Unknown 7	221	7.46	3.82	↑	↓	•

*undetermined disaccharide

**undetermined nucleoside phosphate

Table 5.3. Chemical class, annotation, retention time, t-test p-value, and fold change (FC) values for gonad metabolite profile features differing significantly (p<0.05, FC>2.0) between low TO (≤20) and high TO (>20) male largemouth bass from a Georgia (USA) impoundment. Features are ordered by lowest p-value within each chemical class. On the right, log2 Fold change values are displayed for comparison (green, higher in High TO group; blue, higher in Low TO group). Duplicate annotations are different spectral bins representing the same putative metabolite.

Chemical Class	Annotation	m/z	RT (min)	*p* -value	FC	log2(FC)
Alcohols and polyols	Myo-inositol	613	23.50	0.016	2.1	
	Inositol phosphate	217	23.11	0.028	2.1	
	Myo-inositol phosphate	147	28.19	0.024	3.3	
Amino acids and analogs	Glutamine	73	17.74	0.046	-9.5	
	Creatinine	56	16.73	0.035	-3.4	
	Amino isobutyric acid	89	15.11	0.033	2.1	
	Valine	77	11.00	0.044	2.3	
	Aspartic acid	156	16.64	0.034	2.3	
	Amino isobutyric acid	229	15.28	0.028	2.5	
	Pyroglutamic acid	160	16.35	0.040	3.6	
	Glutamic acid	75	17.68	0.012	7.6	
	Pyroglutamic acid	75	16.36	0.002	8.3	
Carbohydrates and carbohydrate conjugates	Monosaccharide phosphate 1*	305	19.37	0.033	-2.4	
	Methyl N-acetyl-glucosamide	125	15.59	0.026	2.1	
	Ribose 5-phosphate	73	24.68	0.009	2.4	
	Monosaccharide phosphate 2*	73	26.61	0.033	2.7	
	Monosaccharide phosphate 3*	447	23.07	0.044	3.1	
	Monosaccharide phosphate 4*	380	18.03	0.008	3.2	
Carboxylic and carbamic acids	Malonic acid	73	11.49	0.034	2.3	
	Malonic acid	147	11.51	0.045	3.9	
	Carbamic acid	170	7.79	0.035	4.9	
	Lactic acid	66	8.55	0.021	9.8	
	Lactic acid	73	8.59	0.015	11.2	
Hydroxy Acids	Malic acid	190	15.62	0.039	2.4	

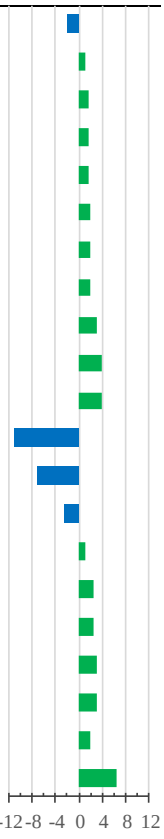


*undetermined monosaccharide phosphate

Continues

Table 3. (*continued*)

Chemical Class	Annotation	m/z	RT (min)	*p* -value	FC	log2(FC)
Nucleosides and derivitives	AXP 2**	191	23.14	0.008	-4.0	
	Uracil	242	13.30	0.048	2.2	
	Uracil	100	13.25	0.038	3.5	
	AXP 3**	243	14.74	0.028	3.5	
	AXP 4**	140	15.89	0.030	3.5	
	Uracil	256	13.23	0.047	3.6	
	Uracil	241	13.27	0.049	3.6	
	Uracil	99	13.24	0.046	3.6	
	AXP 5**	75	17.61	0.026	9.1	
	AXP 6**	147	17.27	0.033	14.0	
	AXP 3**	211	14.72	0.011	14.7	
Phosphates and phosphate esters	Phosphate	299	11.99	0.042	-2072.3	
	Phosphate	72	11.96	0.045	-131.7	
	Phosphate	73	12.01	0.031	-5.8	
	O-Phosphoethanolamine	228	20.64	0.037	2.1	
	O-Phosphoethanolamine	73	20.14	0.008	5.2	
	Phosphate	392	10.31	0.047	5.7	
	Phosphate	301	12.19	0.035	8.0	
	Phosphate	147	12.14	0.018	8.2	
Unknown	Unknown 8	175	7.16	0.035	3.7	
	Unknown 9	57	6.72	0.010	89.4	



**undetermined nucleoside phosphate

CHAPTER 6

SYNTHESIS AND CONCLUSIONS

Introduction

The field of ecotoxicology has not been around for very long compared to other areas within the broader disciplines of ecology, chemistry, and biology. In a matter of decades, a wealth of information and interest in ecotoxicological topics have contributed greatly to our understanding of chemical effects on environmental health, and enacted changes in human land use practices which have inarguably resulted positive changes toward global environmental health. For example, we now understand concepts of chemical bioaccumulation and biomagnification, which have considerably altered the way we view even minute chemical concentrations in the environment. Appreciation of sub-lethal effects, such as those imposed by endocrine disrupting chemical (EDCs) like environmental estrogens, has greatly enhanced our ability to assess ecological risks and hazards, but also given rise to countless questions critical to environmental sustainability, and the inevitable connections with human health. By using a "weight of evidence" approach, specific chemicals/mechanisms of action have been linked to population-level effects in fish and wildlife. The development of the adverse outcome pathway (AOP) framework represents the latest major paradigm shift in ecotoxicology, which emphasizes the need to connect findings from various levels of biological organization in order to assess ecological risk. Advancements in this area are typically achieved in small increments, and comprehensive knowledge of AOPs is not likely to be accomplished. New information regarding existing chemicals, chemical mixtures, interactions with other environmental factors, as well as

the continual development and usage of novel compounds, is a constant source uncertainty. But, it is clear that by gaining a better understanding of the complex relationships of chemical exposure, molecular events, and ecologically relevant effects in organisms, we are better able to enact changes that will benefit human civilization and the environment.

Since the dawn of the industrial age, the mantra, "solution to pollution is dilution," dominated paradigms related to the use of chemicals and their eventual release into the environment. The thought was essentially that if the concentrations of environmental contaminants did not directly result in mass mortality of fish and wildlife, they would not likely cause any harm. When Rachel Carson published the hugely influential work *Silent Spring* in 1962, it sparked an international debate on the use of chemical pesticides. Moreover, global perspectives began to change with respect to the potential for technological advancement to cause irreversible environmental harm. For many, the connectedness of human activity with environmental health came into light; the rate of change observed in the environment was put into perspective with the desire of humans to control it. For example, people began to wonder whether it was worth eradicating a few nuisance insects, if it also meant the permanent eradication of a host of non-target organisms, important for a variety of reasons. Environmentalism very quickly gained a new audience, and ultimately, a grass-roots movement in which the public demanded environmental protection was set in motion. Subsequent ecotoxicological research has unraveled long-standing theories regarding the sustainability of chemical usage and disposal. Today we face a new, yet familiar, set of challenges posed by human hubris, neglect, and lack of understanding. Whereas no amount of data can change the nature of humans to control and manipulate the environment, the overarching importance of ecotoxicology and related environmental fields is to gain an understanding of the environmental

consequences of our actions,such that it contributes to the well-being of both humans and the environment that we share with all other organisms. It is my greatest hope that through scientific work such as that presented in this document, such an understanding can be further clarified. sufficient to enlighten human perspectives so that the wonderous diversity of life that exists on Earth may flourish to its greatest extent for millennia to come.

Synthesis

In Chapter 2, metabolomics analysis revealed evidence that we found evidence that estrogen exposure in juvenile male medaka may lead to prolonged effects on the nonpolar liver metabolome, and changes observed in the liver metabolome could reflect organizational or activation-associated effects of estrogen exposure. Long-term metabolic effects are likely to influence hepatic physiology, as lipids and other nonpolar metabolites play crucial and diverse physiological roles in fish and other organisms. Alterations to the liver metabolome could indicate or mediate adverse effects in the liver and related organ systems that persist throughout the life-cycle of male fish. In Chapter 2, estrogen exposure was also associated with fibrosis in the testis. We hypothesized that intersex would be observed in exposed males, and although intersex was not observed upon termination of the study, it is possible that testicular oocytes resulting from estrogen exposure were sloughed or regressed by the time of termination of the experiment. Thus, fibrosis observed in the testes of exposed males may represent morphological remnants of intersex. Little is known about the regression of testicular oocytes, but it could contribute to changes in the liver metabolome via transport and catabolism of oocyte components. Also, genomic alterations and dysregulation of hormone signaling cascades could have prolonged influence on hepatic metabolism. In the Chapter 2, PIs and PIPs were

significantly higher in E2-exposed males, which have important regulatory functions in cells and often define specialized membrane domains used for membrane transport and protein-binding (Alberts et al., 2015). These results suggest that changes in nonpolar liver metabolites resulting from estrogen exposure may indicate or mediate long-term changes in physiology.

In Chapter 2, the nonpolar liver metabolome of adult male medaka exhibited prolonged effects of early life-stage estrogen exposure, in contrast with the polar liver metabolome, in which very few long-term effects were observed. Metabolite profiling may have utility for ecosystem monitoring, such as assessing recovery from EDC exposure following remediation efforts. In a study of caged adult male fathead minnows exposed to wastewater effluent containing EDCs, Davis et al., (2013) reported rapid recovery of the polar liver metabolome, which suggests that fish may quickly return to a normal physiological state following depuration. However, results of Chapter 2 suggest that effects on nonpolar metabolite profiles may persist for extended periods of time; whereas, long-term effects may not be reflected by the relatively transient polar metabolome. Therefore, monitoring of nonpolar metabolite profiles may offer considerable advantage for long-term assessment of ecosystem recovery. Nonpolar metabolites may be a source of sustained biomarkers of EDC exposure and could have diagnostic utility to complement physiological endpoints indicative of fish health. To better assess the utility of metabolomics for ecosystem monitoring, more information is needed regarding temporal aspects of metabolome recovery following exposure to EDCs. For example, there could be differences in metabolome recovery of longer-lived fish species with different life-histories. Also, the liver was chosen as the source of metabolite profiles in Chapter 2 due to knowledge of extensive hepatic metabolic processes, but similar effects may also persist in other tissues and biofluids. In addition, long-term metabolic effects may also result from exposure to other classes of EDCs.

Due to limitations of mass spectroscopy methods employed in Chapter 2, the specificity as well as degree of certainty pertaining to annotations assigned to features of interest is relatively low. Ideally, high-resolution MS methods capable of detecting accurate mass or tandem MS/MS methods should be used in order to reliably annotate structural details of metabolites, particularly with regard to medium to large molecules. Chapter 2 provides insight to inform future studies investigating long-term metabolomic effects of estrogen exposure in fish, but important information regarding physiological function could be elucidated by detailed structural information, including the identity of fatty acyl components and the amino acid sequence of peptides.

Overall, results of Chapter 2 suggest that nonpolar liver metabolite profiles may be useful for monitoring long-term effects of EDC exposure in fish. In contrast with polar metabolite profiles, which are typically favored for the diversity of metabolites and relative ease of annotation, lipids and other nonpolar metabolites may have greater utility for development of long-term biomarkers of EDC exposure. In addition, changes in the nonpolar metabolome could provide insight into biochemical mechanisms associated with long-term physiological endpoints relevant to adverse outcomes.

In consideration of the potential for long term effects of estrogen exposure in fish reported in Chapter 2, in Chapter 3, the Upper Coosa River (UCR) Basin was used as a model ecosystem to explore factors related to the feminization of wild fish, including estrogen exposure and a broad scope of other biotic and abiotic factors. Portions of the UCR have experienced substantial declines in fish bio-diversity over recent years, and existing evidence suggests that feminizing effects of estrogens observed in imperiled regions may be involved. Statistical models were generated to predict the combined influence of various factors, including sediment

estrogenicity, anthropogenic land use, water quality, and fish taxonomic and morphometric data on the occurrence of intersex (testicular oocytes, TO) among fishes of the UCR. To our knowledge, this is the first study to incorporate such diverse factors from multiple categories into a comprehensive and integrated approach to understand relationships with intersex in wild fish. The purpose of Chapter 3 was not to positively identify a particular factor that causes intersex in fish, but rather to explore the complex relationships among many factors that may be influential to the feminization of fishes in the Upper Coosa River (UCR) Basin, and to provide insight into causative factors of intersex fish within this region and other areas of concern.

In Chapter 3, and in previous studies, members of the family Centrarchidae, and particularly black bass species, had relatively high intersex prevalence and TO counts (number of testicular oocytes detected per individual) compared to other fish taxa. Although the primary taxonomic group targeted in Chapter 3 was the family Centrarchidae, and relatively few individuals from other families were sampled, the vast majority of intersex individuals in this study were centrarchids, and intersex prevalence among this group (25%) was far higher than any other family. The only family other than Centrarchidae with detected TO was Catostomidae, in which in 2 of 73 individuals (2.7%) had TO in the area examined. The prevalence of intersex among centrarchid species was highly variable. The three centrarchid species surveyed in which no TO was detected all had low sample sizes ($n \leq 4$ individuals per species), so it may be possible that all centrarchid species in this region exhibit some intersex. Of the four centrarchid species with an intersex prevalence of greater than 20% (coosa bass, *Micropterus coosae*, largemouth bass, *Micropterus salmoides*, redear sunfish, *Lepomis microlophus*, and spotted bass, *Micropterus punctulatus*), three belong to the genus *Micropterus* (black bass species). This genus is particularly well-documented for intersex incidence in the literature, and the relatively high

prevalence of TO observed among these taxa (47.3%) is consistent with results of previous surveys conducted in the United States. These results support previous suggestions that incidences of TO may be much more likely to occur among centrarchids, and certain centrarchid species including members of the genus *Micropterus*. A higher intersex prevalence may suggest an increased sensitivity to certain effects of EDC exposure, or other causative factors of intersex induction, but may also support suggestions of a natural background prevalence of intersex among certain fish taxa, including centrarchid species.

Variables influential to models explaining intersex suggest the involvement of biotic factors as well as abiotic factors previously suspected to alter endocrine function. Body weight was positively associated with intersex in highly plausible models and was the highest ranked variable of importance for both centrarchids and black bass, suggesting a higher frequency of intersex as fish grow larger over time. The higher probability of intersex predicted as body weight increases (Fig. 3.4A) may potentially be explained by higher body burdens of EDCs and/or longer histories of chemical exposure in larger fish. However, the nature of relationships between TO and fish size/age remains poorly understood. Age has been previously suggested as a potential factor in intersex occurrence in black basses (Hinck et al., 2009), but little is known regarding age distributions of intersex fish. Maximum surface water nitrate concentration was positively associated with intersex for both centrarchids and black bass, and was among the ten highest ranking variables in both cases. Surface water nitrate is often sourced from anthropogenic activity and usually co-occurs with suspected EDCs, but nitrate itself is also suspected as an endocrine disruptor (Guillette and Edwards, 2005; Edwards and Hamlin, 2018; Poulsen et al., 2018), so it may be possible that levels of nitrate influence the incidence of intersex in fish, as suggested by previous reports (Bringolf et al., 2015). Surface water

temperature held very high importance in top-ranking models for centrarchids and black bass, suggesting that exposure to higher environmental temperatures may positively influence incidence of intersex. Average marginal predicted probabilities for intersex among black bass suggest an increased probability of intersex with higher maximum surface water temperatures (Fig. 3.4B).

Intersex may play a role in documented fish population declines within the UCR and elsewhere, but Chapter 3 did not investigate intersex among imperiled species; therefore, direct inferences to populations of concern cannot be made. However, intersex observed among non-imperiled species may be used to speculate the relative likelihood of intersex incidence among coinhabiting fish populations. Incidences of intersex observed in the Conasauga River, where a number of sensitive species occur, were mostly limited to centrarchid species and were not considerably higher than those observed elsewhere in the UCR. Therefore, if similar trends exist among species of concern, intersex does not likely substantially contribute to regional population declines observed in the Conasauga. However, differences in intersex susceptibility may exist in species not surveyed, and the potential threat posed by intersex and other feminizing effects of estrogens in this region cannot be ruled out at this time. The prevalence of intersex among Coosa bass throughout much of the UCR was the highest of any species surveyed (overall prevalence = 64.4%), suggesting that this species may be relatively sensitive to influential factors, or perhaps that some "background" level of intersex exists in this species. As the name suggests, Coosa bass are native to the UCR, and occur throughout much of the region. Surveys of Coosa bass may serve well to identify regions of concern and for further investigations of intersex within the UCR.

The primary goal of Chapter 3 was to better understand relationships between intersex and variables of interest within the UCR, but it may be possible that similar relationships exist in comparable river systems. The UCR is fairly unique in certain aspects, such as its vast biodiversity and extensive use of particular agricultural practices (i.e., widespread application of poultry litter as fertilizer) in certain regions. However, many of the species investigated (e.g. largemouth bass) are found throughout much of North America, and others (e.g. Coosa bass) are closely related to species of particular interest for intersex research (i.e., smallmouth bass). Hence, similar relationships are likely to exist in comparable river systems, particularly those lacking major municipal and industrial effluents, and with extensive agricultural land use within the watershed.

In Chapter 3, some results were unexpected and appeared to contradict presumed hypotheses suggested by previous findings. For example, upstream sites were primarily included for reference, and levels of contaminants and intersex were generally expected to be lower than those detected at downstream sites, reflecting a gradient of anthropogenic land use. Interestingly, there was no clear evidence indicating higher levels of intersex or sediment contaminants at downstream sites. In fact, despite lower levels of anthropogenic land use, some of the highest levels of sediment estrogens and estrogenicity, as well as intersex, were found at upstream sites. These results may suggest the existence of unknown sources of contamination, such as septic system leachate or runoff containing herbicides used for forest management. Alternatively, intersex susceptibility may vary among populations inhabiting different reaches of the river. Also interesting was a negative relationship between intersex and variables hypothesized to positively influence intersex (i.e. sediment estrogens, estrogenicity, and agricultural land use) among top-ranked models. Although total watershed agricultural land use did not appear in candidate model

sets, localized agricultural land use (Ag5) had a negative effect on intersex in models, suggesting perhaps an indirect effect of local agriculture such as the use of riparian buffers.

Due to inherent error associated with histological detection of TO, at least some individuals may have had TO outside the area examined, thereby introducing a chance of false negative determination of intersex. Furthermore, although sampling locations were geographically distant, movement of fish among sites could have skewed results. Whereas centrarchid species are not generally thought to move large distances within riverine habitats (Gatz and Adams, 1994), other taxa (e.g. catostomids) regularly migrate within river systems, particularly during spring months when fish surveys for Chapter 3 were conducted.

Models produced in Chapter 3 indicate a significant association of several measured variables with intersex incidence in fishes of the UCR, and top-ranked models are generally predictive of the intersex condition (Fig. 3.3). However, much variability remains unexplained, and the nature of associations between intersex and predictor variables largely unknown. Directional relationships (positive or negative) with intersex were generally consistent among models for variables of high importance, but some variables (e.g. sediment E1 concentration and spring sediment estrogenicity) displayed ambiguity among models, with a positive effect in certain models and a negative effect in others. These contradictions contribute to uncertainty in interpreting relative variable importance and may suggest the existence of false positives or unknown interactions among explanatory variables of interest. Validation of suggested relationships among variables by additional sampling and inclusion of additional variables and interaction terms in models may aid interpretation of contradictory model results.

Models incorporating multiple sources of biotic and abiotic factors were the most informative for explaining intersex variability. Inclusion of easily discernible factors such as

body weight and surface water temperature were highly important for model plausibility. Despite considerable evidence suggesting the influence of morphometric and physical environmental factors on intersex in fish, such factors are rarely incorporated into comprehensive investigations of potential causes, often in favor of exclusively investigating the roles of xenobiotic exposure and/or anthropogenic land use. Chemical pollutants almost certainly play a role in many incidences of intersex, but extraneous variability observed among and within populations may be partially explained by the inclusion of a broad scope of biotic and abiotic factors. The ecological risk associated with intersex will not likely be realized without first understanding potentially complex interactions among multiple biotic and abiotic factors that likely contribute to its incidence. Results of Chapter 3 suggest that multiple biotic and abiotic factors from various categories are associated with intersex in freshwater fish. Fish weight and surface water temperature appear to be particularly important factors to explain the occurrence of intersex among sunfish and centrarchids. Natural resource management practices to increase shade and reduce stream temperature, such as the use of riparian buffers, may be important for the control of intersex in freshwater fish.

Though it is certainly less than ideal to sacrifice large numbers of wild fish, especially in imperiled regions, conducting lethal histological surveys such as the one outlined in Chapter 3 are currently the only reliable method to investigate effects on gonad morphology associated with feminization. These surveys are extremely valuable for determining the geographic distribution of feminized fish and identifying populations at risk, but ultimately, improved methods of detection are necessary to answer many pertinent questions related to intersex in fish. In Chapter 4, the utility of LC/MS-based metabolomics for the development of intersex biomarkers was explored. Liver was chosen for its rich metabolome, and blood plasma was

chosen because it is possible to collect blood non-lethally. Analysis revealed relationships between the number of oocytes in the testes (TO count) and features of the liver and blood plasma metabolite profiles. Metabolites identified in Chapter 4 may serve as candidates for biomarker development. If successful, such a development could be used to develop high-throughput, non-lethal screening procedures that would have a transformative effect on current methodology for studying intersex fish. If surveys like the one conducted in Chapter 3 could be accomplished non-lethally, large numbers of fish could be spared. In addition, non-lethal techniques to detect intersex could allow for sampling of imperiled species, which may ultimately face the greatest risk of feminization. Furthermore, non-lethal methods would allow for repeated sampling of individuals, which is not possible with established methods. Analysis of other non-lethal biological matrices such as skin mucus may also produce insightful information and further minimize invasiveness of sampling procedures. Urinary metabolites in particular may be informative, given that nitrogenous waste compounds, such as those associated with intersex in Chapter 4, are typically excreted in the urine.

In Chapter 4, I sought to minimize extraneous variability in order to isolate responses associated with TO. Therefore, metabolite profiles of individuals from individual populations of one species were investigated, encompassing a narrow size (and presumably age) distribution. Biomarkers of effect (with intersex being the 'effect') were targeted with this approach, and potential exposure to EDCs was not investigated. Although quantitative information on chemical exposure can be useful for investigating potential causes of TO induction, fish of similar age sampled from confined impoundments are likely to experience very similar histories of chemical exposure during their lifetime. Actively minimizing variability may be useful in the initial stages of biomarker development, but to develop a robust procedure that can be applied under different

circumstances, variability must be addressed and accounted for. Future research could benefit by examining the robustness of these relationships among chemical, spatial, temporal, taxonomic, and genetic factors, and investigating their utility in statistical models that could be used for predictive purposes. Advancement of these techniques could allow for the development of targeted analytical screening procedures that use non-lethal sampling methods to estimate the prevalence and severity of TO among wild fish populations.

Metabolomics analyses of intersex fish in Chapter 4 revealed insight into the potential biochemical mechanisms of intersex induction, a key event that is poorly understood, but is also necessary to determine the role of intersex in AOPs. Fish were collected during the pre-spawn period for metabolomics analysis in this work. Therefore, it is likely that oocytes were actively developing at the time of sampling, and biochemical changes related to intersex in these fish could reflect the mechanisms of action of intersex induction. Putative metabolites of interest included uremic toxins such as guanidinosuccinic acid and citrulline in blood plasma, which suggests differences in the metabolism and/or excretion of nitrogenous wastes in fish with higher TO counts. A considerable number of putative metabolites positively correlated with TO count are urea cycle constituents and are considered uremic toxins. Data on the effects of these compounds in fish physiology are limited or non-existent, but research in other species suggests that uremic toxins are directly and/or indirectly related to cellular pathways that regulate levels of steroid and peptide hormones, as well as normal function, maintenance, development, and differentiation of testicular tissue. For example, uremic (elevated levels of uremic toxins) human male patients with terminal renal failure have higher serum levels of estradiol and luteinizing hormone (LH), and lower levels of anti-mullerian hormone (AMH; Eckersten et al., 2015) Estradiol and LH play critical roles in gonadal development and maintenance, and estrogens

including estradiol and some EDCs are well documented for their feminizing effects on male fish. AMH is an important regulator of testicular function and contributes to the differential regulation of gonadotropins in rats (Garrel et al., 2016). Interestingly, AMH displays male-biased expression patterns in developing fish of several species (Baron and Guiguen, 2003; von Hofsten et al., 2005; Miura et al., 2002; Rodríguez-Marí et al., 2005; Yoshinaga et al., 2004), and is thus suspected for its involvement in testicular differentiation in fish (Schulz et al., 2007). This information suggests linkages between elevated levels of urea cycle constituents and the incidence of TO in LMB, possibly via modulation of sex-specific hormone levels.

Interestingly, particular uremic metabolites identified in Chapter 4 are suspected for involvement in nitric oxide (NO) biosynthesis pathways in vertebrates. NO is a potent signaling molecule that decreases androgen levels by inhibiting several steroidogenic enzymes such as steroidogenic acute regulatory protein (StAR), cytochrome P450-sidechain cleavage enzyme (SCC), and 3β-hydroxysteroid dehydrogenase (3βHSD), (Del Punta et al., 1996; Panesar and Chan, 2000) and can affect the development, motility, and viability of sperm (Del Punta et al., 1996; Kostić et al., 1998; Panesar and Chan, 2000; Ratnasooriya and Dharmasiri, 2001; Rosselli et al., 1995). Both guanidinosuccinic acid (GSA) and citrulline, urea cycle constituents positively correlated with TO count in the present work, have each been noted for their involvement in NO synthesis and signaling. GSA is considered a stable NO mimic which activates NO pathways and NO-mediated effects *in vivo* and *in vitro* (Noris and Remuzzi, 1999). Furthermore, the synthesis of GSA from argininesuccinate has been suggested to stimulate a positive feedback loop of NO synthesis, further potentiating activation of NO pathways in the presence of GSA (Aoyagi et al., 1999). Citrulline is also closely related to NO biosynthesis and NO-mediated pathways. Citrulline is formed as a product in the synthesis of NO from arginine, a reaction catalyzed by

nitric oxide synthase (NOS). Therefore, elevated levels of citrulline in fish with higher TO counts may be indicative of increased NOS activity and subsequently elevated NO concentrations. Citrulline is not only a product of NO synthesis, but can also act as a substrate for NO production, and evidence suggests that citrulline may even be superior to arginine in terms of induction of NO synthesis (El-Hattab et al., 2012). Modulation of NO pathways leading to altered steroidogenesis, could have significant effects on gonadal development in fish, particularly during early developmental stages in which bi-potential gametocytes or germ cells are present. Positive correlations between TO count and the levels of urea cycle constituents Chapter 4 suggest that TO may be associated with uremic toxin-mediated disruption of endogenous NO pathways, potentially resulting in modulation of steroid hormone levels in gonadal tissue. Elevated levels of urea cycle intermediates, such as GSA and citrulline suggest that urea levels may also be elevated. In humans, GSA is thought to be synthesized from arginine when enzymes that facilitate urea production are inhibited by excessive build-up of urea (Noris and Remuzzi, 1999).

Results of Chapter 4 suggest that intersex fish may experience a higher level of physiological stress. Metabolomics analyses of somatic tissues was a useful technique for understanding the physiological state of intersex fish. However, information relevant to molecular initiating events and adverse gonadal physiology may be limited in somatic tissues. Therefore, in Chapter 5 similar metabolomics methods were employed to study differences in biochemical pathways in the testicular tissues of intersex fish.

In Chapter 5, In this study, we detected and putatively annotated features of interest in intersex gonad metabolite profiles and evaluated differences in blood plasma biochemical profiles associated with increased number of TO in largemouth bass. To our knowledge, this is

the first study to demonstrate the utility of metabolomics to identify biochemical differences in gonadal tissues of wild intersex fish. We putatively annotated several testicular analytes that are indicative of altered biochemical pathways in intersex testes and identified plasma biochemicals that indicate the physiological state associated with intersex fish. These results provide novel insight to elucidate cellular pathways underlying the intersex condition in male LMB and to identify candidate analytes for biomarker discovery.

Although differences in blood plasma biochemistry data between high TO and low TO groups of male fish were not significant (FDR-adj $p > 0.05$), results of ROC analyses suggest that ratios of plasma chemistry variables may be useful for discrimination between high and low TO males. The ratio of ALP/AST yielded a mean sensitivity (true positive rate) of 0.8 and 1-specificity (false positive rate) of 0.9, for an AUROC = 0.778 (Fig. 5.1A), indicating moderate accuracy in discriminating between high and low TO individuals (an AUROC of 1.0 with an ROC curve passing through the top left corner of the graph indicates 100% accuracy). This finding suggests that an elevated presence of TO in male LMB is associated with a decrease in plasma ALP and a corresponding increase in plasma AST activity. AST can be indicative of increased amino acid catabolism and energy expenditure in fish exposed to higher temperatures (Savoie et al., 2008) and is an established marker of hepatic ischemia-reperfusion injury, a form of liver damage related to hypoxia (Camargo et al., 1997). In rat cells exposed to hypoxic conditions, ALP showed a significant decrease in activity (Utting et al., 2006). In addition, Atlantic cod (*Gadus morhua*) ALP activity reportedly decreases with increasing temperature (Ásgeirsson et al., 1995). These results suggest that the ratio of plasma ALP/AST in intersex LMB may be indicative of physiological adversities and could serve as a candidate for biomarker discovery. The discovery of blood plasma biomarkers for intersex would be a significant finding,

considering current methods for determination of intersex are lethal, but small amounts of blood could be collected non-lethally. Repeating this work with larger sample sizes of LMB in other populations would be useful to validate these findings, and analysis of nonpolar metabolite profiles may yield additional information. In addition, interspecific comparisons may shed light on taxonomic differences in mechanisms of intersex induction, susceptibility, and related effects.

Metabolomics analysis revealed features of gonad metabolite profiles associated with sexual phenotype and the intersex condition. Separation of sexual phenotype groups in the PLS-DA model was achieved primarily between females from male groups (Fig. 5.3). However, on average high TO males appeared to trend closer to females than did low TO males, suggesting that subtle similarities in gonad metabolomes of females and high TO males may be indicative of increased presence of oocytes within the gonads. To identify these commonalities associated with oocytes, analyte directional response patterns (Table 5.2) increasing or decreasing in an order consistent with the level of oocytes in sexual phenotype groups (i.e. a medial [•] response of high TO individuals) serves to highlight putative metabolites of interest. Glycerol, which was elevated in females and reduced in low TO males is indicative of lipid metabolism and has been shown to be released by adipocytes following exposure to estrogen (Palin et al., 2003). Of note, the putative metabolites glycine, alanine, and pyroglutamic acid were lowest in females and highest in low TO males. These are important components of the fish egg proteins vitellogenin (Reading et al., 2009) and vitelline (Darie et al., 2004). A negative relationship between the relative abundance of these analytes with the number of oocytes in the gonads may reflect increased utilization of free amino acids for egg protein synthesis. Previous studies have reported increased levels of alanine in fish and fish cells exposed to estrogenic EDCs (Davis et al., 2016; Ekman et al., 2008; Teng et al., 2013). Hence, the negative relationship between the level of TO

and levels of testicular alanine observed in the present study suggest that intersex is related to pathways other than those activated by the estrogen receptor.

Pairwise analyses of low TO and high TO males suggest that features of the gonad metabolome associated with increased presence of oocytes in the testes (Table 5.3) may reflect differences in energy metabolism, protein synthesis, and phospholipid production between groups. Levels of amino acids were generally elevated in the gonads of high TO males (with the exception of glutamine and creatinine, which were reduced), which likely reflects differences in protein and peptide biosynthesis. Glutamine, glutamic acid, aspartic acid, and pyroglutamic acid were all significantly different between high TO and low TO groups. These amino acids are primary substituents of gonadins, a class of peptides with demonstrated ability to alter steroid hormone levels in male rats (Ruiz-Alcaraz and del Rio-Garcia, 2005). Altered levels of these amino acids in intersex LMB could be related to differences in gonadin metabolism. Generally, increased levels of phosphorylated carbohydrates in testes of high TO males may indicate differences in glycogenolysis or pentose phosphate pathways. Levels of ribose-5 phosphate, elevated in testes of high TO males, is a product of the pentose phosphate pathway (PPP). PPP plays an important role for oocyte development in other species, including protection of spermatogenic tissue from oxidative stress (Williams and Ford, 2004). In addition, PPP also plays an important role in lipogenesis for oocyte development. For example, female mice exposed to an androgen experienced reduced oocyte lipid content through inhibition of PPP (Jimenez et al., 2013). Thus, activation of PPP in intersex fish could be associated with oxidative stress and/or the production of lipids for oocyte development. Spectral features representing phosphate were not all in agreement, so false annotation of some peaks might have occurred due to errors in alignment and/or unresolved analytes. However, two features putatively annotated as

phosphate were highly reduced in high TO males and showed the largest magnitude of change (131.7- and 2072.3-fold) of any feature measured. Phosphorylated sugars and alcohols, nucleoside phosphates, and O-phosphoethanolamine were elevated in high TO males, which may suggest increased incorporation of phosphates for energy storage and synthesis or modification of more complex biomolecules such as nucleic acids and lipids.

Data presented in Chapters 3, 4, and 5 suggest that TO in LMB and related species is associated with physiological stress. Chemical contaminants were not measured in Chapters 4 or 5, so exposure to unknown chemicals may have played a role in the occurrence of intersex. However, study sites in Chapter 4 and 5 are not known to have a history of chemical contamination, and toxicological factors are not suspected as a cause of intersex in this case. In small, un-aerated impoundments of the Southeastern United States such as those sampled in Chapters 4 and 5, elevated temperatures and hypoxic conditions are fairly common during warmer periods, which can contribute to physiological stress (Esch and Hazen, 1980), particularly when combined with stresses associated with spawning. In a survey of LMB from impoundments of Georgia (USA), including those sampled in Chapters 4 and 5, Bringolf et al. (2015) found intersex to be closely associated with decreased impoundment surface area, suggesting that higher temperatures and/or lower levels of dissolved oxygen typical of smaller impoundments may play a role. Glycogenolysis and glycolysis can be induced by a variety of physiological stressors in fish, including thermal and hypoxic stress (Mukundan et al., 1986). For example, environmental hypoxia induces glycogenolysis (Wright et al., 1989), as well as glycolysis pathways, transgenerational testicular abnormalities, and epigenetic effects in fish (Wang et al., 2016). Therefore, similar mechanisms may be involved with the occurrence of intersex in fish. Signs of elevated glycogenolytic and glycolytic activity associated with intersex

in Chapters 4 and 5, such as elevated levels of testicular sugar-phosphates, lactic acid, and other carboxylic acids, in addition to reduced blood plasma ALP/AST ratio may indicate the involvement of hypoxic and/or thermal stress. Because all fish were collected from the same impoundment, differences observed within the sampled population could be attributed to individual-level variability in sensitivity to thermal and/or hypoxic stress or perhaps variation in habitat selection within different thermal strata of the impoundment.

The potential involvement of temperature (and/or the associated relationship with dissolved oxygen) in the induction of TO would not be surprising, considering evidence that temperature may alter sex steroid production in the gonads (Kime and Hyder, 1983; Kime and Manning, 1986; Manning and Kime, 1985) as well as sex determination and differentiation (Conover and Kynard, 1981; Nomura et al., 1998; Römer and Beisenherz, 1996; Strussmann et al., 1996; Struussmann et al., 1996) in various species. LMB are extremely adaptable to different environments and exhibit a relatively wide range of thermal tolerance (Mulhollem et al., 2015). However, variability is likely to exist among individuals and populations, which may in part be related to intra- and inter-population variability in intersex reported among LMB. Furthermore, interspecies differences in thermal tolerance may help to explain relatively high levels of intersex reported in closely related species (e.g. smallmouth bass, *M. dolomieu*). Centrarchids, which predominate reports of intersex in North America, nest in shallow areas with slow waterflow during in the spring/summer (Bain et al., 1988; Clark et al., 2008), predisposing them to higher temperatures, particularly within small, confined bodies of water. If higher temperatures are related to the induction of intersex, this nesting behavior may partially explain why TO is so common in centrarchids.

In Chapter 5, we identified blood plasma biochemistry variables related to fish health, as well as features of the gonad metabolome associated with increased presence of TO in male LMB. Based on these results, the occurrence of intersex seems to be associated with differences in energy expenditure, gonad lipid and protein metabolism, and thermal/hypoxic stress. This information may aid in explaining the role of intersex in AOPs by revealing adverse physiological states associated with TO in LMB. Only one population of a single species was examined in Chapters 4 and 5, but similar relationships may exist in other incidences of intersex. Methods used in this work could be applied to various species and populations of interest to investigate causative factors and relationships with measures of fish health. For example, I did not investigate EDC exposure in Chapters 4 and 5, but valuable information could be learned by applying metabolomics analysis of gonadal tissues of fish with various exposure histories to link biochemical indicators of apical effects with the occurrence of TO. Indications of thermal and hypoxic stress in individuals with elevated TO counts highlight the need to understand the relationships between intersex, elevated temperatures, hypoxia, and measures of fish health and reproduction. Whether temperature and/or hypoxia are directly or indirectly connected with the incidence intersex in the present work is unknown, but understanding the role of temperature in relation to intersex and other suspected factors could aid in explaining extraneous variability observed in the environment. If elevated water temperature is directly involved in the induction of intersex conditions in fish, it could exacerbate existing feminizing effects of EDC exposure, but could also support suspicions that intersex may occur independently of chemical exposure. Moreover, direct involvement of water temperature and/or hypoxia in the occurrence of intersex would hold implications related to rising global temperatures due to climate change. This work contributes to a growing body of evidence suggesting that global climate change may exacerbate

effects of EDC exposure (Brown et al., 2015; Hooper et al., 2013; Keller et al., 2015), which could adversely affect the health of global fish populations.

Conclusion

In the preceding chapters of this dissertation, a number of biotic and abiotic factors associated with the feminization of male freshwater fish were identified. Methods were developed and adapted from other disciplines to answer complex questions that will inform adverse outcome pathways related to intersex and long-term feminizing effects in fish associated with estrogen exposure. Long-term biochemical, morphological, and physiological effects associated with feminization in fish were identified. These effects are likely influenced by a broad scope of endogenous and exogenous factors, including fish size, surface water temperature and/or hypoxia, and anthropogenic land use. In conclusion, these findings contribute information that may serve to assess ecological risk, monitor ecosystem health, inform natural resource management, and to advance methodologies used to investigate the feminization of fish and other organisms. These results highlight the complexities associated with issues related to endocrine disruption and illustrate the need to consider a variety of biotic and abiotic factors in regulatory and conceptual frameworks.

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