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Effects of 17β Estradiol in the Metabolism and Morphology of Bluegill Sunfish (Lepomis macrochirus)

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EFFECTS OF 17β ESTRADIOL IN THE METABOLISM AND MORPHOLOGY OF BLUEGILL SUNFISH (LEPOMIS MACROCHIRUS)

(TITLE)

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NEETA PARAJULEE KARKI

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ABSTRACT

Fish natural habitats are increasingly contaminated with various estrogenic compounds, including 17β estradiol (E2). This compound causes adverse effects on the reproductive system of male fish; however, the effects of E2 on other aspects of fish metabolism, morphology and histopathological changes in internal organs are not well known. The objective of this study is to evaluate the effects of E2 exposure on the basal and stressed metabolic rate, morphological changes in body shapes, and histological changes in the liver tissues of sunfish species. Fish were held individually in ten gallon tanks under two treatments of 40 and 80 ng/L and one control treatment (no E2). The duration of E2 exposure was 21 days, with E2 being replenished every week based on its half-life. Basal and maximum aerobic scopes were measured using closed respirometry and a chase protocol at the beginning and at the end of the experiment. Lateral pictures of the fish were also taken at these two time points. My working hypotheses are that (1) fish subjected to E2 exposure would experience stress and thus increased oxygen consumption, (2) male dimorphic characters would become less noticeable in exposed fish, and (3) there would be alterations in the structure of liver hepatocytes after E2 exposure. I found that the basal metabolic rate decreased after 21 days in the control group but not in the estradiol exposed groups. In terms of morphological changes, I observed reduction in operculum size and decrease in head size in exposed individuals but not in the control. Some morphological changes in male-related characters in sunfish which eroded male dimorphic characters were caused by E2 exposure. Preliminary histological examination of liver tissues showed that there was a disintegration of hepatocytes in the E2-exposed liver tissues. My research highlights negative effects of

estradiol that are more widespread than simple gonadal alterations which warrant close monitoring of estradiol contamination in the natural water system.

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INTRODUCTION

Natural water resources in the US are increasingly contaminated by pollutants generated from various sources including industries, pharmaceutical companies, sewage management, and livestock industries (Kolpin et al., 2002; Barnes et al., 2008; Focazio ct al., 2008). An extensive study conducted across 139 streams in 30 US states during 1999-2000 showed more than 80% of the streams were contaminated with wastewater chemicals (Kolpin et al., 2002). The most frequent compounds were steroids, caffeine, cholesterol, fire retardants, phosphates and antimicrobial chemicals (Kolpin et al., 2002; Focazio et al., 2008).

Among these contaminants of surface water, steroidal estrogenic compounds, including endocrine disrupting hormones, are of particular importance because of their negative effects on human health and aquatic life (Pal et al., 2010; Fuzzen et al., 2011; Falahatkar et al., 2017). The most abundant estrogenic compounds in the environment are 17β estradiol (commonly known as E2), estrone (E1), estriol (E3), and 17α-ethinylestradiol (EE2) (Cargouet et al., 2004). Among them, E1 and E2 are of major concern because of their potential to cause negative physiological effects in humans, fish, and plants even at lower dosage (Shore and Shemesh, 2003).

The E2 compound is commonly used as contraceptives and as a therapeutic to treat endometrial problems in women. The long-term use of this compound has been associated with breast cancer, developmental fetal deformities, and infertility (Colborn et al., 1993; O'donnell et al., 2001; Adeel et al., 2017). These compounds are excreted in human feces and urine and enter the surface water through the sewage system (Shappell, 2006; Jones-Lepp et al., 2009). In intensive livestock operations, estrogenic compounds are used as a growth promoter, which results in the excretion of these compounds in the

feces and urine of livestock (Kolpin et al., 2002; Adeel et al., 2017). Additionally, these excreta and urine are used as manure in agricultural operations from where estrogenic compounds might leach to the nearby natural water system (Adeel et al., 2017). Thus, with urbanization and pressure from growing populations, intensive agricultural and livestock operations, and increased use for therapeutics and family planning, it is expected that contamination of surface water will increase in the next few decades. E2 has been detected at concentrations from 1 ng/L to 80 ng/L (Wright-Walters and Volz, 2009) in wastewater effluents with 1-10 ng/L detected frequently (Bradley et al., 2009). In Charleston, Illinois, my study area, E2 was detected at the wastewater facility at a concentration from 0 to 25.3 ng/L (Heffron et al., 2016).

This is particularly concerning for conservation of aquatic life including fishes, as several past studies showed the negative effects of E2 compounds in physiology and health of fishes, mainly on the reproductive system (Imai et al., 2005; Ortiz-Zarragoitia and Cajaraville, 2005; Tamschick et al., 2016) even at concentrations as low as 1 ng/L (Wright-Walters and Volz, 2009). Exposure to estrogenic compounds is also known to affect the reproductive system in freshwater snails where females were converted into superfemales, a condition characterized by the formation of the additional female organs (Oehlmann et al., 2000). The exposure to E2 has been shown to cause feminization and reduced gonadosomatic index in male fish while reduced fecundity and decreased reproductive performance in female fishes (Kinnberg et al., 1999; Nash et al., 2004; Brian et al., 2007; Filby et al., 2012). The adverse effects of E2 on the reproductive system of fish have been documented in Longear Sunfish (*Lepomis megalotis*) (Fentress et al., 2006), Bluegill sunfish (*Lepomis macrochirus)* (Wang et al., 2008), and several

other fish species (Kinnberg et al., 1999; Miles-Richardson et al., 1999; Zaroogian et al., 2001; Moncaut et al., 2003; Imai et al., 2005; Kristensen et al., 2005; Ortiz-Zarragoitia and Cajaraville, 2005).

Though the effects of E2 compounds on the reproductive system of fish have been well documented in several fish species, their effects on the general morphology and histopathological changes in major internal organs such as liver are also not well known. In addition, the effects of E2 on other physiological effects are less understood, namely changes in metabolism. These information may be useful to formulate better policies to reduce surface water contamination by compounds such as E2. The Environmental Protection Agency (EPA) of the US and the European Union has already kept estradiol contamination in the drinking water in their watch list (EPA, 2008; Tiedeken et al., 2017).

Changes in the body shapes in fish can be measured using morphometric techniques. Landmarks and semilandmarks are used as source data to estimate changes in the body morphology (Zelditch et al., 2012). Software like .tpsDig and MorphoJ can be used to understand changes in shape that are size dependent. Shape changes can then be further investigated by means of multivariate statistical technique to detect changes in the body morphology (Mitteroecker and Gunz, 2009; Klingenberg, 2011).

Several past studies have shown that internal body organs of fish, such as liver, kidneys, testes, ovary and gills are affected by E2 exposure (Olivereau and Olivereau, 1979; Hinton et al., 1988; Cakmak et al., 2006; Camargo and Martinez, 2007). Histological examination of these internal organs is commonly used as a biomarker to understand the effects of contaminants present in water (Bernet et al., 1999). As the liver

is the primary organ for metabolism and detoxification of chemicals fish are exposed to (Brusle and Anadon, 1996), histopathological examination of liver tissues can offer an early indication of fish exposure to the chemical of interest. Thus, histopathological examination of liver tissues can serve as a biomarker to assess the possible exposure of fish to the contaminants in surface water.

To understand physiological changes in metabolism, estimation of oxygen consumption is the commonly used method (Clark et al., 2013; Roche et al., 2013). Oxygen consumption in fish can be measured through respirometer using oxygen probes. Two of the most common of oxygen consumption measures are the standard metabolic rate (SMR) and maximum metabolic rate (MMR) (Auer et al., 2015). The standard metabolic rate measures the oxygen consumed by fish to maintain life, i.e. when they are at resting condition; while MMR indicates the maximum oxygen consumption (Auer et al., 2015), which is measured after exhausting the fish by chasing. The difference between the MMR and SMR is commonly referred to as aerobic scope (Auer et al., 2015).

For this study, I have chosen bluegill sunfish as my model species. This species was selected due to their wide availability in the local rivers, streams, ponds and local impoundments in Illinois, and their uses as a recreational and aquaculture fish species (Camargo and Martinez, 2007; Gao et al., 2009). The broader objective of my thesis is to improve our overall understanding of the effects of E2 compounds on fish beyond the known direct effects on the reproductive system. The specific objectives are (i) to evaluate the exposure of E2 on the morphological changes in body shapes of sunfish species, and (ii) to evaluate the effects of E2 exposure on the basal and stressed metabolic

rate in sunfish species. My working hypotheses for the above specific objective are that (1) fish subjected to E2 exposure would experience stress and thus increased oxygen consumption, and (2) male dimorphic characters would become less noticeable in exposed fish. Further, I evaluated the effects of E2 exposure on the histopathology of liver tissues in E2-exposed and control fish, which is presented in appendix A.

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CHAPTER 1: EFFECTS OF 17β ESTRADIOL ON BODY SHAPE CHANGES IN BLUEGILL SUNFISH (*Lepomis macrochirus*)

ABSTRACT

Estrogenic compounds including 17β estradiol (E2) are known to negatively affect the reproductive system of fish that includes feminization, altered sex ratio, reduced fecundity and decreased gonadosomatic index. However, much less is known about their effects on the general morphology of fish species. The objective of my study was to evaluate the effects of varying concentration of E2 exposure on the body shape changes in the Bluegill Sunfish species. A mesocosm experiment was set up where fish were individually maintained in ten-gallon tanks and exposed to E2 concentrations of 40 and 80 ng/L or no E2 exposure (the control). The fish were exposed for 21 days, with E2 replenished each week to account for the half-life. Lateral pictures of the fish were obtained and compared before and after exposure. Canonical variate and discriminant function analysis in MorphoJ were used to compare the morphological changes in the fish. The results showed that E2 exposure caused some morphological changes in male-related characters in Bluegill Sunfish towards an erosion of male dimorphic characters. Specifically, there was a narrowing of the caudal peduncle, smaller nape size, reduced opercular flap and pectoral fin, and deeper body shape in the exposed group compared to control fish. This research highlights the widespread effects of E2 on fish health beyond the reproductive system, which could have important conservation implications.

INTRODUCTION

Endocrine disrupting estrogenic hormones are polluting surface waters (Wright-Walters & Volz, 2009) as these compounds are not readily removed during processing at wastewater treatment facilities (Heffron etal., 2016; Jürgens et al., 2002). Commonly used water purification techniques, such as sedimentation, conjugation, and chlorination are ineffective to remove estrogenic contaminants resulting in the potential for chronic low-level exposure of these compounds in animals inhabiting surface waters (Gunatilake et al., 2013). The commonly found estrogenic compounds of environmental concern include estrone (E1), 17β estradiol (E2), estriol (E3), and 17α-ethinylestradiol (EE2) (Cargouet et al., 2004). These estrogenic contaminants have negative effects on the fish reproductive system, even at lower concentrations (Imai et al., 2005; Zaroogian et al., 2001) which might have negative effect on the aquatic ecosystem. For example, exposure to 1 ng/L of EE2 stimulated production of vitellogenin, an egg yolk precursor commonly found in sexually mature female fish, in male rainbow trout (Purdom et al., 1994), while 4 ng/L concentration impaired the development of secondary sexual characteristics in male fathead minnows (Länge et al., 2001). Further, estrogenic exposure resulted in decreased gonadosomatic index, a ratio of gonad to body weight in fish, and observable histological changes in the testes at estradiol concentrations of 0.1 µg/L (Gimeno et al., 1998; Parrott & Blunt, 2005).

Though the impacts of estradiol exposure on gonadosomatic index and other internal body organs are fairly known, their overall effects on body shape changes are poorly understood. Earlier studies showed that fish morphology is affected by a myriad of factors including their ecological conditions (Cooper & Westneat, 2009; Willis et al.,

2005), food availability (Ehlinger et al., 1988), intraspecific competition (Ehlinger, 1997) and sex of fish (Ehlinger, 1997). Differences in body shape of an individual reflect differential local growth rates and temporal shifts in development and morphogenesis (Laurent & Perry, 1991). Some of these changes can be linked to specific adaptation to changes in environmental variables or habitat (Langerhans et al., 2003; Laurent & Perry, 1991). Relatively few studies in the past have attempted to evaluate the effects of estrogenic compounds on the morphology of fish, and most of them used fathead minnows as their species (Elliott et al., 2014; Parrott & Blunt, 2005; Sowers et al., 2009). Very little is known about the effects of the ecologically relevant concentration of E2 exposure on the morphology of bluegill sunfish, one of the native fish species in Illinois.

The development of modern morphometric techniques based on geometry offered a powerful tool to identify the changes in body shape which might be easily missed through traditional morphometric methods and which are also size independent (Bookstein, 1997; Mitteroecker & Gunz, 2009; Park et al., 2013). Outline and landmark-based methods are two commonly used approaches in the geometric morphometric analysis. Landmark-based methods use discrete points, their linear distance and geometric relationships, whereas outline-based approach use perimeter shapes (Cadrin, 2013; Dujardin et al., 2014). In the landmark-based geometric methods, spatial data are collected from different landmarks based on the purpose of the study and analyzed using different univariate, bivariate and multivariate statistical techniques (Mojekwu & Anumudu, 2015). The selections of appropriate landmarks are primarily guided by the purpose of the study. By including different anatomical features, there are increased chances to identify the morphological differentiation (Adams et al., 2013; Farré et al.,

2016). Geometric morphometric analyses namely landmark-based approaches have been increasingly applied to study body shape changes in fish (Cavalcanti et al., 1999; Ibanez et al., 2007; O'Reilly & Horn, 2004; Ramler et al., 2016; Schnitzler et al., 2016; Shukla & Bhat, 2017).

To study the effects of 17β estradiol on the body shape changes, I chose Bluegill Sunfish as a model species due to their relevance in Illinois as well as the exhibition of sexual dimorphism in this species (Ehlinger & Wilson, 1988). The divergence between male and female bluegill sunfish are observed better when their length reaches 100 mm (Bell, 2001). Sexually mature male fish are olive green and yellowish underneath and their body is streamlined while the female are lighter color and are deeper-bodied (Schultz, 2010). Estrogenic exposure leads to vitellogenin production in male fish, which would otherwise be only found in female fish (Jobling et al., 2003). Vitellogenin production can lead to feminization of male fish. I hypothesize that male dimorphic characters would become less noticeable in male fish exposed to 17β estradiol. The objective of this study was to evaluate the effects of 17β estradiol on the body shape changes in bluegill sunfish (*Lepomis macrochirus)* after exposure for 21 days at a concentration of 40 ng/L and 80 ng/L.

MATERIALS AND METHODS

Study subjects

I chose Bluegill Sunfish (*Lepomis macrochirus*) as my model species because they are common in Illinois, and are easily found in the local streams, ponds, and lakes, and have commercial value as a sports fish (Fritts et al., 2016). Moreover, they are more

likely to be affected if surface water sources get contaminated due to their large local population, high site fidelity and wide availability. Due to their abundance in the local water system, they can be used as a biomarker species to study the effects to the aquatic life from water contamination or pollution. I collected sexually mature Bluegill Sunfish from the local impoundments near Charleston by electrofishing in early June to conduct my first cycle of an exposure experiment. The length of fish varied from 146 to 230 mm, while the weight varied from 47 to 203 g. The fish were collected in July and September for the second and third cycle of experiment, respectively. They were transported in well-aerated open buckets to the fish laboratory at EIU and acclimated in the tanks for a minimum of 10 days before dosing began. The sex of the fish was not identified *a priori*, which means both male and female were included in the study.

Experimental design and exposure protocol

I conducted this study from May through December 2016 in three different experimental cycles with each cycle lasting 21 days. Experiments took place under controlled conditions in the fish laboratory at Eastern Illinois University. I set up a mesocosm experiment with 15 10-gallon tanks in each cycle to mimic the best possible natural environment for fish. The temperature of mesocosm was maintained at 21±1°C. Fish were fed soy free pellet food daily. Light cycles were maintained at 12:12hr (day: night). Tank water was replenished using deionized water to ensure no contamination with estrogen, and buffering salts were added to 1 ppt. Filtration was present but carbon cartridges were removed to avoid E2 sequestration. Water quality parameters namely salinity, nitrates, nitrites, ammonia, and pH were regularly monitored. Salinity was kept at <1ppt, pH was kept between 7.0 to 7.5, and nitrates, nitrites, and ammonia were kept at

zero. The tanks were inspected daily to examine fish condition. A protocol to conduct this study was approved by Institutional Animal Care and Use Committees (IACUC Protocol approval number 15-004).

There were two treatment groups and one control group. Among the treatment groups, one group was exposed to 40 ng/L of E2 (T1) and another group was exposed to 80 ng/L of E2 (T2). These concentrations were chosen based on the highest concentration of E2 detected in local wastewater effluents (Heffron et al., 2016). In each group, there were five fish and each of them was kept individually in a 10-gallon tank. The total sample size was 45 fish, with 15 fish in each experiment cycle. As mentioned earlier, experiments were run in three different cycles which lasted 21 days. Fish were exposed to E2 by adding the premixed E2 to the water in tanks to achieve 40 ng/L and 80 ng/L concentrations. A sham treatment of just water was delivered in the control tanks. The E2 were replenished every week based on its half-life to maintain the desired concentration of E2 throughout the experiment by replacing two-thirds of the water in the tank. Fish were gently and humanely handled to minimize pain and discomfort during the experiment.

Morphometrics

Weight and length of all fish were recorded at the beginning and end of each experimental cycle. The left lateral view of each fish was photographed at these two-time points using orthophotography by mounting a Canon digital camera of 16 megapixels on a stand (Figure 2, Table 1). Fish were placed on a moist foam mat at the base to maintain natural configuration. The fins were stretched as much as possible to capture body shape. Images were combined to build a thin-plate spline (TPS) file using .tpsUtil32 software.

The TPS file was then imported into TPS Dig software to digitize the landmarks. In total, I digitized twenty-two landmarks (Figure 3) in each fish to capture the variation across head and body shape. Fish were euthanized and sexed by observation of the gonads at the end of the experiments.

Data analysis

The TPS files were imported to MorphoJ where I used canonical variate (CVA) and discriminate function analysis to test shape changes among the different groups. The CVA differentiates the mutually exclusive groups by analyzing their relative positions. This is done through the construction of a coordinate system called the canonical variates by determining the scores on those axes for all individuals. The discriminate function analysis helps to evaluate the separation between the two groups by creating one or more linear combinations of the predictors for each discriminant function.

RESULTS

There was a slight decrease in the body mass during the 21 days of the experiment while the length of the fish was similar before and after exposure (Table 2). The decrease in the body mass was higher in the E2-exposed groups compared to control groups (Table 2). Among all male fish (n=26), the average mass before exposure was 119.7 g (SD 47.5) while it decreased by nearly 10 g to 109.5 g (SD 49.8) after exposure of 21 days (Table 2). The average length of all male fish before and after exposure was 180.2 mm (SD 23.5) and 179.1 mm (SD 23.9), respectively (Table 2). Among all female fish (n=17), the average mass before and after exposure was 104.4 g (SD 44.2) and 94.3 g (SD 48.1), respectively (Table 2), while the length was 181.2 mm (SD 23.8) and 180.6 (SD 23.8)

before and after exposure, respectively (Table 2). When comparing between male and female fish by groups, I found that the length did not vary before and after exposure, but there was a decrease in weight after exposure in all groups ranging from 4 g to 16 g (Table 2). The greater decrease in body weight was observed among exposed groups compared to control group (Table 2).

Canonical variate analysis (CVA) among eight groups (i.e. control, T1, T2 and female before and after exposure) showed that the data clearly separated into two groups. Most of the variation was explained by the first three canonical variates. The eigen values for the first three canonical variates were 29.7, 5.9 and 3.4, which explained 63.4%, 13.7% and 7.8% of the variance, respectively. The fish from the control before and after exposure as well as T1 and T2 before exposure were grouped together while the fish from female before and after exposure, T1, and T2 after exposure were grouped together along the first canonical variate (Figure 3). Along the second canonical variate, T1 before exposure was separated while female before and after exposure and T2 after exposure were closer to each other (Figure 3). This showed that male fish exposed to higher E2 concentration were found to be closer to females and were grouped together, indicating that the male fish exposed to higher E2 (80 ng/L) concentration tended to be more similar to female fish than fish exposed at 40 ng/L. This supports my hypothesis that the male secondary characteristics erode after exposure to higher concentration of E2.

Further, I combined the male fish from T1 and T2 groups together as a single treatment group. Now, there were four groups (i.e. treatment groups before and after exposure, control male fish, and female fish). The CVA analysis showed that first two canonical variates explained more than 92% of the variations. The first CV explained

83.6% of the variation, while the second CV explained 8.9% of the variation. The fish from control male and treatment groups before exposure were in one direction while the female fish and exposed fish were in the other direction. This indicated that there was a shift of treatment groups towards female fish after exposure. The results of discriminant function analysis showed that female and before exposure (t -square: 5960.62; p <0.0001); female and control (t -square: 1423.62; p <0.0001), and female and after exposure (t -square: 513.63; p= 0.013) were different from each other, while differences were not observed among other pairs (p> 0.05). By identifying the highest Eigen values associated with specific landmarks, I was able to identify as drivers of these differences the following morphological traits: narrow caudal peduncle, smaller nape size, reduced opercular flap and pectoral fin, and deeper body shape in the exposed group (Figure 4).

DISCUSSION

In this study, I evaluated the effects of varying concentration of E2 exposure in the morphology of Bluegill Sunfish. I found that there was a change in morphology of male fish related to secondary sexual characteristics. Specifically, there was a narrowing of the caudal peduncle, smaller nape size, reduced opercular flap and pectoral fin, and deeper body shape in the exposed group. Further, the morphometric analysis showed that the fish were separated into two distinct directions. Fish from control before and after exposure, T1 (40 ng/L), and T2 (80 ng/L) before exposure were grouped in one direction, while the female fish before and after exposure and male fish from T1 and T2 after exposure were grouped in another direction. This indicated that the morphology of male fish was affected by the E2 exposure and was converging towards the female. This effect

was dose dependent, as T2 (80 ng/L) after exposure was found closer to female fish compared to T1 (40 ng/L). These findings were further substantiated when comparisons were made between exposed and unexposed fish before and after exposure.

The observed changes in the general morphology of male Bluegill Sunfish in response to E2 exposure provide an additional avenue to understand the widespread negative effects of E2 that are complementary to their relatively well-known effects in the fish reproduction system (Ibor et al., 2016; Olivereau & Olivereau, 1979; Ortiz-Zarragoitia & Cajaraville, 2005; Wang et al., 2008). The observed changes in the secondary sexual characteristics might be due to the feminization effect of E2 in male fish or it may be a funtion of shifts in energetic costs to cope up with the stress imposed by E2 exposure. As I did not measure plasma cortisol level, a hormone secreted by fish in response to stress, it is difficult to say whether E2 caused stress in fish. However, previous studies have shown that pollutants can cause stress in fish (Love et al., 2013; Meylan et al., 2012). The loss of secondary sexual characteristics in the male fish exposed to estrogenic compounds has also been observed in other studies (Elliott et al., 2014; Länge et al., 2001; Parrott & Blunt, 2005; Sowers et al., 2009). In male fathead minnows (*Pimephales promelas*), severe physical deformities that include anal protrusion with distended belly were observed in 158 days post hatch when exposed to 64 ng/L E2 concentration (Sowers et al., 2009). In addition, they observed that the fish exposed to greater than 4 ng/L E2 never developed secondary male sexual characteristics while those exposed to less than 1 ng/L E2 experienced retardation in their development (Sowers et al., 2009). In another study, fathead minnows exposed to less than 1 ng/L EE2 showed loss of male secondary sexual characteristics, specifically less prominent dorsal pads,

fewer tubercles, and lighter color compared to control fish at 150 days post-hatch (Parrott & Blunt, 2005). Similarly, Elliott et al., 2014 also observed thinner dorsal pads, loss of tubercles, and lighter color in male fathead minnows after exposure to 30 ng/L for six weeks, but did not find significant external morphological changes in bluegill sunfish. In contrast to this finding, I observed morphological changes in the male bluegill sunfish, which may be due to the exposure to higher E2 concentration (40 ng/L and 80 ng/L) compared to 30 ng/L.

Morphological alteration is a complex issue and is influenced by multiple factors. The availability of food, water quality, ecological niche, predation, climatic events such as drought, behavioral traits, such as migration, can affect the morphology of fish (Capelle et al., 2016; Meylan et al., 2012). Though the exact mechanism on how the above factors affect the morphology is poorly understood, it is hypothesized that fish will suffer stress, which results in the release of cortisol. Subsequently, there will be a shift in energy spent that would possibly compromise growth and development to cope up with the stress-induced higher metabolic costs (Bowerman et al., 2017). Environmental E2 affects the morphology of fish possibly through the hormonal effect in male fish and through the mechanism of stress induction. With the increasing contamination of natural water system with estrogenic compounds including E2, it is important to consider the possible role of E2 when evaluating the morphological changes in fish species.

The extent of negative effects on fish health from E2 exposure is determined by the duration and concentration of estrogenic compounds in the water. In addition, it also varies depending on the species of fish. Even the exposure to lower estrogenic concentrations was sufficient to alter the gonadal structure. For example, there is

evidence of induction of vitellogenin, a protein normally produced only by female fish, in male fish as a result of sex alteration with estrogenic concentrations as low as 1 ng/L (Elliott et al., 2014; Purdom et al., 1994). Other reproductive traits such as fecundity and gonad somatic index were shown to decrease with E2 exposure (Akhavan et al., 2015; Elliott et al., 2014). With recent findings on the ability of estrogenic compounds to alter the general morphology of fish, monitoring of natural water systems to assess the level of E2 contamination is more important than ever before.

For future studies, I recommend evaluating plasma cortisol level simultaneously along with plasma estradiol and testosterone levels in the exposed and unexposed fish to tease apart the contributions of sexual hormones and stress levels to changes in body morphology. Further, conducting similar study across the spectrum of E2 concentrations with various exposure times would be helpful to elucidate the trigger point of morphological alteration.

CONCLUSION

This study evaluated the role of E2 exposure on the general morphology of Bluegill Sunfish species by continuously exposing them to 40 ng/L and 80 ng/L of E2 for 21 days in a mesocosm experiment. The findings provide evidence for the loss of secondary sexual characteristics in the male fish and their convergence towards female characteristics. This suggests that the effects of E2 exposure on fish health are widespread beyond the reproductive system. As the morphology of fish is directly related to their reproductive fitness, as well as the ability to tackle predation and survive intra

and interspecific competition, morphological alteration of fish species might have ecological consequences from a conservation standpoint.

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Table 1.1. Description of the anatomical landmarks used for morphometric analysis.

Number of landmarks	Description of the landmarks
1	Anterior tip of premaxilla
2	Posterior dorsal most point of neurocranium
3	Anterior insertion of the spiny dorsal fin
4	Anterior insertion of soft dorsal fin
5	Distal tip of the last dorsal fin ray
6	Posterior insertion of the soft dorsal fin
7	Dorsal indentation of caudal peduncle
8	Ventral maximum curvature of caudal peduncle
9	Posterior insertion of the anal fin
10	Highest point of anal fin
11	Anterior insertion of the anal fin
12	Most distal point of the pelvic fin
13	Anterior insertion of the pelvic fin
14	Tip of the operculum
15	Anterior tip of dentary
16	Anterior margin of the eye
17	Posterior margin of the eye
18	Superior margin of the operculum
19	Points of the maximum extension of the operculum of lateral profile
20	Inferior margin of the operculum
21	Dorsal insertion of the pectoral fin
22	Ventral insertion of the pectoral fin

Table 1.2. Length and mass of fish before and after exposure.

Fish	N	Length (mm) (Std Dev)		Mass (g) (Std Dev)	
		Before exposure	After exposure	Before exposure	After exposure
Male all	26	180.2 (23.5)	179.1 (23.9)	119.7 (47.5)	109.5 (49.8)
Female all	17	181.2 (23.8)	180.6 (23.8)	104.4 (44.2)	94.3 (48.1)
Control male	10	170.4 (23.5)	170.5 (23.1)	109.7 (53.0)	102.4 (51.1)
Control female	5	167.4 (16.6)	166.8 (15.5)	77.4 (36.9)	73.8 (36.8)
T1 male	9	184.6 (27.5)	183.2 (30.1)	131.3 (53.2)	122.0 (59.9)
T1 female	6	180.3 (26.1)	179.0 (25.1)	101.8 (43.8)	92.7 (45.4)
T2 male	7	190.0 (13.2)	186.1 (13.1)	119.3 (32.5)	103.6 (35.6)
T2 female	8	190.5 (24.0)	190.3 (24.9)	123.3 (44.0)	108.4 (56.4)

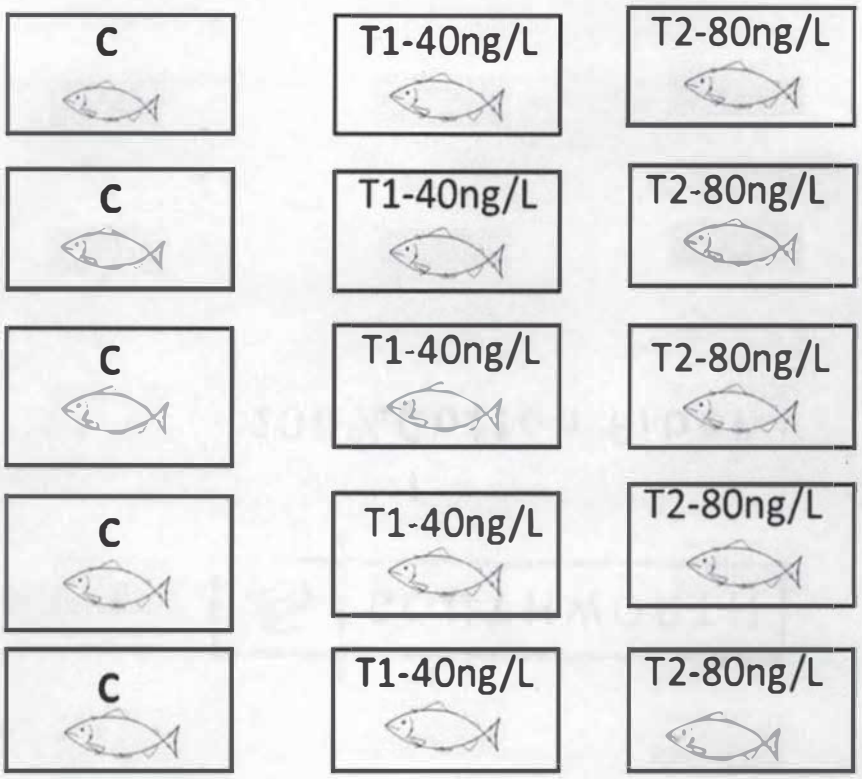

Figure 1.1. Mesocosm experimental setup for exposure experiments (run three times in succession).

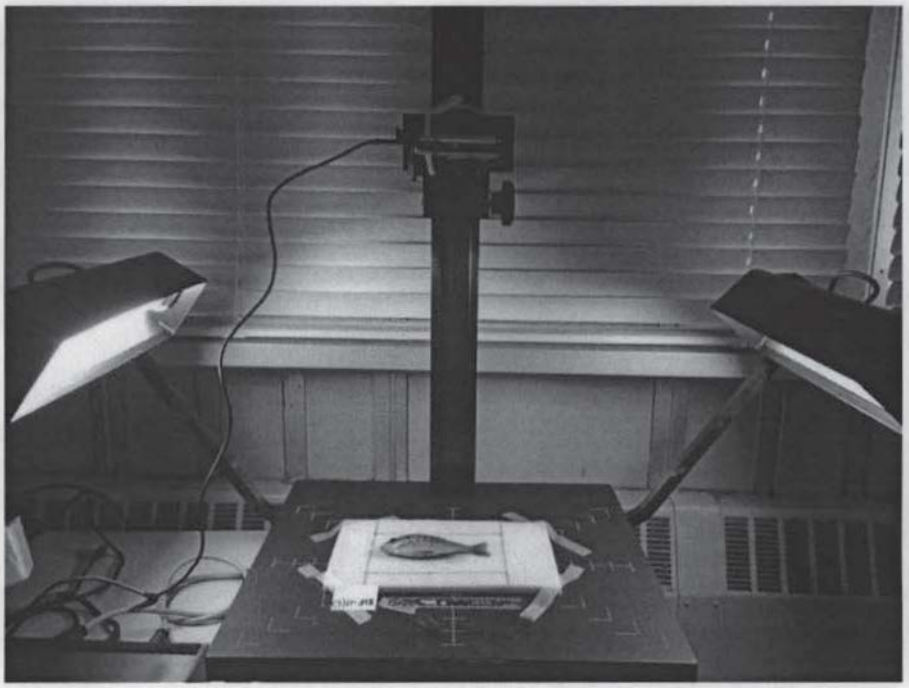

Figure 1.2. Image showing setup for lateral image capture for geometric morphometrics.

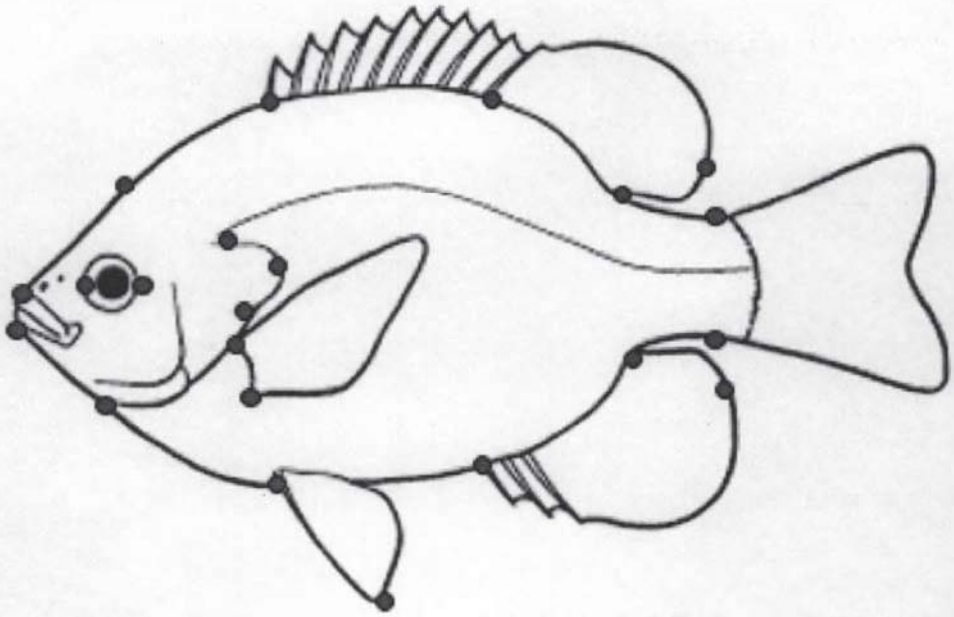

Figure 1.3. A schematic diagram of fish showing the locations of 22 landmarks used for geometric morphometric analysis.

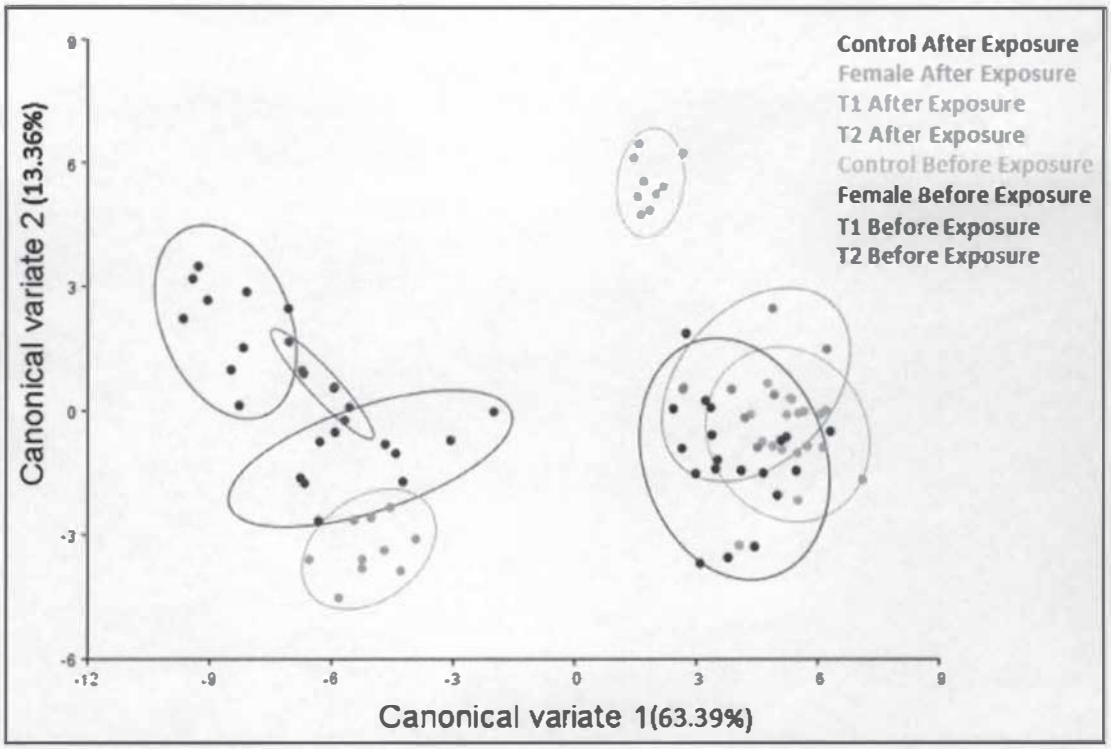

Figure 1.4. Relationship between first two canonical variates among all groups before and after exposure.

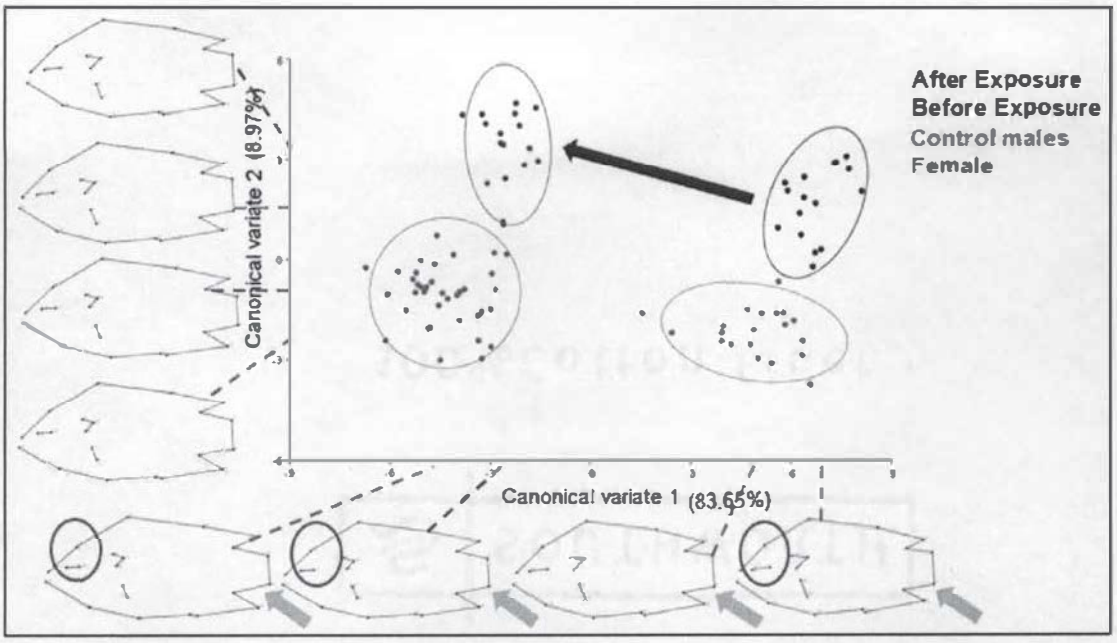

Figure 1.5. Relationship between first two canonical variates for four groups (male fish before and after exposure, control males, and female fish). Large blue arrow represents shift from before to after exposure. Wireframe fish outlines connecting landmarks are shown along each axis to highlight the observed variation (circle and light blue arrows).

CHAPTER 2: EFFECTS OF 17β ESTRADIOL ON METABOLISM OF BLUEGILL SUNFISH (*Lepomis macrochirus*)

ABSTRACT

Fish habitats are increasingly contaminated with estrogenic compounds, including 17β estradiol (E2). E2 causes adverse effects on the reproductive system of male fish; however, its effects on fish metabolism are less known. The objective of this study is to evaluate the effects of E2 exposure on the basal and stressed metabolic rates in Bluegill Sunfish. Fish were held individually in ten gallon tanks under two treatments with varying estradiol concentrations (40 and 80 ng/L) and one control group (no E2). The duration of E2 exposure was 21 days, with E2 replenished every week. Basal and maximum aerobic scopes were measured using a closed respirometer before and at the end of the 21 days of exposure. The results showed that females had 19% higher basal metabolic rates than males before exposure but not after exposure, indicating convergence of basal metabolism in the two sexes. The comparison between groups showed that sex was a significant predictor of difference in oxygen consumption during basal metabolism before exposure but there was no such difference between control, T1 (40 ng/L) and T2 (80 ng/L) groups. This research highlights that negative effects of estradiol are more widespread than simple gonadal alterations.

INTRODUCTION

Various chemical substances found in natural waterways are deteriorating the water quality of rivers, lakes, and ponds, thereby making them increasingly less habitable to aquatic life (Shore & Shemesh, 2003; Wright-Walters & Volz, 2009). One of the several pollutant types found in surface waters that is of growing concern is estrogenic compounds (Cargouet et al., 2004; Kolpin et al., 2002; Shore & Shemesh, 2003). There are several forms of estrogenic compounds that include estrone (E1), 17β estradiol (E2), estriol (E3), and 17α-ethinylestradiol (EE2). Among them, E2 is the most abundant in natural habitats and has the potential to cause negative effects on the fish reproductive system even at lower concentrations of 1 ng/L (Wright-Walters & Volz, 2009). These compounds are found in contraceptives and other therapeutics that are commonly used to treat reproductive system pathologies, such as endometriosis (Vandenberg et al., 2012; Wright-Walters & Volz, 2009).

Another source of estrogenic compounds is intensive livestock production where it is used as a growth promoter in cattle feed (Adeel et al., 2017). As in humans, E2 compounds are excreted in the livestock feces and urine (Jones-Lepp et al., 2009; Kolpin et al., 2002). Though processing at wastewater treatment facilities reduces the load of these compounds (Heffron et al., 2016), still substantial amounts remain in effluents after processing and enter the surface water system (Belhaj et al., 2015; Pessoa et al., 2014). The estrogenic compounds are found in different concentrations in the water effluents depending upon the site, ranging from 1 ng/L to 80 ng/L (Wright-Walters & Volz, 2009), with 1-10 ng/L detected frequently (Bradley et al., 2009). At the wastewater treatment facility located in Charleston, IL, an average of 3.6 ng/L (range 0 to 25.3 ng/L) of estrogenic compounds were detected after processing (Heffron et al., 2016).

Several studies found that E2 compounds cause negative effects on the reproductive system of fish due to their endocrine disrupting properties (Brian et al., 2007; Miles-Richardson et al., 1999; Olivereau & Olivereau, 1979; Wang et al., 2008). The most notable effects of E2 is their ability to cause both sex reversal in male fish and feminization (Tamschick et al., 2016; Vajda et al., 2011). E2 also causes reduced gonadosomatic index, which is the proportion of gonadal tissues compared to their total body weight in male fish (Brian et al., 2007; Pickering, 1993), and erosion of sexual dimorphic characters in exposed males (Chapter 1). The adverse effects of E2 on fish are not limited to male fish. In female fish, E2 causes reduced fecundity and decreased reproductive performance (Miles-Richardson et al., 1999; Olivereau & Olivereau, 1979). This indicates that the long-term exposure of fish species to E2 is likely to alter the population structure of fish species, which will have serious consequences to fish biodiversity (Moyle & Leidy, 1992). Though the effects of E2 and other estrogenic compounds on the reproductive system of fish are fairly well known, their effects on metabolic rate in fish are poorly understood.

The metabolic rate in fish is estimated by measuring the oxygen consumption in two physiological states: resting and exhausted (Auer et al., 2015). Oxygen consumption during resting conditions measures basal or standard metabolic rate, which is the minimum amount of oxygen required to maintain life (MO_2min), while the maximum oxygen that a fish can consume under given temperature and ecological condition is used to measure maximum metabolic rate (MO_2max) (Norin & Clark, 2016; Norin & Malte, 2011). The metabolic rate in fish is increased when fish are subjected to stress. When fish experience stress, the hypothalamus-pituitary-gonadal axis is triggered thereby releasing

a cortisol hormone through the interregnal tissue located in the head-kidney (Pankhurst & Van Der Kraak, 1997; Pickering, 1993). Several factors including exposure to other pollutants, such as bisphenol, cadmium, zinc, mercury, chromium, and lead are known to cause stress in fish and increased oxygen consumption (Authman et al., 2015). Other factors are known to induce stress include predation, intra and inter-specific competition, overcrowding, social condition, food availability, water quality, and temperature, and other climatic conditions including discharge rate (Barton, 2002; Sloman et al., 2001).

I hypothesized that the exposure of fish to E2 would cause significant stress, which would lead to higher oxygen consumption. During stress, cortisol hormone is secreted in the blood stream, which results in increased oxygen demand for osmoregulation and increased metabolism. To test this hypothesis, the objective of this study is to evaluate the effects of E2 exposure on the basal and stressed metabolic rate in Bluegill Sunfish.

MATERIALS AND METHODS

I collected sexually mature Bluegill Sunfish by electrofishing from local ponds near Charleston, IL. After transporting the fish in well-aerated buckets to the EIU fish lab, they were acclimatized for a minimum of ten days before initiating E2 exposure. Both male and female fish were included in the study as sex was not identified *a priori*. The fish were randomly assigned to two treatment groups (40ng/L and 80 ng/L of E2) and a control group with no E2. A protocol to conduct this study was approved by the EIU Institutional Animal Care and Use Committee (IACUC Protocol# 15-004).

Fish were fasted for 24 hours before measuring oxygen consumption to avoid any artifacts caused by digestive metabolism. The transportation of fish from the tanks to the experiment laboratory was performed gently and carefully to minimize handling stress. Weight and length of the fish were measured before placing the fish into a 3.9 L glass respirometry chamber. The chamber was fitted with an optical oxygen probe and sealed with a rubber stopper and parafilm to measure oxygen consumption. The temperature of the water in the respirometer chamber was measured and maintained at 21°±1C. To measure the oxygen consumption during resting (or basal metabolism), the fish was acclimatized for one hour in the chamber with the lid open before starting the trial. After acclimatization, the lid was sealed and a YSI ProODO optical oxygen probe was inserted (Figure 1). Trials lasted 30 minutes and the oxygen partial pressures were always above 75%. To measure the oxygen consumption under stressed conditions (or maximum metabolism), a chase protocol was followed. I chased the fish for five minutes intensively with a net to exhaust the fish (Ferguson et al., 1993). After that, the fish were immediately placed in the respirometry chamber to measure the oxygen consumption following the same procedure as in basal metabolism.

The oxygen consumption rate was calculated as a difference in dissolved oxygen concentration over time, taking into account the weight of the fish and the volume of the chamber, using the following formula:

$$(1)\ MO_2 = \frac{(DO\ initial - DO\ final) * V}{(T * W)},$$

where, MO_2 is the mass corrected oxygen consumption rate ($mgO_2h^{-1}\ kg^{-1}$), DO initial is the concentration of dissolved oxygen in the chamber at the beginning of measurement in mgO_2/L, DO final is the concentration of dissolved oxygen in the chamber at the end of

measurement in mgO_2/L, V is the volume of respirometer chamber in liters, T is the total time elapsed in hours and W is the weight of fish in kilograms.

Statistical analysis

First, I performed descriptive statistics (mean and standard deviation) to understand the oxygen consumption pattern before and after E2 exposure during different physiological stages (basal and maximum) and across sex. The variables considered were basal metabolic rate before exposure (basal before), basal metabolic rate after exposure (basal after), maximum metabolic rate before exposure (maximum before) and maximum metabolic rate after exposure (maximum after). The normality and homogeneity of variance of these four variables were tested by conducting Shapiro-Wilk W tests and Levene's tests respectively. An independent sample t-test was used to compare the oxygen consumption rate between male and female separately for the four variables mentioned above. Paired t-tests were used to compare the before and after oxygen consumption for those four variables separately for male and female fish. Finally, I conducted two-way ANOVA tests to compare the oxygen consumption rate between two treatment groups and control group taking into account the sex for all those four variables. Post-hoc tests were conducted if there was a significant result in the ANOVA tests.

RESULTS

Among all male fish, the average oxygen consumption during basal metabolism before and after E2 exposure was 128.5 $mgO_2h^{-1}kg^{-1}$ (95% CI: 112.5- 144.5 $mgO_2h^{-1}kg^{-1}$) and 93.2 $mgO_2h^{-1}kg^{-1}$ (95% CI: 77.5- 108.9 $mgO_2h^{-1}kg^{-1}$), respectively. There was a

decrease in oxygen consumption by 27% which was statistically significant (t-value= 3.88, p-value=0.0006). The oxygen consumption in male fish during maximum metabolism before and after exposure also decreased by 13% (172.7 $mgO_2h^{-1}kg^{-1}$, 95% CI: 156.3- 189.1 $mgO_2h^{-1}kg^{-1}$ versus 149.3 $mgO_2h^{-1}kg^{-1}$, 95% CI: 129.5- 169.1 $mgO_2h^{-1}kg^{-1}$), which was statistically significant (t-value= 3.06, p-value= 0.005).

The average oxygen consumption among all female fish during basal metabolism before E2 exposure was 158.6 $mgO_2h^{-1}kg^{-1}$ (95% confidence internal (CI): 139.7- 177.5 $mgO_2h^{-1}kg^{-1}$, Table 1). However, the average oxygen consumption among all female fish during basal metabolism after E2 exposure was only 106.9 $mgO_2h^{-1}kg^{-1}$ (95% CI: 86.5- 127.4 $mgO_2h^{-1}kg^{-1}$, Table 1), indicating 32% decrease in the average oxygen consumption after E2 exposure. The difference in the oxygen consumption during basal metabolism among females before and after E2 exposure was statistically significant (t-value= 4.38, p-value= 0.0004). Though there was a significant drop in oxygen consumption during resting stage before and after exposure, no such difference was found for the maximum metabolism in female fish (t-value= 1.21, p-value= 0.24). The average oxygen consumption during maximum metabolism among female fish before and after E2 exposure was 178.9 $mgO_2h^{-1}kg^{-1}$ (95% CI: 160.8- 197.0 $mgO_2h^{-1}kg^{-1}$) and 163.2 $mgO_2h^{-1}kg^{-1}$ (95% CI: 141.9- 184.6 $mgO_2h^{-1}kg^{-1}$), respectively.

The comparisons between average oxygen consumption in all females and all male fish before exposure indicated that the basal metabolism in female fish was higher by 19% compared to male fish, which was statistically significant (t-value= 2.54, p-value= 0.01, Figure 2). However, there was no such difference between female and male fish in the oxygen consumption after exposure (t-value= 1.11, p-value= 0.26, Figure 2).

For maximum metabolism, no differences were found between female and male fish both before and after exposure. When all treatments were combined in two groups based on sex, I found that basal and maximum metabolism decreased by 42% in males and 20% in females after exposure.

After comparing overall male and female fish, I evaluated the effects of E2 exposure by groups, namely control, T1, and T2 (Figures 3 and 4). In general, I found that the oxygen consumption was higher in female fish across all groups and physiological stages but the magnitude of the difference was higher during basal metabolism compared to maximum metabolism. The results of the two-way ANOVA showed that sex was a significant predictor of differences in the oxygen consumption during basal metabolism before exposure but there was no such difference between control, T1 and T2 groups. However, oxygen consumption was not significantly different either by sex or groups during basal metabolism after exposure and maximum metabolism both before and after exposure (Tables 2 and 3). Among all fish, the aerobic scope increased after exposure (t-value= -3.12, p-value= 0.003). When compared to sex and treatment groups, neither the aerobic scope before exposure and after exposure, nor their difference, vary significantly.

DISCUSSION

This study evaluated the role of 17β estradiol (E2) on the oxygen consumption during basal and maximum metabolism in Bluegill Sunfish by continuously exposing them for 21 days at two ecologically relevant E2 concentrations. I found that male and female fish are differently affected by E2 exposure. The influence of E2 exposure was also more pronounced on the basal metabolic rate compared to maximum metabolism.

The basal metabolic rate in female fish before exposure was higher than their male counterparts but there was no such difference after E2 exposure. This finding suggested that after the E2 exposure, there was a gradual convergence between male and female fish resulting in similar oxygen basal consumption irrespective of their sex. This may be due to the feminization of male fish caused by the E2 exposure (Chapter I). Several studies have shown that E2 leads to feminization in male fish (Blázquez et al., 1998; Filby et al., 2012; Gorshkov et al., 2004; Heffron et al., 2016).

In this study, both the basal and maximum metabolism decreased after E2 exposure. This finding was contrary to my hypothesis, as I had expected that E2 would cause stress to the fish, which generally leads to increased oxygen consumption. Stress causes elevated cortisol levels in fish, which in turn results in increased oxygen consumption (Barton, 2002; Jentoft et al., 2005). For example, higher cortisol levels and subsequently higher oxygen consumption were recorded in juvenile Steelhead (*Salmo gairdners*) after physical stress (Barton & Iwama, 1991). Likewise, higher cortisol levels and increased oxygen consumption were found in subordinate fish compared to dominant aggressive fish in Brown Trout (*Salmo trutta*) due to social stress (Sloman et al., 2001). The contrast findings in my study may indicate that the exposure duration of 21 days at the chosen concentration might not be sufficient to exert enough stress in fish to caused increased oxygen consumption. As I did not measure the cortisol level, stress in fish could not be measured, which I recommend should be done in future studies.

In this study, I did not find significant changes in the maximum metabolic rate and aerobic scope before and after exposure. This indicates that basal metabolism is more sensitive to the effects of E2 exposure. Previous studies evaluating the effects of the

environmental contamination of E2 compounds on fish focused mostly on the reproductive system of fish. Very few studies have so far evaluated the effects of E2 exposure on metabolic rates. The findings from this study provided additional evidence that E2 is capable of affecting metabolism, primarily basal rate. As metabolic activity in fish is directly related to fish growth and reproduction, it can impact the health of fish populations. Additionally, the affected fish might be more prone to the predation due to stunted growth. As this study was conducted in a mesocosm controlling for several factors, such as water quality parameters, it would be interesting to evaluate how E2 is impacting populations in natural settings.

CONCLUSION

In summary, I evaluated the effects of E2 exposure on the basal and stressed metabolic rate in Bluegill Sunfish. The result indicated that after the E2 exposure the basal metabolic rate in male and female fish became similar while it differed between sexes before the E2 exposure. This indicates the convergence of male and female fish due to E2 exposure. As metabolism of fish is affected, additional effects on other aspects of fish may be observed, such as growth and reproduction, due to shift in the energetic costs. This finding suggests that the effect of environmental contamination of E2 on fish might be beyond the reproductive system and needs further investigation with larger sample sizes. The close monitoring of E2 levels in natural water systems and stricter guidelines for E2 removal in wastewater treatment plants may help curb the negative effects of E2 compounds on fish and other aquatic fauna.

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Table 2.1. Descriptive results of oxygen consumption in overall female and male fish

Variable	N	Mean	SD	QI	Q3	Minimum	Maximum
Female							
Basal before	17	158.6	36.8	136.6	174	90.5	245.4
Maximum before	17	178.9	35.3	150.9	198.9	118.8	255.8
Basal after	17	106.9	39.8	89.4	125.7	41.7	177.3
Maximum after	17	163.2	41.5	128.4	190.6	94.8	239.7
Male							
Basal before	26	128.5	39.6	107.3	143.4	67.4	229.9
Maximum before	26	172.7	40.6	138.9	204.5	94.3	252.5
Basal after	26	93.2	38.9	65.6	106.2	40.6	213.6
Maximum after	26	149.3	49	113.3	180.1	58	246

N= Sample size; SD=Standard deviation; Q1= First quartile; Q3=Third quartile

Table 2.2. Two-way ANOVA results for basal oxygen consumption before and after E2 exposure

	Sum of squares	F-value	p-value
Basal before			
Group	29	0.008	0.991
Sex	9222	5.71	0.022
Group: Sex	1143	5.71	0.704
Residuals	59705		
Basal after			
Group	47	0.013	0.986
Sex	1863	1.1	0.301
Group: Sex	484	0.143	0.867
Residuals	62646		

Table 2.3. Two-way ANOVA results for maximum oxygen consumption before and after E2 exposure

	Sum of squares	F-value	p-value
Maximum before			
Group	1090	0.348	0.708
Sex	513	0.327	0.571
Group: Sex	2067	0.66	0.522
Residuals	57933		
Maximum after			
Group	2990	0.669	0.518
Sex	2381	1.06	0.308
Group: Sex	1989	0.445	0.644
Residuals	82642		

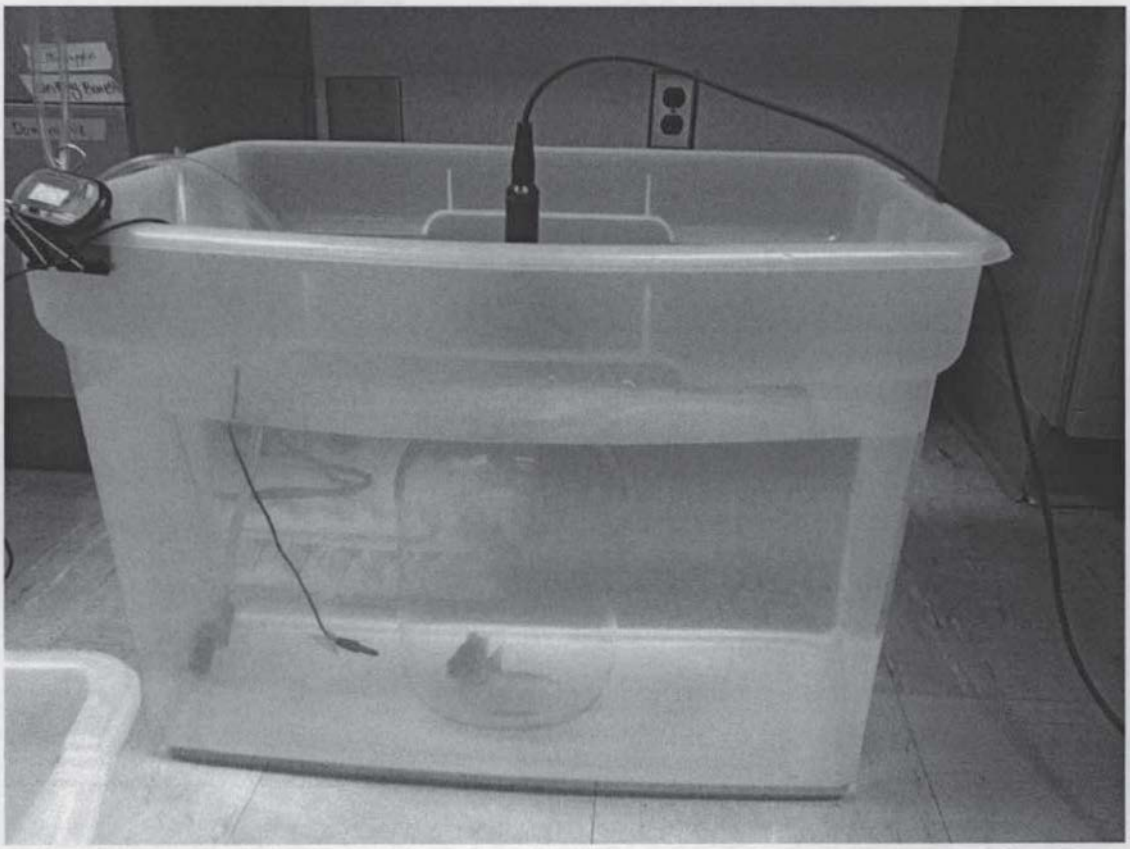

Figure 2.1. Experimental apparatus for measuring oxygen consumption in fish using a closed respirometer chamber fitted with an optical oxygen probe.

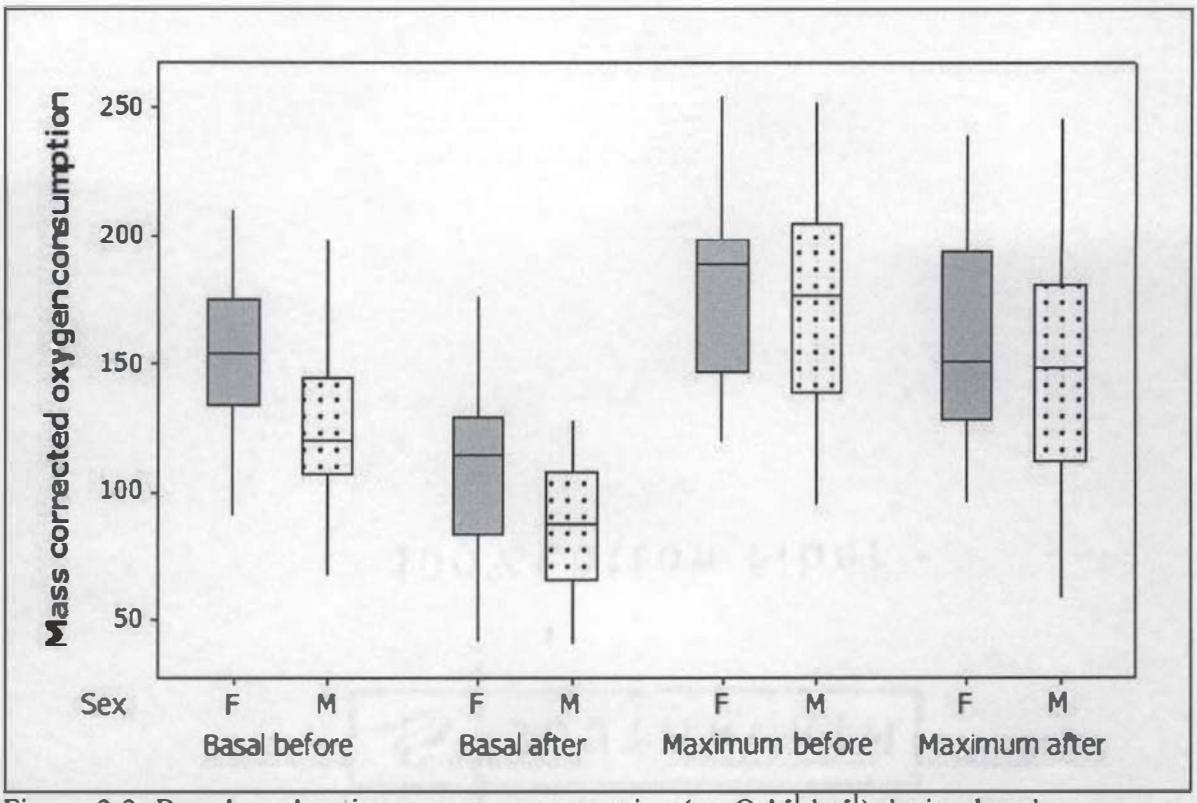

Figure 2.2. Boxplots showing oxygen consumption (mgO_2h^{-1} kg^{-1}) during basal metabolism before and after exposure in male and female fish in control and treatment groups.

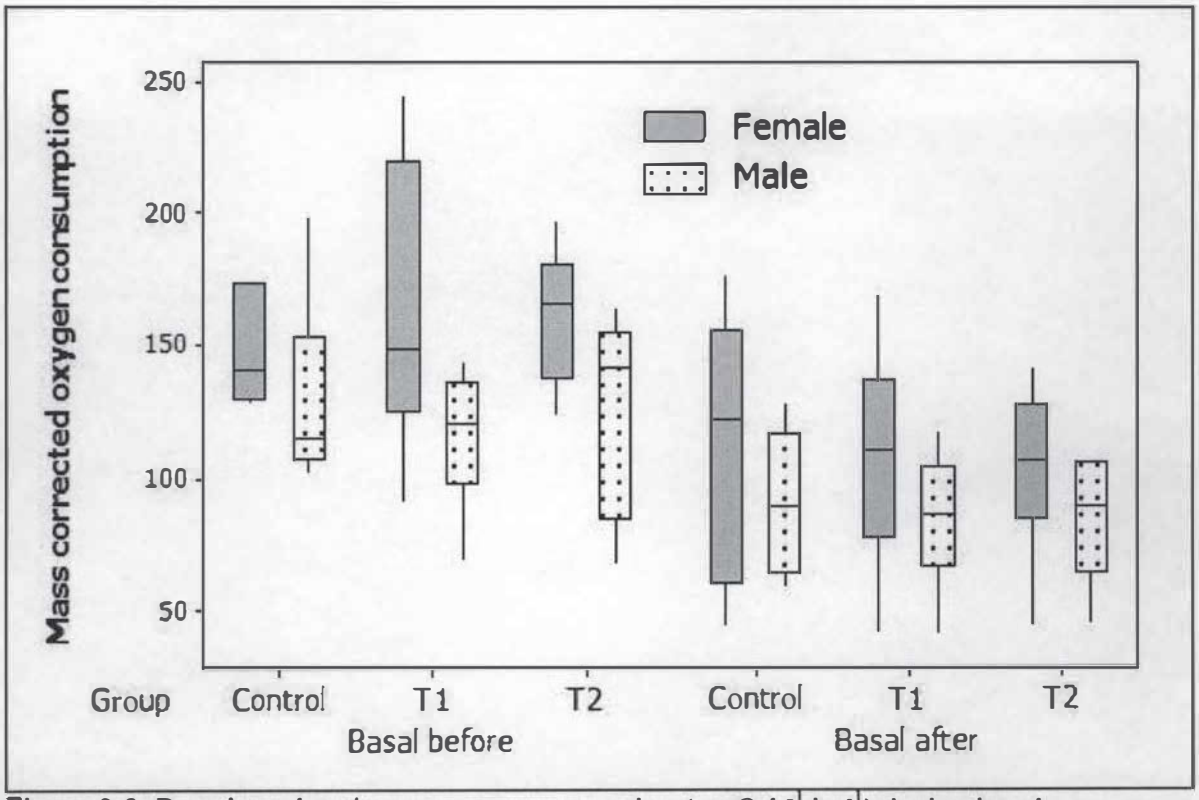

Figure 2.3. Boxplots showing oxygen consumption ($mgO_2h^{-1}\ kg^{-1}$) during basal metabolism before and after exposure in male and female fish in control and treatment groups. T1 indicates treatment group 1 (40 ng/L E2) and T2 indicates treatment group 2 (80 ng/L E2).

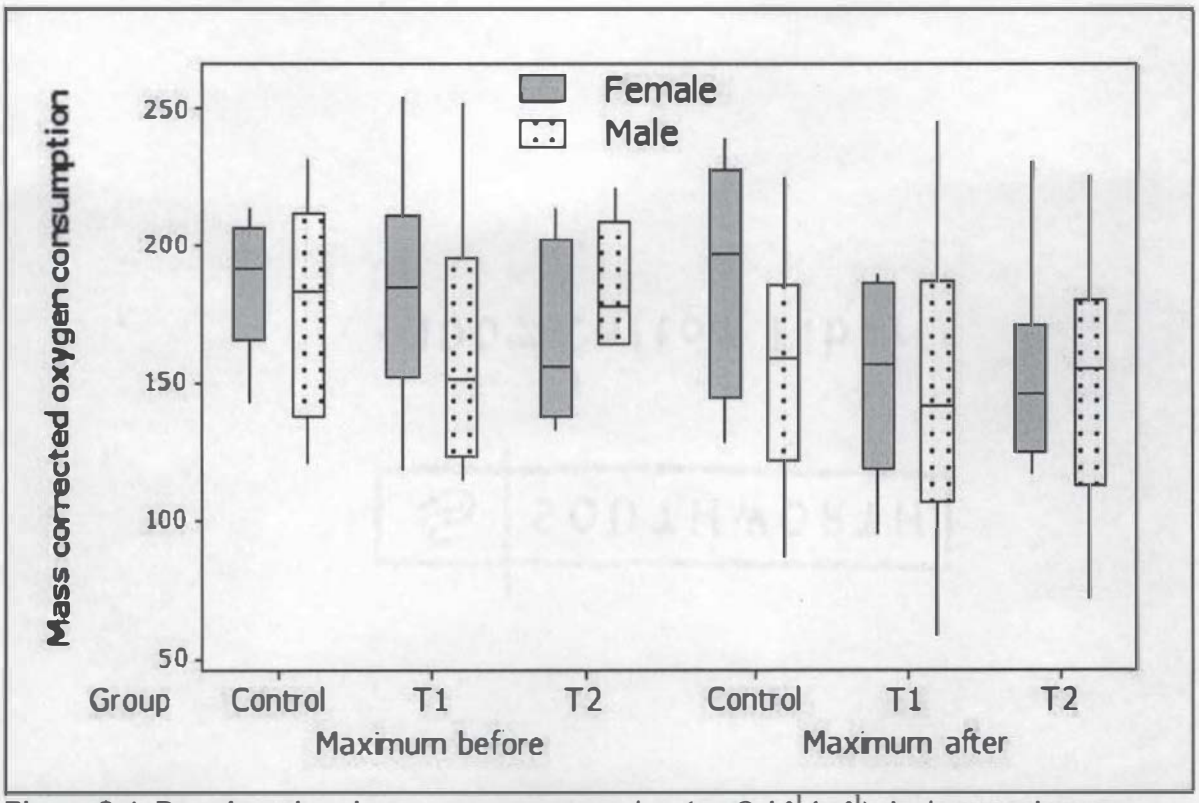

Figure 2.4. Boxplots showing oxygen consumption (mgO_2h^{-1} kg^{-1}) during maximum metabolism before and after E2 exposure in male and female fish in control and treatment groups. T1 indicates treatment group 1 (40 ng/L E2) and T2 indicates treatment group 2 (80 ng/L E2).

CONCLUSIONS, FUTURE RESEARCH DIRECTIONS, AND RECOMMENDATIONS

Estrogenic compounds, including 17β estradiol (E2), cause adverse health effects in fish. Specifically, they are known to affect reproductive systems of fish that includes feminization of male fish, alteration of sex ratio, reduced fecundity, decreased gonadosomatic index and changes in gonadal structure. Due to these unwanted effects of estrogenic compounds, contaminations of surface water with these compounds are of growing concern. However, there have been very few studies to understand the effects of estrogenic compounds other than the reproductive system. To address this gap, in this study, I evaluated the effects of E2 exposure on the basal and maximum metabolic rate, body shape changes, and histology of liver tissues in Bluegill Sunfish species by exposing the fish at ecologically relevant concentrations of E2 for three weeks.

In this study, I found that the fish exposed to E2 compounds negatively affects basal metabolism in male fish, causes loss of male secondary characteristics and alters the histological structure of liver tissues. Specifically, the basal metabolic rate in male and female fish was different before the exposure but was similar after the exposure indicating convergence of male and female fish after E2 exposure. The morphometric analysis also showed that male fish after exposure were converged towards female fish while unexposed male fish were aggregated in the other direction. This provides additional evidence that E2 exposure causes convergence between male and female fish. Histological examination revealed that there is a disintegration of hepatocytes in the liver tissues exposed to E2, which indicates that histological examination of liver tissues may be used as a biomarker to evaluate the E2 contamination in surface water. In summary,

this study showed that the effects of E2 exposure on male fish are widespread and beyond the reproductive system.

As this study was conducted in a mesocosm controlling for different variables that could affect fish metabolism and morphology, such as the water quality, temperature, and food availability, it would be interesting to evaluate how the E2 exposure will affect metabolism and morphology in the natural environment. In real life, fish have to deal with all the stressors mentioned above and as well as predation. The effect of E2 compounds on fish in the presence of several other stressors would be worth evaluating. In this study, we examined the effects of only two concentrations of E2 (40 ng/L and 80 ng/L) for 21 days. In future studies, I recommend evaluating the effects of E2 over a wider spectrum of concentrations and duration, which will help determine to know what dose of E2 and duration of exposure would trigger the negative effects in fish metabolism, morphology and histological changes in the liver tissue. As only qualitative evaluation of changes in liver histology was conducted in this study using only one liver sample from each group (control, 40 ng/L and 80 ng/L), I recommend conducting liver histological examinations with larger sample sizes from each group, which will help to quantify the changes in the liver tissues.

Nevertheless, the findings from this study provide important guidelines for understanding the adverse effects of E2 exposure in a native fish species, Bluegill Sunfish, which was not previously investigated. As this fish species has both aquaculture and recreational values in addition to being an important member of the local ecosystem, the contamination of Illinois surface water should be considered seriously. I recommend monitoring of Illinois surface water to assess the level of E2 contamination because

increasing levels of this harmful contaminant is likely to negatively affect the native fish species in Illinois. To make wise use of limited resources, monitoring around the wastewater treatment facilities both upstream and downstream would be helpful as effluents from these facilities have been linked to contaminating the surface water with E2. If there are enough resources, I would also recommend using modern technologies in wastewater treatment facilities that could process estrogenic compounds thereby preventing leaching into the surface water. Further, monitoring of fish health and diversity upstream and downstream of the discharge point may provide important clues with respect to the possible role of contaminants entering the surface water. Finally, public awareness programs to educate local residents and policy makers about the adverse effects of estrogenic compounds on fish health and local biodiversity would be extremely helpful to make suitable policies to mitigate the encourage usage of estrogenic compounds and monitoring of these compounds in the surface water in Illinois.

Appendix A

PRELIMINARY ANALYSIS OF HISTOLOGICAL CHANGES IN THE LIVER TISSUES OF BLUEGILL SUNFISH (*LEPOMIS MACROCHIRUS*) AFTER EXPOSURE TO 17β ESTRADIOL

MATERIALS AND METHODS

Bluegill Sunfish were collected from local impoundments near Charleston and randomly divided into two treatment groups (40 ng/L of E2 and 80 ng/L of E2). The details on the experimental design, E2 exposure, and fish husbandry are detailed in Chapter 1 of this thesis. The fish were euthanized at the end of an experiment of each cycle and preserved in a -20°C freezer. From these preserved specimens, one fish from each treatment group and control group were selected for histological analysis to qualitatively assess the changes in the liver tissues between E2-exposed and control fish.

Histological procedures

Liver samples of one preserved male fish from control and two treatment groups (40 and 80 ng/L E2 exposed) were excised and fixed immediately in Formaldehyde Acetic Acid Ethanol (FAA). They were kept in fixative for four days and preserved in 70% ethanol. Specimens were cut to a size of 5mmx5mm using a sharp razor blade, which was then transferred to scintillation vials for dehydration. A paraffin embedding technique followed by Hematoxylin and Eosin (H&E) staining were done to prepare the specimens for microscopic examination (Culling et al., 2014). The stained sections were viewed under a bifocal microscope in 50x and 400x magnifications.

RESULTS AND DISCUSSION

The examination of histological slides of liver tissues under 50x and 400x magnification showed differences in the structure of hepatocytes and other liver cells between control and E2 exposed groups (Figure A.1. A-F). In general, the fish livers that were exposed to E2 appeared fattier in appearance compared to control groups. Under the 50x magnification, the liver of control fish showed the distinct trabecular appearance of hepatocytes and reticular sinusoids of male fish (Fig. A.1. A). In addition, the central venule was visible with little or no connective tissue in their cell wall. However, in the fish exposed to an E2 concentration of 40 ng/L (T1), central venules were reduced in size and sinusoids were intermediate sized (Fig. A.1. B). Portal venules with indistinct bile ductules and arterioles were observed. In the group exposed to 80 ng/L E2 (T2), severe global loss of hepatocyte architecture with obstruction and disintegration of sinusoids, diffused hepatocellular basophils, and minimal cytoplasmic vacuolization was observed (Fig. A.1.C).

Under the 400x magnification, in the control group, nuclei were observed clearly stained with Hematoxylin and Eosin. Hepatocytes and sinusoids were distinct. Nuclei were more uniform and round in shape (Fig. A.1. D). However, in the T1 fish, moderate generalized disruption of hepatocytes were observed (Fig. A.1. E). In the T2 fish, severe generalized disruption of hepatocyte structure with an intracytoplasmic lipid accumulation was observed (*) (Fig. A.1. F). Nuclei pyknosis and karyorrhexis were common and moderate multifocal accumulations of inflammatory cells with occasional and locally diffused necrosis of hepatocytes were seen. In some areas, there was

thickening of the endothelial lining of sinusoids (indicated by elbow double arrow connector in Fig. A.I. F at the right end corner).

The histological changes of liver tissues of Bluegill Sunfish after exposure to E2 were examined to understand the effect E2 has on liver tissues. It was found that the E2 exposure had a negative effect on the histology of liver tissues that includes disintegration of the hepatocyte structure and other important cells of liver tissues such as the sinusoids, central venule, portal vein, and bile duct. The observed changes in the histology of liver tissues in the E2 exposed group may be due to the endocrine disruptive ability of the estrogenic compounds. The E2 might also cause stress in fish, which may lead to disruption in the bile duct and ultimately metabolism.

Several other studies conducted in different fish species also indicated a negative effect of E2-related compounds in the histology of liver tissues. In Streaked Prochilod (*Prochilodus lineatus*) living in disturbed urban streams, hypertrophic hepatocytes with degeneration of cytoplasm and nuclei were observed (Camargo & Martinez, 2007), while many hepatic nuclei were observed near the sinusoids in Channel Catfish (*Ictalurus punctatus*) that were exposed to estrogenic compound through feed (Gannam & Lovell, 1991). Vacuolated hepatocytes were also observed in Summer Flounder (*Paralichthys dentatus*) (Zaroogian et al., 2001) after E2 exposure, similar to our observations in Bluegill Sunfish. In the adult male Zebrafish (*Danio rerio*) spherical peroxisomes near liver hepatocytes were found after 15 days of E2 exposure at 10 µg/L (Ortiz-Zarragoitia & Cajaraville, 2005), which we did not observe in our study. However, their concentration was 1000-fold higher than our exposure concentration. There is also the possibility that the effects of E2 are more prevalent in younger fish that have higher

metabolic rates. An increase in sample size with observations of multiple slides from each group might help to capture the broader histopathological changes.

One of the reasons for the observance of the fatty liver in E2 exposed group may be related to the ability of E2 to convert male fish into a female. In general, female fish tend to have more fatty livers compared to male fish. The possible conversion of male to female tissues under E2 exposure might have caused an accumulation of fats in the liver. Similar to our study, increased hepatic lipids were observed in hepatic cells in rainbow trout (*Oncorhynchus mykiss*) (Cakmak et al., 2006).

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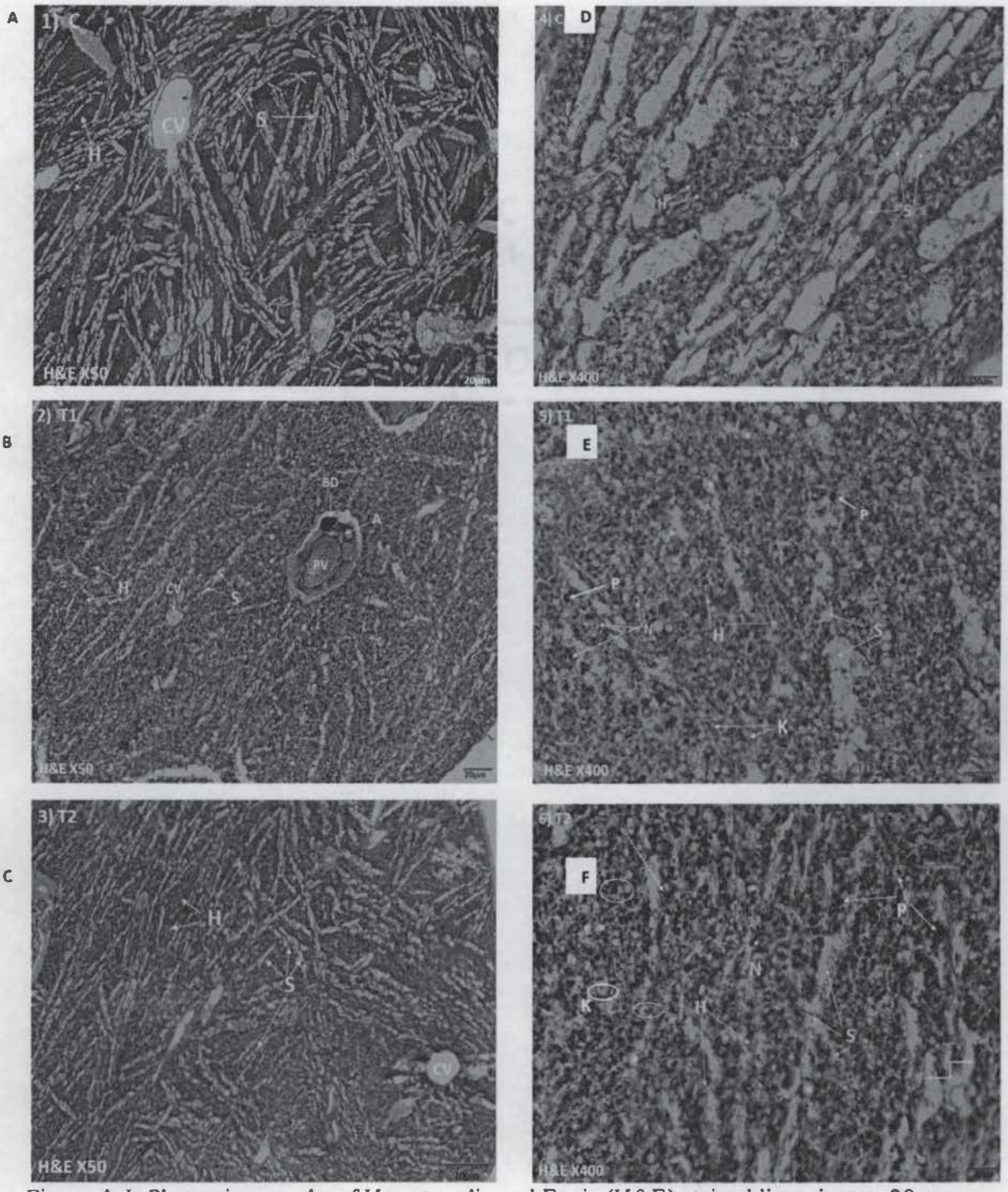

Figure A.1. Photomicrographs of Hematoxylin and Eosin (H&E) stained liver tissues, 20 μm scale bar: A: Control fish liver tissue at 50x; B: Treatment 1 (40 ng/L E2) fish liver tissue at 50x; C: Treatment 2 (80 ng/L E2) fish liver tissue at 50x; D: Control fish liver tissue at 400x; E: Treatment 1 (40 ng/L E2) fish liver tissue at 400x; F: Treatment 2 (80 ng/L E2) fish liver tissue at 400x.

A= Arteries; BD= Bile duct; CV= Central vein; H= Hepatocytes; K= Karyyorrhexis; P= Pyknosis; PV= Portal vein; N=Nuclei; S= Sinusoids;