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Assessment Of Streams And Aquatic Organisms In The Vicinity Of Birmingham, Alabama For The Presence And Biological Activity Of Endocrine-Disrupting Chemicals

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ASSESSMENT OF STREAMS AND AQUATIC ORGANISMS IN THE VICINITY OF BIRMINGHAM, ALABAMA FOR THE PRESENCE AND BIOLOGICAL ACTIVITY OF ENDOCRINE-DISRUPTING CHEMICALS

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ABSTRACT

Freshwater ecosystems play a central role in the environment. Through various processes such as nutrient recycling, groundwater recharging, and the attenuation of many pollutants these biological systems help to maintain environmental health. Unfortunately, many recent studies have identified emerging threats to these special environments and the life they sustain. One source of mounting concern is a group of compounds that interact with the endocrine systems of humans and wildlife. Known as endocrine disrupting chemicals (EDCs), these natural and synthetic chemicals may mimic or interfere with the action of natural hormones—thus disrupting the endocrine system. Multiple studies have reported negative effects associated with EDCs on both the health of humans and wildlife; however more information is needed on the reproductive effects that EDCs may pose to wildlife, in particular fish inhabiting these freshwater ecosystems. Fish serve as a useful indicator of the health of an aquatic ecosystem. To this end, the scope of the current investigation provides an integrated approach in assessing the streams and aquatic organisms in the vicinity of Birmingham, Alabama for the presence and biological activity of EDCs. It follows a preliminary study conducted at several sites along the Cahaba River in which a yeast estrogen screen (YES) detected sufficient estrogenicity in water samples to imply the possible feminization of fish. Repeated samples were collected between 2012 and 2013 providing an assessment to the extent of

seasonal and annual variation in estrogenic activity. Using the same YES assay as employed previously, it was determined that water samples collected at several wastewater treatment plant (WWTPs) outfalls contained estrogenic components in concentrations sufficient to cause endocrine disruption in aquatic organisms. Water samples were further analyzed using tandem liquid chromatograph/mass spectrometry and the predominant estrogenic components identified. At WWTPs where habitats were suitable, specimens of largescale stoneroller (*Campostoma oligolepis*) were collected and biomarkers of endocrine disruption evaluated. Such biomarkers included the presence of intersex, reduced gonad size in males and/or females, and reduced secondary sex characteristics within males. In each study described within this dissertation, chemical and biological evidence suggests that environmental estrogens were present at low-level concentrations during the sampling window represented here. Environmental estrogens detected in water samples from the WWTPs investigated illustrated non-significant seasonal influence of environmental concentration. Neither LC/MS nor the YES assay detected significant estrogen loading into receiving bodies of water via wastewater effluent. Additionally, biomarkers for endocrine disruption evaluated within *C. oligolepis* failed to detect significant differences in either histology or morphology between WWTP present and WWTP absent sites. We conclude that the WWTPs assessed in this study are not currently contributing environmental estrogens to the receiving waters in concentrations sufficient to produce discernible effects upon the fish populations within Jefferson County, Alabama; this is primarily due to their presence at low-level concentrations and intermittent persistence within receiving bodies of water.

Keywords: Cahaba River, Endocrine Disrupting Chemicals, Environmental Estrogens, Largescale Stoneroller, Wastewater Treatment Plant, Yeast Estrogen Screen

DEDICATION

Science by its virtue is a community effort. I have been more than fortunate to have ‘my community’ encourage me from start to finish. For me, this community has taken the form of my *family, mentor, and friends*. I dedicate the work represented here, both seen and unseen, to them. I only hope I can pay it back.

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LIST OF ABBREVIATIONS

AA	Amino Acid
E2	17β-Estradiol
EA	Estrogenic Activity
EDC	Endocrine Disrupting Chemicals
EE2	17α-Ethinylestradiol
EEQ	17β-Estradiol Equivalents
ER	Estrogen Receptor
LC/MS	Liquid Chromatography/Mass Spectrometry
ONPG	Ortho-Nitrophenyl-β-Galactoside
TRP	Tryptophan
VTG	Vitellogenin
WWTP	Wastewater Treatment Plant
YES	Yeast Estrogen Screen
YNB	Yeast Nitrogen Base

GENERAL INTRODUCTION

When considering water's vital role in sustaining life, it is of no real surprise that mankind's dependence on this natural resource encompasses far more than merely sustenance. In fact, water has been long utilized due to its physical characteristics as a universal solvent, mode of transportation, source of generating electric power, and for the maintenance of hygiene (Alcamo et al., 2003; Jenkins et al., 2006; Postel et al., 1996). Our collective dependence on such a versatile resource has allowed for civilizations to develop and advance. For example, in as early as 1200 B.C. Phoenicians erected some of the first aqueducts in what is now present day Cyprus (Wright, 1992). By channeling water from nearby mountain ranges, social centers were able to thrive, allowing for the development of government, religion, and commerce. From this example it becomes evident that the societal relationship with water is one born out of necessity.

In more recent times our dependence on water has only increased (Berger & Finkbeiner, 2010; Hoekstra & Chapagain, 2006; Ridoutt & Pfister, 2012). This is primarily due to the exponential growth of the Earth's population, and the subsequent use of water in both industry and agriculture (Baroni et al., 2007; Sellin et al., 2009; Tsegaye et al., 2006). Approximately 71% of the Earth's surface is covered with water. While it would seem to be an almost limitless resource, that is not the case. Only 2.5% of the water on earth is fresh water available and suitable for human use (Miserendino et al., 2011; Reimann & Banks, 2004; Stackelberg et al., 2004). Moreover, 70% of all available

fresh water is used directly by the agricultural industry, leaving a much more limited reserve of potable water resources (Barber, 2014; Baroni et al., 2007). And, while the demand for water increases with population growth, it is becoming ever more apparent this natural resource is showing signs of its overuse (Barber, 2014; Miserendino et al., 2011; Singer et al., 2002). Here overuse of such a resource is defined as the unavailability of potable water reserves and diminished water quality, such that it poses a threat to the wellbeing of humans and wildlife.

Historically, interest in water quality and its conservation originated from practical concerns. Most notably, in the mid-1800's Michael Faraday took it upon himself to investigate the unsanitary conditions of the River Thames in London, England. It was here that Faraday related the polluted condition of the River Thames with a rise in cholera outbreaks (Okun, 1996). Later it would be determined that it was the advent of the modern flush toilet, and the ill-equipped sewer systems which largely contributed to this epidemic (Newsome, 2005; Stanwell-Smith, 2010). However in more recent times, as public knowledge of water quality advanced, the scientific community became aware of other, less apparent, factors that also negatively influence water quality.

Near the end of the Second World War, a newly industrialized United States repurposed many chemical and ammunition manufacturing plants to commercial production of pesticides and insecticides. In certain instances, the very same proprietary formulas which served as nerve-gas agents and combat irritants were simply reformulated and sold to the general public for fighting home invasions of the insect variety (Katz, 2010; Robbins & Sharp, 2003; Vitols, 2010). And as the baby-boomer generation moved into suburbia, the demand for such products and their use grew accordingly. In time more

effective methods of pest eradication were required. Compounding this consumer demand were the international interests, which grew largely during the Pacific Front of the Second World War, to combat mosquito-borne illnesses such as malaria and yellow fever (Benton et al., 1994; Read et al., 2009). Here, Swedish chemist Paul Hermann Müller had a Nobel-Peace prize winning answer, dichlorodiphenyltrichloroethane (DDT) (Perkins, 1978). Hailed as the most effective contact poison for use against arthropods, it seemed the adage "better living through chemistry" held true. However, in less than two decades concerns would be mounting regarding the risks DDT posed to not only the health and safety of wildlife, but also to humans.

Though the direct effects of overexposure to specific toxicants are relatively uncomplicated to assess, in an environmental setting multiple variables generate challenges for an investigation of potentially harmful chemicals and their effects on living organisms (BERAC, 2010; Bosker et al., 2009; Wolf et al., 2014). Such factors as seasonality, environmental location, and temporality contribute to increased complexity in the investigation process (Cheng, 2003; Munn et al., 2009; Smith et al., 1999). To this end, investigators often receive reports of aberrant environmental conditions from the general population. And while this speaks to the vigilance of public awareness, the post hoc nature of such findings implies that it is too late to prevent the already-observed effects. A prime example such events was evidenced in the 1962 publication *Silent Spring*, where public reports regarding diminished avian populations ultimately contributed to the interdiction of DDT (Daston et al., 2003).

DDT is a polychlorinated compound which has detrimental effects upon the vertebrate endocrine system (Hinck et al., 2008; Kelce et al., 1995; Turusov et al., 2002).

DDT, like many other environmental toxicants, interacts agonistically with the estrogen receptor (ER) (Gray Jr. et al., 2005; Kristensen et al., 2007). Moreover, DDT is environmentally persistent, with a soil half-life of up to 15 years and a half life in aquatic systems of up to 150 years. Due to DDT's ability to persist in the environment storm events contribute to the rapid transport of it, as well as other toxicants, from terrestrial habitats into receiving bodies of water (Petersen & Tollefsen, 2011). Upon entering a body of water the natural progression of the hydrologic cycle can potentially move these contaminants into environments far from the original site of introduction. This is evidenced by the presence of DDT in the milk of lactating polar bears and the blubber of certain species of Artic seals who live thousands of miles from any direct sources of DDT (Chiu et al., 2000; Muncke, 2009; Vos et al., 2000). And, while the long-term effects of low body burdens of pesticides are unknown, numerous laboratory studies have shown that acute exposure to DDT and its breakdown products, dichlorodiphenyldichloroethane (DDD), and dichlorodiphenyldichloroethylene (DDE), impair hormonal regulation (e.g. lactation, gestation, and fertility) in a wide variety of animal classes, including humans (Hinck et al., 2009; Madenjian, 2011; Turyk et al., 2007). By December of 1970, motivated primarily by the work of Rachel Carson and pressure from the public, the United States government formed the Environmental Protection Agency (EPA) in order to enforce environmental regulations passed by Congress with the intent to protect the health of humans and wildlife (EPA, 1992). In less than two years, the EPA deemed DDT as a persistent bioaccumulative toxin (PBT) and banned its use and sales within the United States and its territories (Maguire & Hardy, 2009). However, it would not be until

two decades later that DDT would be identified as a member of a relatively new class of environmental contaminants.

In the early 1990's growing evidence indicated the existence of a previously unrecognized group of environmental contaminants with the ability to disrupt the homeostatic function of the endocrine system (EPA, 2014). One of the best known example cases during this time period involved Lake Apopka, Florida. The Tower Chemical Company was cited by the EPA for improperly discharging and storing a mixture of industrial chemicals, including DDE, near Lake Apopka (Guillette et al., 1994; USEPA, 2010). Within a decade researchers provided evidence correlating reproductive disorders observed in various fish, avian, and reptile species with the presence of those specific chemicals discharged by the Tower Chemical Company. For example, male American alligators (*Alligator mississippiensis*) from Lake Apopka were observed to exhibit elevated plasma hormone levels, and to have impaired hepatic function, reduced gonad and phallus size—indicative of overexposure to estrogens (Guillette et al., 1994; Guillette et al., 1995; Lind et al., 2004). Based upon these and other findings, the United States Congress issued the 1996 amendments to the Food Quality Protection and Safe Drinking Water Act in which the EPA was mandated to screen for the presence of endocrine disrupting effects in pesticides which could impair the health of humans and wildlife (USEPA, 2014). With this congressional authorization, the EPA's Endocrine Disruptor Screening Program (EDSP) formally acknowledged the existence of endocrine disrupting chemicals (EDCs) (2014).

An EDC is defined as: "an exogenous agent that interferes with the synthesis, secretion, transport, binding, action, or elimination of natural hormones in the body that

are responsible for the maintenance of homeostasis, reproduction, development, and/or behavior" (Kavlock et al., 1996). More recently the SWITCH Consortium of the European Union, a professional panel of researchers and water utilities consultants, recommended that EDCs be placed into one of three broad groups for further classification: 1) synthetic hormones or chemicals designed to deliberately alter the endocrine system, 2) natural compounds and their metabolites, and 3) human-made chemicals which, by chance, disrupt the endocrine system (Katarzyna Kujawa-Roeleveld, 2007). However, these classifications of a toxicant are relatively novel and the lists of such compounds are ever mounting. Combating this growing list of compounds the EPA's EDSP has developed a comprehensive screening program whereby chemicals deemed to possess the potential to interfere with normal endocrine function may be properly evaluated, and the appropriate regulatory policy, if needed, be applied (USEPA, 2014). Currently the EDSP has proposed a three-tier screening process where Tier1 identifies chemicals which could potentially meet the criterion of an EDC, Tier 2 assesses the endocrine-related effects produced by each chemical and establishes dose dependent responses in animal models (i.e. fish, amphibian, and mammal), and Tier 3 serves to create appropriate federal policies governing the use and application of the identified EDC (USEPA, 2014). At present, approximately 10,000 chemicals have been selected to undergo the EDSP's comprehensive screening process, which includes over 6,000 drinking water contaminants and over 1,000 pesticides (USEPA, 2012). Unfortunately, the comprehensive regulatory testing was initially set forth during the 1996 Congressional amendments to the Food Quality Protection and Safe Drinking Water Act, and did not commence until November of 2012. As of May 22, 2014 only two of the

10,000 chemicals had been screened for potential endocrine-related effects (USEPA, 2014; USEPA, 2012).

In order to enhance the rate of assessment of potential EDCs, the EDSP has advocated the enlistment of stakeholders into the EDSP program (USEPA, 2014; USEPA, 2011). Professionals from academia and industry with backgrounds in environmental toxicology have been sought out to provide input and expertise on the topic of endocrine disruption. Additionally, the EPA has created an Endocrine Disruptor Methods Validation Subcommittee (EDMVS) where relevant findings in the field can be discussed, scientific methods evaluated, and concerns related to EDCs addressed (USEPA, 2011).

One of the best approaches to furthering the objectives of the EDSP lies in regional investigations of the presence and effects of EDCs along the country's waterways. Studies have been conducted by the United States Geological Survey (USGS) to investigate the presence of EDCs in rivers and streams in the U.S., although the streams they studied have been primarily located in the Upper East Coast, Mid-West, and North Pacific regions (Barber et al., 2011; Colborn & Thayer, 2000; Godbout et al., 2009). Currently, few published studies exist on the possible presence of EDCs in waters flowing through the Southeastern states, including Alabama.

As early as the 1940's, reports began to surface concerning problems with the reproductive health of game fish in the United States. For example, reproductive abnormalities were observed in several largemouth bass taken from the Embarras and Sangamon watersheds of Illinois (James, 1946; Lin & Raman, 1991). Male bass from both sites possessed intersex characteristics that ranged from the presence of several

scattered oocytes per testis to the occurrence of functional testis and ovary (ovotestes) in the same individual (James, 1946). Both watersheds are located within the heavily farmed Midwestern corn belt and have a history of receiving both urban and agricultural runoff. The Embarras and Sangamon watersheds provide sinks for much of region's mixed runoff. What is more, numerous investigations report the presence of EDCs in fresh water ecosystems and the ill-effects they exert upon fish reproduction, behavior, and development (Colborn & Thayer, 2000; Gross-Sorokin et al., 2006; Jobling & Tyler, 2003; Solé et al., 2003; Sumpter, 2002). Because of these findings, it was demonstrated that pesticides (dicofol and DDT and its metabolites DDE and DDD) mimicked the female sex hormone estradiol (E2). In fact, a growing category of EDCs, known as environmental estrogens, appear to be the most pervasive class of EDCs threatening the reproductive fitness of aquatic organisms (Hinck et al., 2009; Kidd et al., 2007; Kostich & Lazorchak, 2008; Schramm et al., 2008). Unfortunately, by the time the presence of these EDCs are detected in a body of water, the exposure of wildlife has already occurred. Thus, it is important to determine the major sources of these compounds within an aquatic ecosystem and establish early indicators of endocrine disruption that can be detected and serve as warning signs before significant reproductive impairment has occurred.

For some time, fish have been considered good indicators of the quality of an aquatic ecosystem (Brammell et al., 2010; Oceanic, 1988; van der Schalie et al., 1999). As they are restricted to the aquatic environment, fish serve in the role of sentinels for the body of water they inhabit (Everaart et al., 1993; Hinck et al., 2008; Oceanic, 1988; Saaristo et al., 2010). To this end, measurable biological features may be assessed in

order to gauge the health and condition of the fish, and thus accurately reflect the status of the watershed of interest (Burger et al., 2007; Hanson, 2009; Lentz et al., 2003; Suter, 1990). A biomarker can be defined as: "any xeniobiotically induced variation in cellular or biochemical components, processes, structures or functions that are measurable in a biological system or samples" (National Research Council, 1987). Traditionally, biomarkers have been used in both invertebrate and vertebrate model systems as indicators of exposure to various toxicants (National Research Council, 1987). Because much of a fish's reproductive development and function is governed by endogenous hormones, specific biomarkers related to endocrine function can be monitored as indicators of exposure to EDCs. Hence, alterations of natural reproductive processes may reflect exposure to environmental EDCs. One biomarker that has been effective at indicating exposure to environmental estrogens is the egg yolk protein precursor vitellogenin (VTG) (Hinfray et al., 2010; Wunschel et al., 2005). Expression of the VTG gene is estrogen dependent and, as such, is normally limited to females. Its detection in the blood of male fish or immature fish is an indicator of exposure to an estrogen or estrogen mimetic. Other popular biomarkers of EDC exposure include abnormal secondary sex characteristics, altered reproductive behavior, reduced gonad size, and the presence of hermaphroditism in organisms that normally have separate sexes (Edwards & Guillette Jr., 2007; Guyón et al., 2012; Hayes et al., 2002; Rhee et al., 2011).

With the risk that environmental estrogens may pose to the health of humans and wildlife, a growing number of U.S. and European studies are concluding that current bioassessments of watersheds should include both chemical screens for estrogenic activity (EA) (e.g. Yeast Estrogen Screen (YES) assay, Liquid Chromatography/Mass

Spectrometry (LC/MS)) and reproductive biomarkers of fish (Allen et al., 2008; Becker et al., 2014; Bistan et al., 2012; Houtman et al., 2007). By identifying locations within watersheds where the signs of endocrine disruption are most prevalent, the major sources of EDCs can be identified.

One point source of EDCs that has been reported by numerous studies is the effluent from wastewater treatment plants (WWTPs) (Blewett et al., 2013; Fernandez et al., 2008; Saaristo et al., 2014; Sousa et al., 2010; Wick et al., 2009). Present WWTP designs have been shown to be rather inefficient at removing various organic containments from their influent—including EDCs (Kostich & Lazorchak, 2008). In fact, studies worldwide have consistently shown that treated WWTP effluent has estrogenic activity, identifying WWTPs as significant sources of environmental estrogens in waterways (Jenkins et al., 2009).

A major objective of this study was to analyze water samples from selected streams in the vicinity of Birmingham, Alabama for the presence of biologically active concentrations of EDCs - specifically environmental estrogens. Additionally, fish living in those streams were evaluated for biomarkers of endocrine disruption. As WWTPs have been identified as potentially important sources of EDCs, streams receiving effluent from Jefferson County, Alabama's municipal WWTPs served as the field sites for both water and fish collections. Many of these WWTPs discharge into the Cahaba River, a biological treasure supporting aquatic species diversity that surpasses any other temperate river of its size in the world (Onorato et al., 2000); or directly upstream of intakes for public drinking water treatment plants.

Part of this investigation followed up on a previous study, conducted at several sites along the Cahaba River, where estrogenic activity was detected in water samples, thus indicating that EDCs could be a problem in local rivers (Jackson, 2010). In the current study, seven major Jefferson County, Alabama public wastewater treatment plants were studied to investigate (1) the possible presence of EDCs in their effluents, and (2) possible endocrine disruption in fish living in the receiving waters. Water samples were collected and characterized two ways: 1) analysis using Liquid Chromatography/Mass Spectrometry (LC/MS), allowing for the identification and quantification of specific environmental estrogens and 2) analysis using a Yeast Estrogen Screen (YES) assay which provides a measure of the total EA present within a given water sample, but does not identify the bioactive chemicals. Specimens of the largescale stoneroller (*Campostoma oligolepis*) were obtained downstream from selected WWTPs to evaluate biomarkers of endocrine disruption, specifically, the presence of ovotestes in males, reduced ovary size in females, skewed sex ratios, Fulton's condition factor, and both hepatosomatic and gonadosomatic indices - all potential biomarkers of endocrine disruption.

The overall objectives of this study were to investigate whether environmental estrogens are present in the effluents of WWTP effluents in Jefferson County, Alabama in concentrations sufficient to comprise a significant threat to the reproductive health of fish in the receiving waters and to determine whether WWTPs are a significant route by which EDCs enter local waterways. The results have been composed as two manuscripts to be submitted for publication in the peer-reviewed scientific literature. The first paper reports on analyses of water samples using both the YES and LC/MS to quantify the

amount of estrogenic activity in water samples and to analyze the concentrations of specific estrogens. The second paper reports on the evaluation of biomarkers in largescale stonerollers (*Campostoma oligolepis*) collected downstream of WWTPs for evidence of endocrine disruption.

ASSESSMENT OF WATER SAMPLES FROM MULTIPLE MUNICIPAL WASTEWATER TREATMENT PLANTS FOR PRESENCE OF ESTROGENIC COMPOUNDS IN JEFFERSON COUNTY, ALABAMA

by

BRYAN S. ARWOOD AND ROBERT A. ANGUS

IN PREPARATION FOR ENVIRONMENTAL SCIENCE & TECHNOLOGY

FORMAT ADAPTED AND ERRATA CORRECTED FOR DISSERTATION

ABSRACT

Numerous investigations have clearly demonstrated the presence of endocrine disrupting chemicals (EDCs), specifically environmental estrogens, in the effluent of wastewater treatment plants (WWTPs). While most studies in the United States have focused efforts of detecting environmental estrogens around the Potomac River and throughout the North West, few studies have been performed in the southeastern U.S. In this study, we conducted a two year (2012-2013) survey investigating the possible presence of environmental estrogens in the effluents of six municipal WWTPs located in Jefferson County, Alabama. This study was one portion of a multi-tiered EDC survey which used the Yeast Estrogen Screen assay in addition to liquid chromatography/mass spectrometry. Although estrogens were sporadically detected in the outfall water from several WWTPs, the concentrations were well below those expected to cause endocrine disruption in aquatic organisms in the receiving waters.

INTRODUCTION

For decades the unrestricted use of many anthropogenic compounds has been correlated with environmental contamination and detrimental effects on the health of humans and wildlife (Daston et al., 2003; Doney, 2010; Matthiessen, 2003). In recent times, the most noteworthy were the 1978 Love Canal incident, the 1976 Seveso dioxin cloud of Milan, and the decades of unrestricted application of DDT for insect control. However, it was not until the early 1990's that the compounds involved in these incidents were properly classified by the scientific community based on their modes of action (Hotchkiss et al., 2008a). The term endocrine disrupting chemical (EDC) was first used in

1993 to classify a growing group of compounds exhibiting the potential to disrupt the homeostatic endocrine function of living organisms. In 1996 the United States Environmental Protection Agency (USEPA) accepted the definition of an EDC as: "any exogenous agent that interferes with the synthesis, secretion, transport, binding, action, or elimination of natural hormones in the body which are responsible for the maintenance of homeostasis, reproduction, development, and/or behavior" (Kavlock et al., 1996). Still, over two decades after the conception of the term, little is known about the long term *in vivo* effects of these compounds on the health of humans and wildlife (Greally, 2011; Hood, 2005; Thomas et al., 2007).

Recognized as significant routes of nutrient overloading to aquatic ecosystems, wastewater treatment plants (WWTPs) have also been identified as primary contributors of EDCs (Chang et al., 2011; Ra et al., 2007; Stackelberg et al., 2004; Wright-Walters &Volz , 2007). Although the complex mixture of organic compounds contained in the influent are subject to multiple physical and biological processes to remove contaminants before being discharged, numerous studies have identified EDCs remaining in the effluent of WWTPs (Celiz et al., 2009; Jürgens et al., 2002; Liu et al., 2009; Robinson & Hellou, 2009). Current findings indicate that, when operating effectively, WWTPs achieve only an average 85% reduction in EDCs present in the influent (Manickum & John, 2014). Additionally, other investigations have detected the presence of EDCs in the receiving waterway as far as 10 km downstream of the WWTP effluent discharge site (Balaam et al., 2010). Several studies have also shown that certain classes of EDCs may remain bound in nearby sediment, becoming shielded from degradation, as well as leading to the accumulation of high concentrations of these containments in relatively

confined localities (Grund et al., 2011; Luo et al., 2011; Peck et al., 2004; Reddy & Brownawell, 2005; Rickson, 2014). Added concern stems from rain events which may mobilize the sediments and liberate the sediment-bound EDCs, thus rendering them bioavailable (Jenkins et al., 2009). Given the known risks of environmental EDCs to aquatic wildlife and humans, considerable efforts have been made to detect and quantify EDCs in treated wastewater (Carvan et al., 2008; Celiz et al., 2009; Savage & Diallo, 2005).

Both national and international efforts have focused on the investigations of EDCs in the proximity of WWTPs, however a variety of factors make this task challenging. In almost every instance EDC concentrations in sewage are associated with human activity, thus an inherent variability exists in the concentrations of EDCs found in WWTP effluent discharged by a municipality at a given time (Carlisle et al., 2009; Hotchkiss et al., 2008a; Sumpter et al., 2006). Furthermore, some investigations have noted a correlation between the specific EDCs in treated wastewater and characteristics of the community providing the influent. For example, the prevalence of various environmental androgens, originating from the musks of colognes, has been shown to occur with higher frequency around urban townships; or, the occurrence of environmental estrogens (i.e. EE2, E2, and E1) in the effluent of female school dormitories (Boyd et al., 2004; Carballa et al., 2004). Weather and geography have also been shown to complicate detection efforts (Jonkers, et al., 2009; Rickson, 2014; Shore et al., 2004; Shumway et al., 2007). A primary example the temporal influence on wastewater characteristics is variability in the retention time of WWTPs; whereby both the wastewater processing time of the plant and variations in human activity generate differing outputs of detectable

organics in effluent streams (Collier, 2008; Hemming et al., 2004; Veach & Bernot, 2011; Wittmer et al., 2010). In addition, seasonal variability in effluent EDC concentrations may be affected by the amount of rainfall (Burger et al., 2007; Ferguson et al., 2008; Jin et al., 2011; Katsiadaki et al., 2012). Stormwater, if it enters the sewer system, will dilute human waste and any EDCs contained therein. In addition, rain has been shown to resuspend EDCs in the sediments and to modify stream channel geometry, thus altering sediment deposition and particle binding (Duong et al., 2010; Peck et al., 2004; Trimble, 1997). Many studies also identified temporal and seasonal variation in the concentrations and types of EDCs present in wastewater effluent (Alvarez et al., 2009; Choi et al., 2008; Fernandez et al., 2008; Wittmer et al., 2010). Despite the variability in WWTP effluent makeup, studies have consistently detected one particularly pervasive class of EDC in municipal WWTP effluents: estrogens (Elsby et al., 2000; Rankouhi et al., 2004; Sumpter & Jobling, 2013; Thorpe et al., 2001).

On every continent, environmental estrogens have been detected either in soil and water samples, or accumulated in the adipose tissue of multiple species (Betts, 2010; Christen et al., 2010; Kidd et al., 2007; McLachlan et al., 2006). The concentrations of environmental estrogens reported vary. However even very low concentrations may be cause for concern. *In vitro* assays assessing human granulosa cell function have concluded at least one type of environmental estrogen, 2,3,7,8-tetrachlorodibenzo-p-dioxin , can disrupt steroidogenesis by inhibiting mRNA production and steroid secretion at the femtomolar range (Baldridge et al., 2015; Schlecht et al., 2006; Sheehan et al., 1999; Snyder et al., 2008; Welshons et al., 1999). The ubiquity of environmental estrogens results largely from synthetic estrogens present in pharmaceuticals, chemicals

with estrogenic activity in cosmetics and plastics, and through the catabolism of endogenous estrogens where metabolites are found in excreta (Chen et al., 2012; Muncke, 2009; Turusov et al., 2002). Further risk associated with the presence of this class of EDCs is due to the conservation of the estrogen receptor (ER) across a wide variety of species, and its relative promiscuity in binding ligands (Richter et al., 2007; Rider et al., 2009; Shanle & Xu, 2010). These characteristics further confound the ability to accurately identify which chemicals will bind to the estrogen receptor and, as a consequence, be added to the ever growing list of new environmental estrogens known as estrogen mimetics (e.g. Bisphenol-A, Triclosan, Nonoxynol-9) (Lin & Janz, 2006; Silva et al., 2007; Watson et al., 2007).

Numerous studies, done both in the United States and Europe, have investigated the effects of environmental estrogen exposure on various animals (Alvarez et al., 2013; Hannah et al., 2009; Hinck et al., 2008; K+¦hler et al., 2007). With some of the earliest reports linking environmental estrogens to impaired endocrine function in fish populations, it is of no surprise these organisms have been popular model organisms for studies of endocrine disruption and detection (Kidd et al., 2007; Sumpter, 1998). Due to their water-dependent life cycle, fish have long been considered good indicators of the overall water quality of a region, and to provide insight to the particular chemical pollutants in a body of water (Carvan et al., 2000; Oceanic, 1988; van der Schalie et al., 1999). However, the use of fish as test organisms for environmental screening is not without limitations. While various health indices such as Fulton's Condition factor (K), the gondosomatic index (GSI), hepatomsomatic index (HSI), and histopathology provide valuable information useful for detecting endocrine dysfunction, they alone do not

indicate that an EDC is the agent of causality (Hanson, 2009; Sepulveda et al., 2002; South & Ensign, 2013; Wolf et al., 2014).

In order to conduct a more complete investigation into the possible presence and effects of EDCs, it is becoming standard practice to integrate both chemical and biological water quality analysis (Johnson et al., 2009; Wolf et al., 2014). Chemical methods of water analysis, e.g. High-Performance Liquid Chromatography (HPLC), Gas Chromatography (GC), and Mass Spectrometry (MS), allow for the detection and quantification of specific compounds of interest, but when coupled with biological assays (e.g. Yeast Estrogen Screen (YES) assay, MCF-7 cell proliferation assay) much stronger conclusions may be made regarding the possible biological effects on the health of wildlife in a given watershed (Swart et al., 2011). Additionally, utilizing a combination of two or more techniques increases the likelihood of detecting unsuspected EDCs. For example, while LC/MS can detect extremely low concentrations of known EDCs, this method of chemical analysis is restricted to the detection of only those compounds for which an internal standard has been prepared. However, of the compounds that are detected, a high confidence exists in the validity of both the identification and estimated concentration of that chemical. Similarly, biological testing, like that of the YES assay, offers comprehensive screening of a water sample, allowing whichever contaminant(s) may be present in a water sample to be detected as long as they interact with the appropriate cellular receptor. Biological assays do not identify the precise contaminants. However, due to the promiscuity of the estrogen receptor, the biological assay has a greater chance of detecting novel compounds that might be missed in the more specific chemical analyses.

In this study, we investigated the presence of estrogenic activity (EA) in the effluents and nearby receiving waters of six municipal WWTPs in Jefferson County, Alabama. While other investigations have been performed in the United States, few have been conducted in Southeast region (Chambers & Leiker, 2006; Jackson & Sutton, 2008; Kolpin et al., 2002; Oblinger et al., 2007). Moreover, many studies have focused on short-term investigations with periodic sampling, the current study consisted of sampling each plant every month (when not prohibited by weather conditions) for the span of two years using the integrated analysis approach (Hinck et al., 2006, 2007, 2008). The objective of this study was to determine whether environmental estrogens are present in the receiving waters of local WWTPs, and whether the WWTPs are contributing to the overall estrogenicity of these waters downstream of each plant. This was done by testing water samples for estrogenic activity using a modified version of the YES assay and identifying specific estrogens present in the samples by analysis using LC/MS.

MATERIALS AND METHODS

Sample Collection and General Preparation

Water samples were collected in 1L acid-washed, glass bottles. Thoroughness of the washing procedure was validated using reverse-phase high pressure liquid chromatography (HPLC) and a subsequent YES assay to assay washed glassware which had previously contained deionized water with E2. At each plant surveyed, water samples were collected at three locations: 50 meters upstream, 50 meters downstream, and directly from the effluent source (prior to outfall mixing) (Table 1). Water samples were

collected as close as possible to mid-stream and mid-depth so as to best capture mean up- and downstream environmental conditions. Sampling was not conducted within 72 hours of a rain event of one or more inches, or during an active rain event. Immediately following collection, water samples were spiked with 0.75 mL methanol (HPLC-grade Fisher Brand) and stored on ice in a dark container to prevent potential degradation of organic components via bacterial metabolism and UV photolysis.

The water samples were processed immediately upon arrival at the laboratory. To remove suspended particles, the samples were first passed through 1.76µm and then 0.46µm disposable, woven glass filters (Millipore). The filtered water samples were passed through solid-phase extraction (SPE) cartridges (10mL, C-18; Varian Walnut Creek, CA, USA) which had been primed with 1mL methanol and 1mL deionized (DI) water. C-18 SPE were selected for this experiment as they have the highest binding affinity for the broadest range of non-polar organics and, lowest binding affinity for polar compounds. The columns were then sealed with Parafilm and stored at -13°C until elution. Organics were eluted from the SPEC columns with 6mL of HPLC grade methanol. Extraction efficiency for this procedure was determined in a previous study to be 88.9% (Arwood et al., 2011). Each sample was then dried under a nitrogen blower at 17ºC and stored in airtight sterile containers at 4ºC until further analysis.

Yeast Estrogen Screen

YES assays were performed using a modification of a protocol from Fox et al. (2008). The YES assay used a strain of genetically engineered yeast cells, kindly

provided by Charles Miller, with a plasmid containing the gene for the human estrogen receptor α, an estrogen response element (ERE), and the β galactosidase reporter gene.

The yeast cells used in the YES assay were *trp 1* deletion mutants. The cells were grown in a yeast nitrogen base (YNB) medium containing all essential free AAs, except TRP. Because the plasmid contains a functional *trp 1* gene the tryptophan-lacking medium contraselects any yeast cells that have discarded the plasmid. At the beginning of an assay, a single colony of yeast cells was transferred from solid media to 3mL of liquid media consisting of 100x AA –TRP-glucose YNB and incubated overnight at 30ºC. After the yeast cells reached saturated growth phase, the cell density was diluted to produce a final cell concentration of $4x10^5$ cells/ mL in each well of the culture plate.

The dried extracted water samples were reconstituted with a volume of dimethyl sulfoxide (DMSO) such that the final concentration after inoculating a well with 2µL of the sample produced a concentration 2X environmental. For each 96-well plate loaded with water samples a standard curve was run concomitantly by inoculating a series of cells with a known concentration of E2, ranging from 5ng/L to 0 ng/L at their final concentration. After incubation for 18 h, cell density was measured using a Bio Rad iMark microplate reader at 600 nm. The cells were lysed using a Lac z buffer containing the chromogenic substrate, ortho- nitrophenyl-β-galactoside (ONPG). The tissue plate was incubated for up to 30 minutes with agitation at 37ºC. The incubation was ceased by addition of 50 µl 1M of sodium carbonate when positive control wells were noticeably yellow and the absorbance of each well was read at 405 nm.

The amount of estrogenic activity in each plate well was quantified as Lac z units using the formula (Fox et al., 2008):

$$\text{Lac z units} = \frac{1000 \times (\text{ABS}_{405}\ \text{test sample} - \text{ABS}_{405}\ \text{negative control})}{\text{min} \times \text{mL} \times \text{ABS}_{600}}$$

Where ABS_{405} and ABS_{600} are the absorbances of the wells at 405 and 600 nm, respectively, min. is the development time in minutes, and mL is the volume of the exposed cells and medium which was added to the Lac z buffer (Fox et al., 2008). The estrogenic activity of each sample, as estrogen equivalents (EEQs) was calculated by comparison to the Lac z values of a standard curve, run concurrently, with known concentrations of E2. Lac z units were obtained using the formula above for each water sample collected. Next, from the standard curve the lowest E2 dose to generate the maximum Lac z (Lac z MAX) value was selected in order to quantify the EEQ for each Lac z value obtained. The EEQ for each sample was EEQ was derived by multiplying the concentration for the lowest dose to cause the Lac Z MAX with the average Lac z units of a given sample as a percent of 100. Each sample was assayed in quadruplicate and a mean and standard deviation was calculated. Due to preliminary results indicating estrogenic activity below the detection limit (BDL), yeast cells were exposed to environmental samples twice the environmental concentration to ensure proper ligand binding for assay reporting. Table 2 report the annual means of EA at twice the environmental concentration for the 2012-2013 seasons as reflected by the YES assay.

Liquid Chromatography/Mass-Spectrometry

Those samples which were designated to undergo LC/MS analysis received 100µL addition of D4-estradiol/D3-estriol (5 ng: 5 ng) to serve as internal standards (IS). By adding standards to the samples prior to processing, analyte loss during the extraction process from the SPEC cartridge could be quantified. Additionally, samples were derivatized using 200 µL dansyl chloride after elution from the SPEC cartridge. Processing of samples through the SPEC cartridges followed the same procedure as above, except that organics bound by the filter were extracted using 6 mL of 10% methanol in tert-Butyl-methyl ether (TBME) solution. Following the extraction process, samples were immediately dried using a nitrogen blower at 13ºC and reconstituted in 400µL 20:80 5mM ammonium acetate/acetonitrile. Separation of the derivatized estrogen compounds was performed using a Kinetics C18 column with a linear gradient consisting of 5 mM ammonium acetate buffer and acetonitrile; each contained 0.5% formic acid (400 µL/min). Peaks generated between a retention time of 9-14 minutes were evaluated against a library of estrogen standards (E1, EE2, αE2, βE2, E3 and IS) added to each analysis, both the standards and IS where used during each analysis conducted. Sample detection was performed with an AB Sciex 4000 QTRAP mass spectrometer employing positive ion electrospray ionization (450°C at 5kV) and mixed-reaction-monitoring. This method of analysis allowed for characterization both the peak area and unique retention time of the peak(s) of interest for each water sample analyzed. Sample detection yielded up to 100% accuracy in analyte recognition. This was validated by comparing those peaks of interest present within environmental samples to the series of estrogen standards mentioned above at known concentrations ranging from 0.5 ng/L to 20 ng/L thus, serving

as a calibration curve for each sample analysis. Finally, analyte identification and quantification was performed by comparing the fixed concentration of the IS as a proportion between chromatogram peaks.

Statistical Methods

All summary statistics are expressed as mean ± standard error. In the YES assay, estrogenic activity was measured in Lac z units and represented in 17β-estradiol equivalents (EEQs), thus reflecting EA of the sample. To determine whether or not each WWTP was contributing to significant EA loading into receiving bodies of water mean EA activity was compared between each collection zone per plant over the 2012-2013 seasons. This was performed by determining the difference in EA between upstream and downstream, and effluent and upstream samples. After differences in EA between collection zones were established, statistical analysis could proceed. In this study both a 1-sample t-test and its non-parametric equivalent the Wilcoxon signed rank test were used to evaluate the statistical significance of EA stream loading per WWTP surveyed. Initial analysis employed use of a 1-sample t-test to detect whether or not EA activity contributed to each sampling zone was significantly different than 0 ng/L. Because parametric tests are appropriate only for normally distributed data, the Wilcoxon signed rank test was also used as a compliment to the 1-sample t-test analysis. Confidence in a statistical conclusion is improved if both parametric and non-parametric methods of data analysis reach the same conclusion. For all statistical tests, the cut off for statistical significance was set at $p \leq 0.05$.

Results

YES Assay

In this study both the parametric 1-sample t-test and the non-parametric Wilcoxon signed rank test were utilized to determine whether or not WWTPs were contributing to EA activity in receiving bodies of water at concentrations significantly different than 0 ng/L. Environmental estrogen loading for each plant was determined by comparing the difference in EA between sampling zone (i.e. effluent EA- downstream EA; effluent EA-upstream EA). From the outcome of these tests it was established that WWTPs within Jefferson County, Alabama were not contributing to EA significantly different from 0 ng/L during the 2012-2013 sampling seasons even when reported at twice the environmental concentration (Table 2). Persistent, low-level concentrations of EA were consistently detected at every WWTP surveyed during this investigation and reported at twice the environmental concentration (Table 2). As it was determined that no one plant contributed mean EA concentrations significantly different than 0 ng/L—variation attributed to either season, weather, or plant design could not be determined.

LC/MS Analyses

LC/MS results (Table 3 a & b) indicated that, while analogs of estrogen were detected; they appeared to occur sporadically in an unpredictable manner, with no discernible pattern. Data from LC/MS analysis were consistent with results from the YES data in that, none of the WWTPs appeared to be contributing estrogens to receiving bodies of water in very high concentrations. The highest concentration of an estrogen detected in this study was reported for estrone at 8.74 ng/L, which was detected in the

effluent of Plant 5. Samples containing the absence of estrogen analytes or samples with analytes below the detection limit (0.5 ng/L) occurred more frequently than did samples where those compounds were detected. Of the five estrogen analogs investigated, estrone occurred most frequently at all WWTPs surveyed over both collection seasons.

DISCUSSION

The current study is the first of a two-part investigation assessing the presence of estrogenic activity in the effluent and receiving waters of seven WWTPs over a two-year period (2012-2013). When weather permitted, water samples were taken directly from the effluent of each WWTP, as well as upstream and downstream of the outfall. Each sample was screened for total estrogenic activity using a using a YES assay and analyzed for specific estrogenic compounds using LC/MS.

The results from this study were similar to other investigations reporting low concentrations of EA and an intermittent occurrence of environmental estrogens in the receiving bodies of water near WWTPs (Roberts et al., 2015). While it was determined that mean WWTP contributions of EA did not significantly differ from 0 ng/L in the receiving bodies of water between 2012-2013, consistently low levels of EA (<.1 ng/L EEQ) were detected in across all WWTPs surveyed. For this reason, inferences regarding the significance of seasonal variability, weather, or plant design could not be made. Near uniform consensus among numerous investigations indicate the levels of EA reported in the current study are well below concentrations known to cause abnormal reproductive, physiological, or behavioral biomarkers for environmental estrogen exposure (Ankley et al., 2010; Melzner et al., 2009; Miller et al., 2013; Saaristo et al., 2010). For example, in

one recent study Kidd et al. reported the near collapse of a fathead minnow (*Pimephales promelas*) in an experimental holding pond from the chronic addition of EE2. From their report, daily doses of EE2 at 5-6 ng/L over a seven year period led to a virtual collapse of the entire fish community (Kidd et al., 2007). In reports like these the persistent addition of potent, high-dose EDCs confirm those negative effects associated with environmental estrogens; however the inconsistent, low-dose concentrations of estrogens reported in our investigation do not appear to pose such risks.

While the YES assay employs an efficient means of evaluating total EA for multiple environmental samples, it lacks the specificity required to quantify individual compounds of interest—for this LC/MS was implemented (Miller et al., 2009). During both sampling seasons no discernable trends for environmental estrogen loading into receiving bodies of water could be detected using LC/MS. Additionally, of those water samples evaluated in this study, samples lacking the detectable presence of environmental estrogens occurred more frequently than water samples where environmental estrogens were present. The discrepancy among these two methods of detection is likely due to those unit processes employed at WWTP for the reduction of organic waste. While modern WWTP are not designed to remove emerging contaminants like environmental estrogens, certain methods of containment reduction have proven effective at reducing EDCs within treated wastewater. For example, during the activated sludge process ,which most WWTP implement, microbes present within sludge basins reduce EDC activity by degrading such compounds through reduction-oxidation (redox) reactions (Jenkins et al., 2003; Kirkwood et al., 2006; Liu et al., 2009). During pilot plant trials activated sludge has proven to exhibit an almost complete removal of EDC from wastewater (Hashimoto

et al., 2007; Petrović et al., 2003; Thayanukul et al., 2010). Additionally, reductions in EDC activity may also be due to WWTPs that utilize UV-sanitization during the final stages of wastewater reclamation, which were present at all WWTPs surveyed. Through the use of UV light remaining organic contaminates, including EDCs, have been proven to become photo oxidized within plant effluent, with near complete removal of EA (Arwood et al., 2011; Kunz & Fent, 2006; Liu et al., 2003). Findings from a previous published study utilizing advanced oxidation techniques (AOTs) to degrade EE2 indicated that briefly exposing estrogens to similar processes proved to effectively reduce EA. Interestingly, while reductions in EA activity were noted via a ligand-binding assay, signature peaks synonymous with EE2 were detected through high-performance liquid chromatography (HPLC) (Arwood et al., 2011). Findings such as these indicate redox reactions affect ligand-receptor binding through minor distortions of the steroid structure which cannot be completely detected under chromatographic analysis (Arwood et al., 2011). As both activated sludge basins and UV-sanitization were used during the wastewater reclamation process at these plants this is a probable scenario for water samples where the presence of estrogen was detected in LC/MS, but EA was absent or reduced in the YES assay.

Since the inception of the term EDC in 1993, hundreds of studies have reported the presence of environmental estrogens in the effluents of WWTPs (Hotchkiss et al., 2008). Currently, EDCs and environmental estrogens found in the effluent of WWTPs have been deemed to be an epidemic (Carreau, 2000; Christen et al., 2010;Kidd et al., 2007; Muncke, 2009). However, even in studies where low-dose or intermittent detection of environmental estrogens are reported multiple factors must be considered in order to

reduce potential threats to the health of humans and wildlife. The finding of high concentrations of estrogenic activity in treated wastewater appears to occur most frequently in regions which are highly urbanized, have impaired, minimal, or nonexistent wastewater infrastructure, or lack any active environmental regulatory body (Boyd et al., 2004; Jackson & Sutton, 2008). To date, some of the lowest concentrations of environmental estrogens reported in treated wastewater were in locations with well-maintained WWTPs, which are only moderately urbanized, and pursue proactive approaches to environmental conservative policy (Krein et al., 2012; Roberts et al., 2015; Servos et al., 2005). In this study, none of the plants surveyed consistently discharged effluent containing EA greater than environmental quality standard (EQS) set forth by the European Union for EE2 (0.035 ng/L), with the highest measured concentration of EA determined to be 0.03ng/L EEQ at twice the environmental concentration (Johnson et al., 2013). However, these data alone do not indicate a reduction in environmental estrogens exclusively due to current Jefferson County WWTPs. Other factors also account for relatively low levels of EA in municipal WWTP effluent (Kasprzyk-Hordern et al., 2008; Roberts et al., 2015).

As permission to obtain influent samples was not granted by Jefferson County Environmental Services, we cannot report the removal efficiency of EDCs for the WWTPs reported in this investigation. Because similar investigations have illustrated physiologically relevant levels of EA discharged from the effluent of WWTPs it is likely multiple factors may be influencing environmental estrogen detection. From past investigations and industry reports multiple factors my negatively impacted the removal efficiency and, subsequent contaminant breech of a WWTP (Black &Veatch, 2014; Clara

et al., 2005a; Clara, et al., 2005b). Primarily these failures in waste removal from effluent flow stem from sub-par plant management, structural failures, and design flaws (Black &Veatch, 2014). Indirect impacts on WWTP waste removal efficiency may also include increase population growth and flooding which exceeds a particular plant's design specifications for proper day to day operations (Konrad & Survey, 2003). Within this study, it is possible that such factors as structural failures and design flaws account for the EA reported here. Presently, Jefferson County has been undergoing extensive renovations, estimated at $3.3 billion dollars, in repairs and upgrades to the sewer systems (Jefferson County Commission, 2010). These repairs include major renovations to those sewer systems which carry influent to WWTPs. With structural failures like this, it is well established groundwater and rainfall lead to substantial increases in influent volume and stress plant processes through infiltration. Of special note is the unique sewer design which Jefferson County utilizes for municipal waste transport. Here, all WWTPs surveyed (except Plant 4) are set up on a 'spoke-and-wheel' design where WWTPs are linked through an interconnected sewage system which services numerous townships. As such, through the act of back-flow and mixing, the waste generated by one service region has the potential to be discharged in-part by a WWTP at another location. With these sewage designs, influent samples, and thus effluent samples may not be characteristic of a specific nearby community's waste output.

While modern WWTP infrastructure typically has high removal efficiencies, in this study potential exists for alternative factors to account for the low EA observed (Desforges et al., 2010; Kostich et al., 2013; Writer et al., 2010). Since numerous studies utilizing both chemical and biological methods of analysis have detected estrogens in

treated wastewater globally, one may conclude that the concentrations detected in this study represent a best-case scenario (Balaam et al., 2010; Björkblom et al., 2008; Terasaki et al., 2009). It is therefore expected that that studies comparable to this one will continue to report the presence of EDCs, like environmental estrogens (Brown et al., 2005; Keller et al., 2006; Short et al., 2005). What is more, reports detailing high concentrations of EA in treated wastewater at locations in heavily populated service regions will likely be the norm. As of 2014, the World Health Organization reported 54% of the world's population to reside in dense urban populations (WHO, 2015). The most heavily urbanized locations occur in third world countries, which lack the resources necessary for construction and adequate upkeep of WWTP facilities. If current trends continue, urban population growth will increase at annual rate of ~2% between the years 2015-2020 (WHO, 2015). This leads to the expectation of more investigations reporting concentrations of environmental estrogens in wastewater (treated or not) at levels exceeding the threshold for safety.

In conclusion, the present study consistently detected low concentrations of estrogenic activity in the effluent and in the receiving bodies of water near multiple municipal WWTPs in Jefferson County, Alabama. These results indicate that these WWTPs do not currently contribute measurable EA to the local aquatic environment. However, it would be prudent to continue with similar investigations as the population density in Jefferson County continues to increase.

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Table 1. Six of the largest WWTPs in Jefferson County Alabama, along with the population size serviced at each facility.

Plant ID	MGD	Estimated Population Size
1	12	65,000
2	30	73,000
3	2	5,500
4	5	30,000
5	85	220,000
6	60	230,000

Table 2. The annual mean EEQ (ng/L) are shown for each WWTP at each designated collection zone

2012					2013				
Plant ID	**Sampling Zone**	**Sample Size (n)**	**±SEM**	**Mean EEQ (ng/L)**	**Plant ID**	**Sampling Zone**	**Sample Size (n)**	**±SEM**	**Mean EEQ (ng/L)**
3	Down	5	0.0112	0.0135	3	Down	5	0.0076	0.0278
	Up	5	0.0102	0.0114		Up	5	0.0061	0.0197
	Effluent	5	0.0107	0.0226		Effluent	5	0.0122	0.0208
6	Down	1	*NA*	0.0065	6	Down	3	0.0018	0.0084
	Up	1	*NA*	0.0057		Up	3	0.0005	0.0023
	Effluent	1	*NA*	0.0047		Effluent	3	0.0025	0.0032
5	Down	2	0.0080	0.0149	5	Down	6	0.0021	0.0172
	Up	2	0.0029	0.0158		Up	6	0.0029	0.0090
	Effluent	2	0.0009	0.0158		Effluent	6	0.0015	0.0101
2	Down	2	0.0059	0.0179	2	Down	6	0.0089	0.0220
	Up	2	0.0067	0.0172		Up	5	0.0075	0.0248
	Effluent	2	0.0030	0.0150		Effluent	6	0.0044	0.0342
4	Down	5	0.0027	0.0201	4	Down	6	0.0041	0.0078
	Up	5	0.0045	0.0212		Up	6	0.0061	0.0129
	Effluent	5	0.0042	0.0208		Effluent	6	0.0085	0.0141
1	Down	5	0.0054	0.0149	1	Down	6	0.0020	0.0130
	Up	4	0.0016	0.0157		Up	6	0.0022	0.0143
	Effluent	5	0.0009	0.0132		Effluent	6	0.0010	0.0121

Table 3 a and b. *Annual means ±SEM for each WWTP investigated for 2012 (a) and 2013 (b). Estrogen analytes are represented in ng/L. Analyte ranges, as depicted by minimum and maximum values, include values below the limit of quantitation (BQL) or, <0.5ng/L. BQL were replaced with zero to estimate the annual means.*

a.)

ID	Zone	n	E1(ng/L)	±SEM	Min.-Max.	EE2 (ng/L)	±SEM	Min.-Max.	17 α E2 (ng/L)	±SEM	Min.-Max.	17 β E2 (ng/L)	±SEM	Min.-Max.	E3 (ng/L)	±SEM	Min.-Max.
1	Down	6	0		0	0		0	0		0	0		0	0		0-BQL
	Up	5	0		0	0		0	0		0	0		0	0		0-BQL
	Effluer	6	0		0	0.3250	0.3250	0-1.95	0		0	0		0	0		0
2	Down	3	0		0	2.1767	2.1767	0-6.53	0		0	0		0	0		0-BQL
	Up	3	0		0	0		0	0		0	0		0	0		BQL*
	Effluer	3	0		0	0		0	0		0	0		0	0		0
3	Down	7	0		0	0		0	0		0	0		0	0		0
	Up	6	1.2900	1.2900	0-7.74	0.1700	0.1700	0-1.02	0		0	0		0	0		0
	Effluer	6	0		0	0		0	0		0	0		0	0		0
4	Down	7	0		0	0.6657	0.6657	0-4.66	1.8429	1.8429	0-12.9	0		0	0		0-BQL
	Up	5	0		0	0		0	0		0	0		0	0		0
	Effluer	6	0		0	0		0	0		0	0		0	0		0
5	Down	6	0		0	0		0-BQL	0	0.8867	0-5.32	0-BQL		0	0.7620	0.5984	0-BQL
	Up	6	0		0	0		0	0		0-BQL	0		0	0.2788	0.1795	0-BQL
	Effluer	7	0		0	0		0	0.1471	0.1471	0-1.03	0.2214	0.2214	0-1.55	1.4963	1.2186	0-8.74
6	Down	2	0			0			0			0			0.3710	0.3710	0-0.742
	Up	2	0			0		0-BQL	0			0			0		
	Effluer	2	0			0			0			0			0		

b.)

ID	Zone	n	E1(ng/L)	±SEM	Min.-Max.	EE2 (ng/L)	±SEM	Min.-Max.	17 α E2(ng/L)	±SEM	Min.-Max.	17 β E2(ng/L)	±SEM	Min.-Max.	E3(ng/L)	±SEM	Min-Max
1	Down	4	0		0	0		0	0		0	0		0	0		0
	Up	4	0		0	0		0	0		0	0		0	0		0-BQL
	Effluent	4	0		0	0		0	0		0	0		0	0		0
2	Down	5	0.3528	0.2268	0-1.1	36.6	36.0000	0-183	0		0	0		0-BQL	0.2352	0.1440	0-0.595
	Up	5	0		0	0		0	0		0	0		0	0		BQL*
	Effluent	5	1.408	0.8822	0-4.11	0		0	0		0	0		0-BQL	0.57	0.3493	0-1.47
3	Down	4	0		0	0		0	0.182	0.1820	0	0		0	0		0
	Up	5	0		0	0		0	0		0	0		0	0		0
	Effluent	5	0		0	0		0	0		0	0		0	0		0-BQL
4	Down	5	0		0	0		0	0		0	0		0	0		0-BQL
	Up	4	0		0	0		0	0		0	0		0	0		0-BQL
	Effluent	5	0		0	0		0-BQL	0		0-BQL	0		0	0		0-BQL
5	Down	3	0		0	0		0	0		0	0		0	0		0-BQL
	Up	3	0		0	0		0	0		0	0		0	0		0-BQL
	Effluent	3	0		0	0		0	0		0	0		0	0		0-BQL
6	Down	3	0		0	0		0	0		0	0		0	0		0-BQL
	Up	3	0		0	0		0	0		0	0		0	0		0-BQL
	Effluent	3	0		0	0		0	0		0	0		0	0		0-BQL

CAMPOSTOMA OLIGOLEPIS A NOVEL FISH SENTINEL IN THE DECTECTON OF THE POTENTIAL EFFECTS OF ENVIRONMENTAL ESTROGENS FROM MULTIPLE WASTEWATER TREATMENT PLANTS THROUGHOUT JEFFERSON COUNTY, ALABAMA

by

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IN PREPARATION FOR ENVIRONMENTAL SCIENCE & TECHNOLOGY

FORMAT ADAPTED AND ERRATA CORRECTED FOR DISSERTATION

ABSTRACT

For decades fish have served as model organisms for the investigation of a wide variety of aquatic toxicants, both in and out of the laboratory. Over the last two decades fish have become popular as experimental models and environmental sentinel organisms for the detection of endocrine disrupting compounds in the environment, specifically estrogens. In many studies where environmental estrogens have been detected, exposed fish population have been demonstrated to possess a variety of behavioral and physiological abnormalities. These included intersex, impaired physical and reproductive fitness, atypical sex ratios, and reduced courtship displays. In the current study a minnow, the largescale stoneroller (*Campostoma oligolepis*), was used as a sentinel species for investigating the possible presence of environmental estrogens in the vicinity of several wastewater treatment plants (WWTPs) in Jefferson County, Alabama. This study was one portion of a two-part investigation using both chemical and biological methods of water analysis. Stonerollers were collected over two breeding seasons (2012-2013) and gonad histology as well as several biomarkers for health measured. The results in this study are consistent with chemical data indicating that environmental estrogens were not present at concentrations sufficient to cause endocrine disruption in aquatic organisms in the receiving waters.

INTRODUCTION

Presently, 56% of the drinking water in Alabama is drawn from rivers (Arello et al., 1998). Unfortunately, many recent studies have identified emerging threats to these potable water resources, which, in turn, threaten the health of humans and wildlife. One

relatively new source of concern is a group of compounds that interact with the endocrine systems of humans and wildlife (Buchinger et al., 2013; Daston et al., 2003; Gagne et al., 2004; Larsson et al., 1999; Writer et al., 2010). Known as endocrine disrupting chemicals (EDCs), these natural and synthetic chemicals may mimic or interfere with the action of natural hormones—thus disrupting the endocrine system. An EDC has been defined by the U.S. Environmental Protection Agency (EPA) as "an exogenous agent that interferes with the synthesis, secretion, transport, binding, action, or elimination of natural hormones in the body which are responsible for the maintenance of homeostasis, reproduction, development, and/or behavior" (Kavlock et al., 1996). Numerous studies have reported the negative effects associated with EDCs on both the health of humans and wildlife; however more information is needed on the reproductive effects that EDCs may pose to wildlife, in particular fish inhabiting these freshwater ecosystems (Aksglaede et al., 2006; Becker et al., 2014; Colborn et al., 1996; Hotchkiss et al., 2008; USEPA, 2011). Many studies have shown correlations between the presence of known EDCs in freshwater habitats and an increase in myriad endocrine-related dysfunctions; as indicated by atypical sex ratios, delayed/inhibited sexual development, reduced courtship displays, decreases in the sizes of historically abundant fish populations, and the presence of intersex characteristics (Barnhoorn et al., 2010; Cameron & Dalerum, 2009; Kidd et al., 2007; Larsen et al., 2008; Larsson & Forlin, 2002; Maack & Segner, 2003; Park & Kidd, 2005; Saaristo et al., 2010). Of the EDCs present within aquatic ecosystems, most act similarly to estrogens (Chang et al., 2011; Hannah et al., 2009; Pérez et al., 2012; Reyhanian et al., 2011; Sumpter & Jobling, 2013). The ubiquitous nature of these

compounds has provoked many investigators to investigate the origins of these potential threats and their routes of exposure.

EDCs are an extremely varied class of compounds. Currently, the U.S. EPA's Endocrine Disruptor Screening Program (EDSP) identifies 10,000 potential EDCs, 6,000 of which are classified as water contaminants; of these, 1,000 are pesticides which are in current use (USEPA, 2011). To this end, while many of the compounds exhibiting the potential to disrupt homeostatic endocrine function appear obvious, many are not. Here, the SWITCH Consortium of the European Union, a professional panel of researchers and water utilities consultants, recommends that EDCs be placed into one of three broad groups for further classification: 1) synthetic hormones or chemicals designed to deliberately alter the endocrine system, 2) natural compounds and their metabolites, and 3) chemicals which, by chance, disrupt the endocrine system (Kujawa-Roeleveld & Mahmoud, 2007). And, while natural compounds can present some risk to the health of humans and wildlife, many findings indicate it is likely Group 1 and Group 3 pose the greatest risk. This is primarily due to the fact that synthetic hormones are designed to elicit responses at low concentrations (e.g. EE2, Trenbolone, DES) (Doyle et al., 2013; Heldring et al., 2007; Korshin, et al. , 2006; Schiffer et al., 2004). And, chemicals that alter endocrine function simply by chance often times are found in common consumer products that are manufactured in considerable amounts (e.g. triclosan, nonylphenol, bisphenol) and enter the environment long before their potentials to cause deleterious effects are identified (Ellis, 2006; Kasprzyk-Hordern et al., 2008; Snyder et al., 2009).

Compounding the challenges faced when identifying potential EDCs and the effects they pose, are their diverse routes of exposure. With personal care products

(PCPs) and certain pharmaceuticals contributing to many of EDCs observed within aquatic ecosystems and freshwater resources, wastewater treatment plants (WWTPs) that have been designated as a significant routes of exposure to the overall presence of estrogenic activity within nearby waterways (Bain et al., 2014; Behera et al., 2011; Brown et al., 2006; Chang et al., 2011; Sowers, 2009). This is primarily due to the typical designs of WWTPs and the incomplete removal of these types of compounds from the discharged effluent. Conventionally, wastewater containing a mixture of compounds enters a plant and is treated to remove the majority of organic contaminants and subsequently discharged into a nearby receiving body of water at a relatively constant rate (Chen et al., 2007; Jobling et al., 2009; Sarria et al., 2011; Sun et al., 2009). What is more, WWTPs have been designed to cope with traditional contaminants (e.g. solid waste, DO, pathogens, and excess nutrients) and not specifically to eliminate "emerging" contaminants, such as PCPs, pharmaceuticals and EDCs which are biologically active at very low concentrations.

Historically, the health of resident fish has been considered a good indicator of the overall biological health of a an aquatic ecosystem; due to their dependence on water factors associated with development, reproduction, and population age structure provide valuable insight to the health of a body of water (Oceanic, 1988; van der Schalie et al., 1999). Furthermore, it was due to reports of disrupted endocrine function in various species of fish that scientists first became aware of the presence of EDCs in the effluents of WWTPs (Jobling et al. ,1998; Jobling & Tyler, 2003; Pinkney et al., 2001; Vigano et al., 2001). Members of the minnow family (Cyprinidae) have been popular study organisms in numerous investigations where the effects of EDCs (i.e. environmental

estrogens) have been detected in the waterways near WWTPs (Johnson et al.,2009; Martinoviæ et al., 2007; Rickwood et al., 2006; Vigano et al., 2001; Yang et al., 2011). In the current study, we examined populations of a minnow species, the largescale stoneroller (*Campostoma oligolepis*), inhabiting bodies of water downstream of several municipal WWTPs in Jefferson County, Alabama during the breeding seasons of 2012 and 2013. Fish from the different sites were compared for gonad histology and various health indices in order to assess potential differences between populations inhabiting waterways receiving effluent potentially containing environmental estrogens versus those at the control site.

MATERIAL AND METHODS

Animals

Two investigations were conducted. The animals used were adult and juvenile, male and female largescale stonerollers (*Campostoma oligolepis).* A previous study indicated that *C. oligolepis can* serve as a useful indicator of environmental estrogens by exhibiting a dose dependent response to laboratory exposures to E2 (Legg et al., 2014). The fish were collected via electrofishing near the outfalls of WWTPs, as wells as at a control site, Five Mile Creek in Tarrant, upstream of any WWTP outfalls and free from the potential effects associated with malfunctioning septic systems. The fish serving as the experimental subjects were collected no more than 100 yards downstream from multiple municipal WWTPs in Jefferson County, Alabama (see Table 1).

Collection dates coincided with the spring breeding season for the largescale stoneroller in Alabama (i.e. April of 2012 and May of 2013). Fish were collected and handled in accordance with protocols approved by the UAB Institutional Animal Care and Use Committee (IACUC) and guidelines set forth by the Organization for Economic Co-operation and Development's (OECD's) panel on the uniform analysis of endocrine-related histopathology in fish gonads. Immediately subsequent to capture, fish were placed into aerated holding tanks with a sedating concentration (120mg/L) of the anesthetic tricane methanesulfonate (MS-222; Finquel®) and transported immediately to the laboratory. In the lab, the fish were allowed to acclimate briefly before being euthanized by immersion in 300 mg/L MS-222 until respiratory movements ceased.

Morphological measurements

Immediately following euthanasia, each fish was blot dried , weighed to the nearest 0.01 gm, and both the standard length and total length measured using digital calipers to the nearest 0.01 mm. Fulton's condition factor (K) was calculated as body weight as a percentage of the total length^3 where length is expressed in cm (Nash et. al., 2006). The liver and gonads were then removed and weighed to the nearest 0.001 gm. Gonadosomatic (GSI) and hepatosomatic indices (HSI) were calculated as organ weight as a percentage of the total body weight (i.e. before any organs had been excised) (Diniz et al., 2005; Everaarts et al., 1993; Yeh et al., 2003). Finally, the gonads were fixed using Davidson's fixative for 24 hours and transferred to 10% neutral buffered formalin for storage in opaque, air-tight Nalgene® containers until histopathology and gender assignment could be performed (Humason, 1962; Johnson et al., 2009).

Histopathology

Infiltration and Embedding

Excised gonads were placed in a tissue cassette and transferred to 10% neutral buffered formalin at least 24 hours followed by a brief rinse with tap water. Infiltration of clearing agents followed as prescribed in Humason et al. (1962). Following infiltration, each tissue specimen was embedded in paraffin for sectioning.

Microtomy

The paraffin-embedded gonads were sectioned using a microtome (American Optics, Model 820) with new blade during each sectioning period. Each section was cut at 8 µm in thickness. Approximately seven slides were made for each fish, with six to ten sections per slide, depending on gonad diameter.

Staining and Tissue Characterization

Hematoxylin & eosin (H&E) staining was performed as describe by Humason et al. (1962). After staining, the slides were dipped briefly in xylene, and a few drops of Permount (Fisher®) added to each slide. Cover slips were applied and the slides allowed to dry at room temperature for several days.

Characterization and tissue identification followed a modified version of gonad assessment as developed by Johnson et al. (2009) and Smith (2004). With recent developments announced by Wolf et al. (2014) regarding the increased risk/prevalence of misdiagnosis attributed via complex grading schemes, a dichotomous grading system was developed to reduce misrepresentation of specimens. Utilizing this method of classification, gonad tissue was graded as either "normal" or "abnormal." Final determination of specimen quality depended upon the prevalent tissue grouping over 6-7 tissue section per individual. Each tissue section was compared to known gonad sections exhibiting normal microarchitecture.

Statistical Methods

All summary statistics are expressed as a mean ± standard error. Health indices were compared between fish collected at WWTP sites and the controls using the nonparametric Kruskal-Wallis test, followed by Dunn's pair-wise comparisons (Dunn, 1964). Sex ratios were compared between sites using 2x2 contingency tables with Yates' correction for continuity; when expected values were low a Fischer's exact test was utilized. Gonad tissue histology was quantified by comparing frequencies of normal and about normal tissue between collection sites using a Fischer's exact test. The cutoff for statistical significance was $p < 0.05$.

Results

Population sex ratios

Figure 1 (a and b) illustrates the proportions of different sex phenotypes in the largescale stoneroller (*Campostoma oligolepis*) populations at the sampling sites where individuals were available for collection. Using a 2x2 contingency table with Yates' Correction for continuity it was determined all 2012 sample populations differed significantly from a 1:1 sex ratio—including the designated Control site. During the 2013 sample season the Plant 7 site did not significantly differ significantly from a 1:1 sex ratio. For the Plant 4 site during the 2013 season, a Fischer's exact test was employed and determined that the observed sex ratio did not differ significantly from the expected 1:1 sex ratio. Because juvenile stonerollers possessed undifferentiated reproductive tissues individuals exhibiting indistinguishable or, underdeveloped, gonad morphology were not included in these tests.

Health Indices and Biomarkers

For the 2012 and 2013 sampling seasons biomarkers indicating the potential effects of endocrine disruption via environmental estrogen exposure were screened: Fulton's condition factor (K), hepatosomatic index (HSI), and gonadosomatic index (GSI). When fish were available (they did not occur at all study sites) these health indices were recorded and evaluated using the Kruskall-Wallis test followed by Dunn's pair wise comparisons assessment. Per each biomarker screened (i.e. K, HSI, and GSI) comparison were made between both control (WWTP-absent) and WWTP-present sites (Figure 1 and 2). For the 2012 season, both male and female individuals exhibited no significant differences in condition factor among groups sampled. However, among juvenile/undifferentiated individuals, significant differences were found between Plant 2 and Plant 7 for K. For the 2012 season on the measure of HSI no significant differences in HSI were detected in either the adult male or juvenile individuals obtained, yet differences were observed for adult females. Among females during the 2012 season , significant differences in HSIwere noted between the Control and Plant 7 sites. With respect to GSI during the 2012 collection season, significant differences were observed between the Control and Plant 7 sites for adult females. During the 2013 season overall fish abundance was reduced relative to 2012, the abundance of undifferentiated juveniles (n = 1) was insufficient to conduct statistical analysis. Furthermore while all biomarkers showed elevated values among groups compared to the previous collection season, the 2013 season exhibited fewer significant differences across biomarkers—except GSI. Significant differences were detected in GSI between females at the Control and Plant 7 sites.

Histopathology Quality Assessment

During the 2012 and 2013 sampling seasons a dichotomous grading scheme was utilized to assess gonad histology and microarchitecture for *C. oligolepis* when reproductively mature individuals were present during the breeding seasons (Table 2). Because expected values were low, statistical analysis was performed using a Fischer's exact test to compare the numbers of fish in the two categories between groups for each collection season. While abnormalities were detected across both seasons, malformations to the chorion of cortical alveolar oocytes were the single most frequent pathology detected (Figure 4). No abnormalities were observed in male testes over the 2012-2013 sample seasons. While no significant abnormalities were detected within the 2013 sample season, a significant increase in the proportion of chorion malformations was seen at Plant 2 when compared to both the Control and Plant 7 sites during the 2012 season.

DICUSSION

Here we report the results of one portion of a two part investigation into the potential presence and effects of environmental estrogens in the effluents of WWTPs in Jefferson County, Alabama. This is the first such investigation conducted in North-Central Alabama. Analyses of effluent and stream water reported in the companion study found concentrations of environmental estrogens that were either low or non-detectable. The current study of fish inhabiting the receiving waters in the vicinity of the WWTPs did not find any consistent indications of endocrine disruption. While populations surveyed during the 2012 sampling season deviated from 1:1 sex ratio, the 2013 sampling season illustrated populations which did not significantly deviate from a 1:1 sex ratio

across all sites. Few significant differences were found in various measures of fish health, such as HSI, GSI, and K between fish from the different study sites. A significant difference was observed between females from the control and Plant 7 groups for the HSI and GSI indices during the 2012 season. However, both indices were greater in the fish from the Plant 7 site (potentially exposed to EDCs) than those from the control site. This may indicate that those individuals collected from Plant 7 illustrate little to no sign of endocrine disruption for the health parameters mentioned here. As Plant 7 individuals have an elevate mean K value it is likely their robust physiology, relative to the control site, stems from the abundance of algae observed within the outfall of the fertile discharge stream. Also, during the 2012 sampling season a significant difference was detected in K values between the juveniles collected at the Plant 2 and Control groups. During the 2013 sampling season the only significant difference detected occurred between females at the Plant 7 group for GSI, where differences detected seem likely to have occurred due the elevated nutrient content of the discharge stream.

An ever-increasing body of investigations have reported the occurrence of intersex among a variety of freshwater and marine fish (Blazer et al., 2007; Iwanowicz et al., 2009; Jobling et al., 1998; Tetreault et al., 2011). Historic data indicate such events occurring as early as the 1940's (James, 1946). To date, organizations such as the U.S.EPA, the United States Geological Survey (USGS), and U.S. Fish and Wildlife have launched multiple investigations focusing on the presence of EDCs within aquatic habitats and the potential effects these compounds have on fish communities (Harding et al., 2006; USGS, 2009; Zajicek et al., 2000). Moreover, international studies have reported analogous findings wherein various fish species exhibited an array of endocrine-

related pathologies including: abnormal courtship behavior, skewed sex ratios, population collapse, intersex individuals, lowered fecundity, and decreased measures of health (Baatrup, 2009; Barron et al., 2000; Colman et al., 2009; Grim et al., 2007; Kidd et al., 2007; Kristensen et al., 2007).

In light of recent reports indicating that endocrine disruption is occurring, investigators have noted that improvements in study methods are needed (Johnson et al., 2009; Sumpter & Jobling, 2013; Wolf et al., 2014). Recent reports have pointed out the misdiagnosis of intersex among species of fish and have particularly pointed out errors in several U.S. National Park system studies (Wolf et al., 2014). Those reports indicated statistically significant occurrences of intersex where, in fact, none or few cases existed. For example, one study found up to 100% of the female salmon in populations at several U.S. National Parks in Alaska and throughout the West were intersex (Schreck and Kent, 2012). Subsequently, careful re-evaluation of these tissue samples determined that the intersex findings were incorrect (Wolf et al., 2014). Reasons for such misidentifications have been offered ranging from the lack of experts specializing in fish histopathology to inadequate quality control measures, and methods of statistical analysis which limit or confine the descriptions of tissue specimens. The prevalence of incorrect findings has led to the formation of expert panels that seek to standardize histological studies investigating the potential effects of EDCs (Johnson et al., 2009; USEPA, 2011; Wolf et al., 2014). In the current study, we have used a modified version of these proposed criteria.

As a biomarker of exposure to EDCs, gonad tissue is typically observed for abnormalities in microarchitecture compared to a control site (Johnson et al., 2009; Leino

et al., 2005; Webb, 2009). Here we detected significant differences between female fish found within the effluent of Plant 2 when compared to the control group and the Plant 7 group during the 2012 season. This could not be reevaluated during the 2013 season as individuals were entirely absent at the Plant 2 site in 2013. Malformations in the chorion have been associated with a variety of EDCs, specifically organophosphorus insecticides (e.g. fenitrothion) (Miyashita, 1984; Oh et al., 2007; Adamski & Ziemnicki, 2005). However, this specific class of compounds was not investigated as part of our chemical analysis and it would only be speculative to assume it to be a causative agent.

In many reports the presence of EDCs, specifically environmental estrogens, poses a significant risk to the stability of fish communities (Ankley et al., 2009; Filby et al., 2010; Jobling & Tyler, 2003). Either through the breakdown of mechanisms governing sexual reproduction, the disruption of normal gonad formation, and/or the general decline of health, EDCs have been established in many laboratory settings to negatively impact a wide range of organisms, including vertebrate and invertebrate species (Panter et al., 2006; Sarria et al., 2011). This includes a seven-year study in which low dose concentrations (5-6 ng/L^{-1}) of EE2, the active ingredient in most birth control medication, effectively extirpated an entire local population of fathead minnow (*Pimephales promelas*) in northwestern Ontario, Canada (Kidd et al., 2007). Additionally, the observations are not limited to experimental testing, but have also been shown in numerous international reports where impaired communities and signs of intersex have been detected in fish populations living downstream of WTTPs (Cargouet et al., 2004; Hou et al., 2011; Jackson & Sutton, 2008; Pawlowski et al., 2003). To this end, one significant observation implicating an impending collapse a fish community is

sex ratio strongly skewed toward females (Larsson & Forlin, 2002; Piferrer, 2001; Vogl et al. , 1999). In species, such as *C.oligolepis*, where populations have been shown to exhibit a 1:1 sex ratio, significant deviations from this ratio could serve as an indicator of endocrine disruption (Osmundson, 2006). In the current study, significant departures from 1:1 sex ratios were observed during the 2012 season at the Control and Plant 2 location. It is presumed that the observed departure from a 1:1 sex ratio at the Control site was due to sampling error as gonad histology, historical data, and subsequent sex ratio reports were all normal. Also, during the 2013 sampling season a 1:1 sex ratio was observed at the control site, while no fish were detected whatsoever at Plant 2. Between seasons it was also noted that no fish were ever observed during water chemistry sampling at the Plant 2 location.

Historically, measures of health have served as reliable biomarkers for the biological health of living organisms (Han & Fang, 2010; Nash et al., 2006; Rashleigh, 2009). Such measures as body length, weight, and organ-to-body relative weight provide insight to a specific organism and its pharmacologic response to a particular compound, or combination of compounds. In vertebrate models this has long served as the basis for gauging acute toxicity exposure, fitness, and the sub-lethal effects observed with various toxicants (Di Giulio & Hinton, 2008). Here, we applied similar measures of health comparing overall measures of fitness (K) along with organ-to-body relative weight measures (GSI and HSI) to determine the overall health among sample populations found both at WWTP-influenced sites and a WWTP-free site. As exposure to environmental estrogens has been associated with hepatic enlargement, altered gonad size, as well as suboptimal health, these biomarkers assess the potential sub-lethal effects that exposure

to environmental estrogens have been reported to cause. While it was noted that during the 2012 season significant differences were observed between the females present at the Control site and Plant 7 for GSI and HSI, this failed to indicate physiological differences based upon the effects of endocrine disruption. Significant differences were also detected in K between the juveniles collected at Plant 2 and Plant7, however this difference is once again likely due to the abundance of vegetative growth present at Plant 7. Vegetative growth, and therefore a viable food source, appeared wholly absent from Plant 2. During the 2013 sampling season female specimens collected at the Plant 7 site maintained a significantly higher mean GSI relative to Plant 4 females. This observed difference does not appear to indicate signs of endocrine disruption so much as expected dissimilarities among the phenotypes of separate populations experiencing different environmental factors related to resource availability and maturation rates.

One of the many benefits of conducting EDC screening studies in a laboratory is the ability to control a wide range of variables to better reduce variation among the sample population, as well as to more accurately account for the effects observed. Unfortunately, this level of control is not achievable in field studies where a variety of environmental factors contribute to variability in the data (BERAC, 2010; Carvan et al., 2008; Celiz et al., 2009). In addition to varying physical conditions, toxicants in nature do not occur singly. This includes the complex mixture of organics found commonly with WWTP effluents. In view of this inherent challenge, surveys such as this one should enlist a variety of metrics to appropriately determine whether or not a population is exhibiting signs of endocrine-related disorders. It is now becoming standard practice to incorporate both biological and chemical means of analysis as complements in the

investigative process (Alvarez et al., 2013; Maurìcio et al., 2006; Wolf et al., 2014). With each method of analysis having its own strengths and limitations, investigators may more accurately diagnosis any effect observed within a given population and perhaps discern the causative agent. It is therefore prudent to draw conclusions about a given fish community only when a variety of metrics uniformly indicate plausible outcomes similar to those studies where a single compounds effect has been empirically observed.

In conclusion, the results of the present study indicate that, of seven Jefferson County municipal WWTPs studied, only individuals at Plant 2 indicate some abnormal findings when evaluating histomorphology of the gonads. Nevertheless, this finding alone does not indicate WWTPs present a direct threat to the local fish communities through the introduction of environmental estrogens. Further research should be aimed at monitoring the long term health of *C.oligolepis* as well as other fish species at this location. While noteworthy departures from the controls were detected at other sites, the lack of significant findings across all variables measured did not yield as much confidence in the observed effects recorded. Finally, based upon these findings we suggest for enhanced aquatic environmental risk assessment for environmental estrogens, and an integrated approach to investigating EDCs especially when conducting in-field studies.

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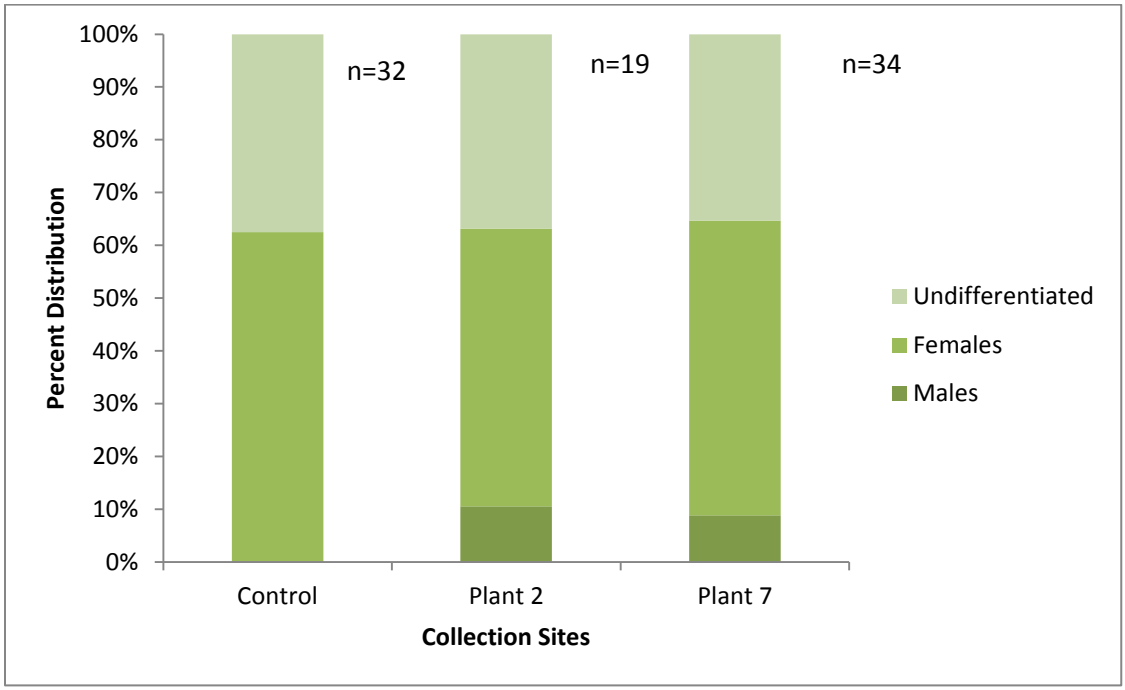
Table 1. Seven of the largest WWTPs in Jefferson County Alabama are listed below. Along with each plant the estimated service population is provided and the average effluent discharged from each plant in terms of millions of gallons per day (MGD).

Plant ID	MGD	Estimated Population Size
1	12	65,000
2	30	73,000
3	2	5,500
4	5	30,000
5	85	220,000
6	60	230,000
7	8	20,368

a.)



b.)

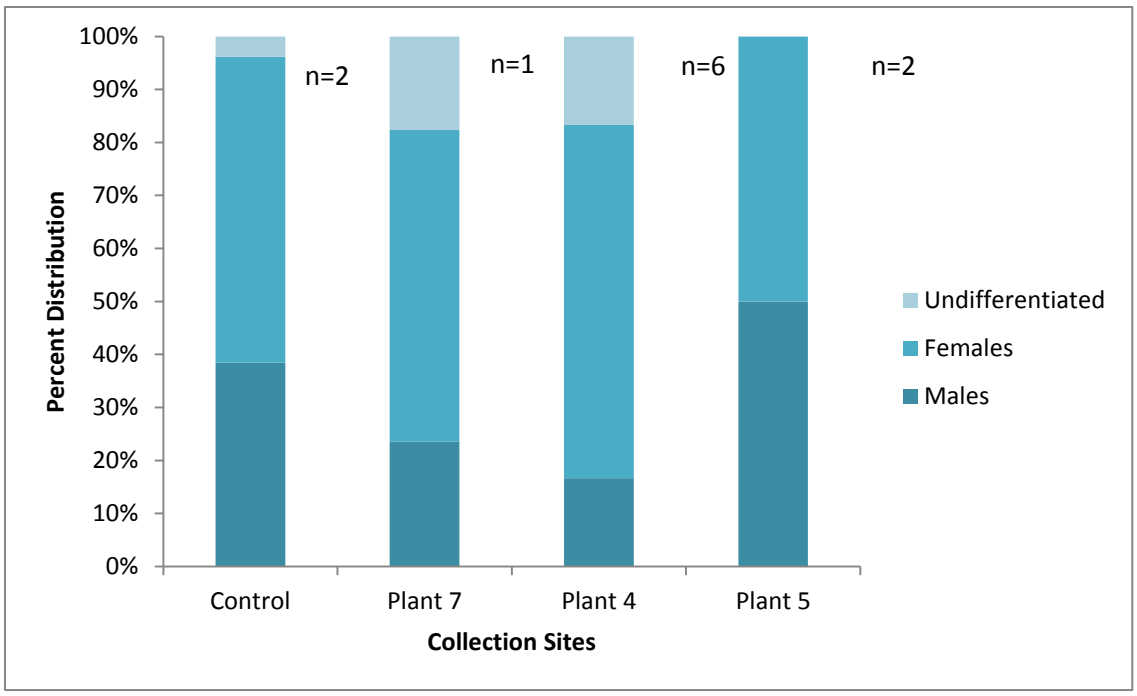
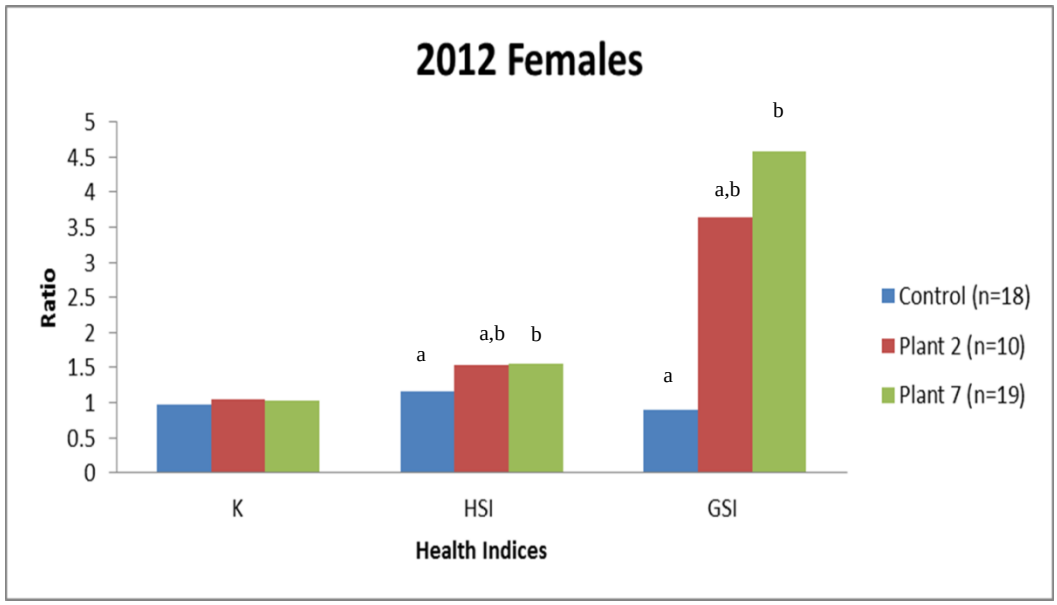
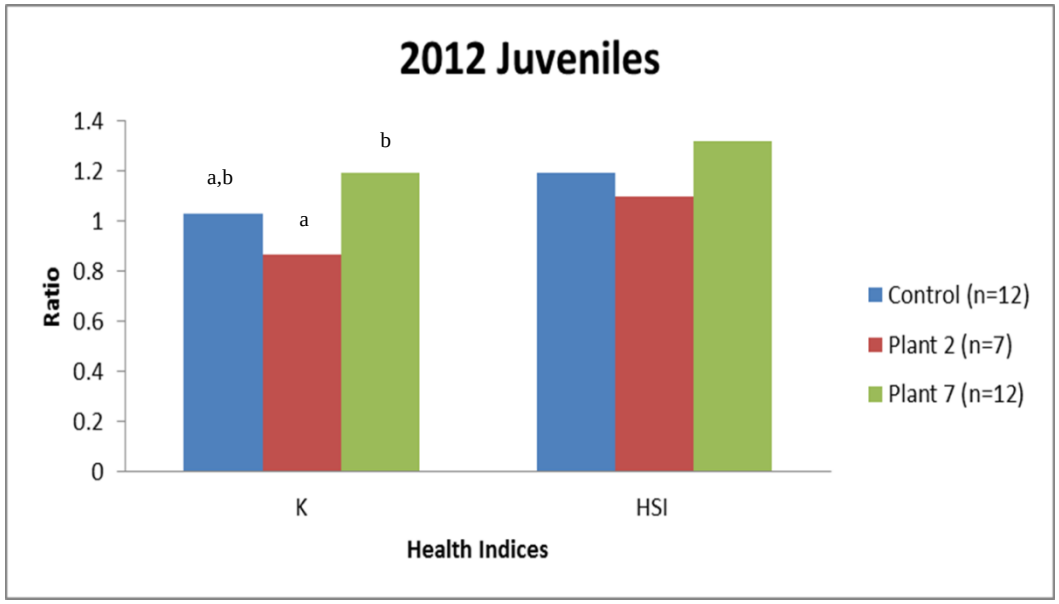


Figure 1. Sex ratios at the different study sites during 2012 (a) and 2013 (b).

a.


2012 Females
Ratio
5
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
a
a,b
b
a
a,b
b
K
HSI
GSI
Health Indices
Control (n=18)
Plant 2 (n=10)
Plant 7 (n=19)

b.


2012 Juveniles
Ratio
1.4
1.2
1
0.8
0.6
0.4
0.2
0
a,b
a
b
K
HSI
Health Indices
Control (n=12)
Plant 2 (n=7)
Plant 7 (n=12)

c.

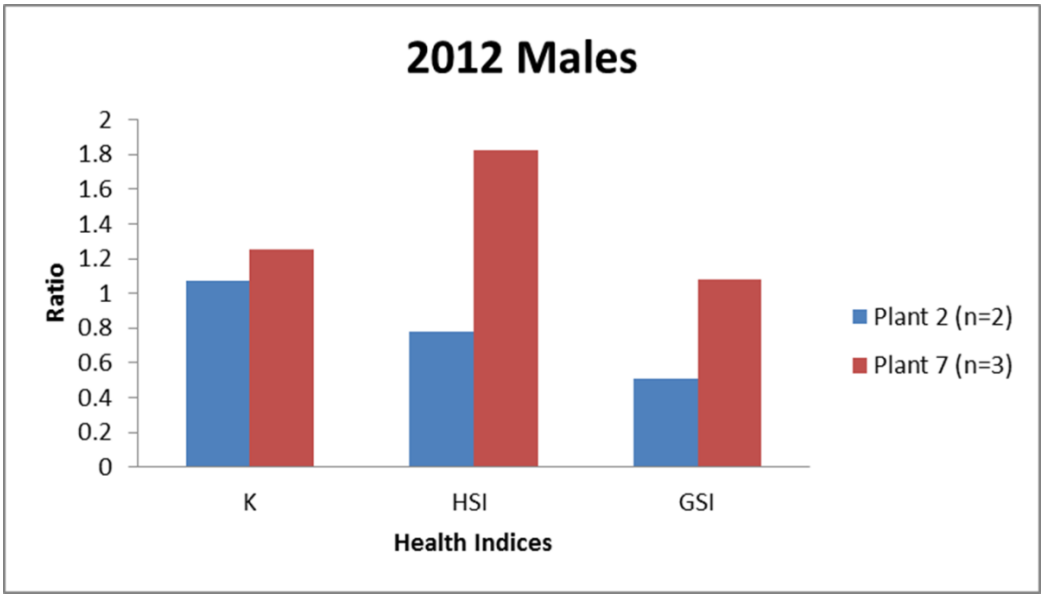
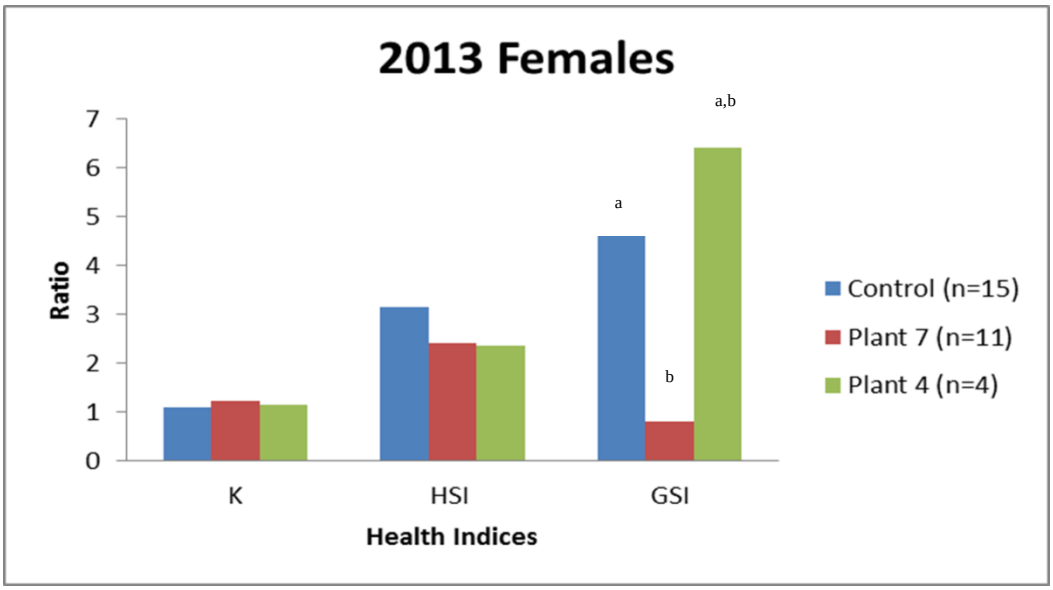


Figure 2 a-c. Biomarkers for female, juvenile, and male largescale stonerollers (*Campostoma oligolepis*) are shown, respectively, in 2012.

a.



b.

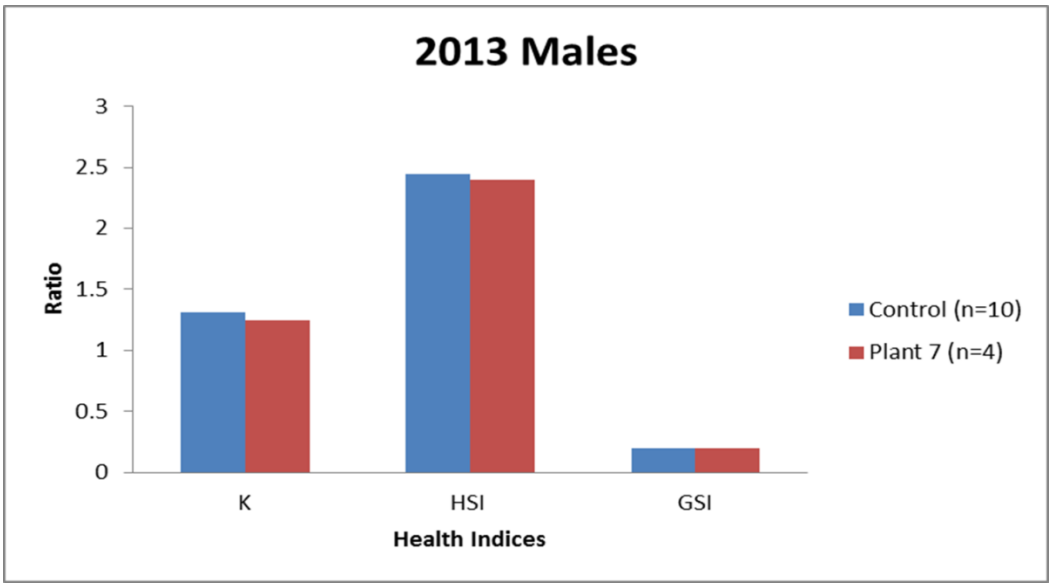


Figure 3 a-b. Biomarkers for female and male largescale stonerollers (*Campostoma oligolepis*) are shown, respectively, in 2013.

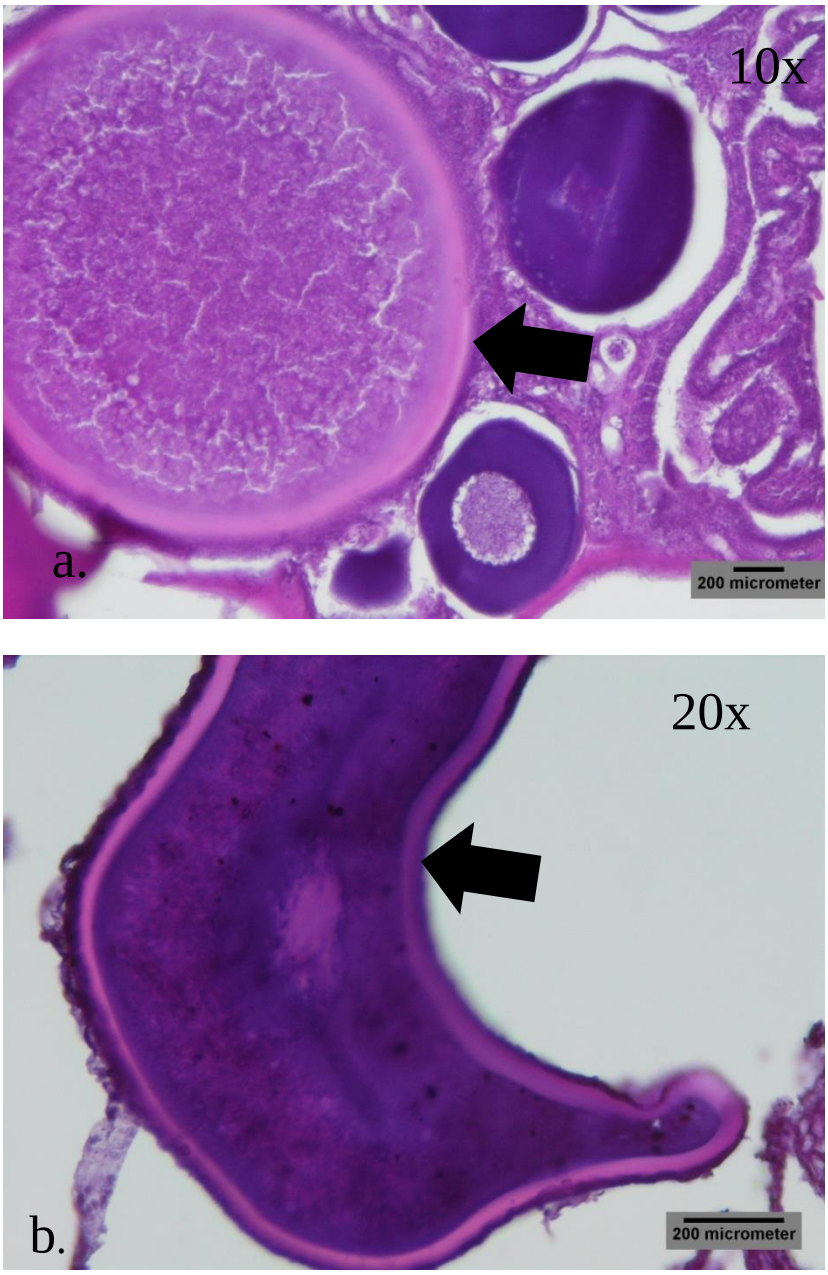


Figure 4. *Campostoma oligolepis* ovarian cross-section: Both frames are H&E stained, 8µM thick section of female ovary. Frame a indicates the presence of normal ovarian tissue, as characterized by the occurrence of cortical alveolar oocytes in the late-vitellogenic phase and perinuclear oocytes. Frame b illustrates the presence of abnormal ovarian tissue with the presence of one cortical alveolar oocyte with asymmetrical margins, or malformed chorion. The chorion in both picture has been denoted by the black arrow.

Table 2 a and b. Below the 2012 (a.) and 2013 (b.) seasons for histopathology are shown. Grading followed a dichotomous grading scheme, where tissue fit either a normal or abnormal classification. When one phenotypic sex was observed, but the other absent this annotated by NA or, not available.

a.

Site	*Gender*	*Normal Count*	*Abnormal Count*
Control	**Female**	16	0
	Male	1	0
Plant 2	**Female**	7	4
	Male	NA	NA
Plant 4	**Female**	1	0
	Male	NA	NA
Plant 7	**Female**	18	1
	Male	NA	NA

b.

Site	*Gender*	*Normal Count*	*Abnormal Count*
Control	**Female**	10	0
	Male	8	0
Plant 4	**Female**	5	0
	Male	1	0
Plant 5	**Female**	1	0
	Male	1	0
Plant 7	**Female**	11	1
	Male	6	0

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APPENDIX

INSTITUTIONAL ANIMAL CARE AND USE COMMITTEE APPROVAL

NOTICE OF RENEWAL

DATE: February 15, 2013

TO: ROBERT A ANGUS, Ph.D.
CH -378A 1170
FAX: (205) 975-6097

FROM: Judith A. Kapp
Judith A. Kapp, Ph.D., Chair
Institutional Animal Care and Use Committee (IACUC)

SUBJECT: Title: Investigation of Possible Endocrine-Disrupting Chemicals in Local Waterways
Sponsor: Internal
Animal Project Number: 130209343

As of February 23, 2013, the animal use proposed in the above referenced application is renewed. The University of Alabama at Birmingham Institutional Animal Care and Use Committee (IACUC) approves the use of the following species and numbers of animals:

Species	Use Category	Number in Category
Fish	A	550

Animal use must be renewed by February 22, 2014. Approval from the IACUC must be obtained before implementing any changes or modifications in the approved animal use.

Please keep this record for your files, and forward the attached letter to the appropriate granting agency.

Refer to Animal Protocol Number (APN) 130209343 when ordering animals or in any correspondence with the IACUC or Animal Resources Program (ARP) offices regarding this study. If you have concerns or questions regarding this notice, please call the IACUC office at (205) 934-7692.