AN INTEGRATED ASSESSMENT OF ENVIRONMENTAL ESTROGENS

IN THE UPPER CONASAUGA RIVER, GA, USA

by

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(Under the Direction of Robert Bringolf)

ABSTRACT

The current study evaluated the effects of estrogens on fishes in the upper Conasauga River (UCR) and identified tributaries with the highest estrogen inputs. Estrogen concentrations were higher in sediment than surface water. However, vitellogenin, a biomarker of estrogen exposure, was higher in male fish exposed to UCR water than those exposed to UCR sediments, suggesting estrogens in the water were more bioavailable than those in the sediment. In a survey of wild adult fishes from the UCR, 13 (7.5%) of the 174 male fishes (27 species) collected had testicular oocytes. Additionally, gonopodia of Western Mosquitofish collected from a UCR farm drainage ditch were shorter compared to mosquitofish from other areas of the basin with different land uses. Larval fish exposed to sediment from Mill Creek and Sugar Creek had lower growth compared to controls. Results indicate estrogen compounds in the UCR may be linked to declines in fish populations.

INDEX WORDS: Endocrine disruption, estrogen, vitellogenin, Conasauga River, Fathead Minnow, Tricolor Shiner, Polar Organic Chemical Integrative Samplers, Mosquitofish, testicular oocytes

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A.B., The University of Georgia, 2010

A Thesis Submitted to the Graduate Faculty of The University of Georgia in Partial Fulfillment of the Requirements for the Degree

MASTER OF SCIENCE

ATHENS, GEORGIA

2013

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December 2013

DEDICATION

To my parents: for their unwavering support, love, and encouragement

ACKNOWLEDGEMENTS

I would like to thank my advisor, Dr. Robert Bringolf, and my graduate committee members, Drs. Cecil Jennings and Brett Albanese, for their mentoring and advice, and Fish and Wildlife Service for funding the project. I also owe a great deal of gratitude to Bob Ratajczak, Pete Lasier, Kristen Kellock, and Matt Urich for their assistance during my graduate degree. The work on this project was conducted under the auspices of the University of Georgia Animal Use Permit A2010-06-104-R2.

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CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

Conasauga River

The Conasauga River is part of the Coosa River Basin in northwest Georgia (Figure 1-1). The river stretches 145 kilometers before converging with the Coosawattee River to form the Oostanala River near Calhoun, GA (USFS 2000). The Conasauga River is home to an exceptionally diverse aquatic fauna with approximately 77 extant native fish species and 15 introduced fish species (Walters 1997). However, many of the native species are in peril: six fish species are presumed to be extirpated (Walters 1997), three are federally protected, and an additional nine are state protected in Georgia and/or Tennessee (Table 1-1; Skelton and Albanese 2006). Furthermore, of the 37 freshwater mussel species that are native to the Conasauga River, only 19 remain (Novak et al. 2004). To protect and restore the remarkable aquatic diversity of the river, the U.S. Fish & Wildlife Service has proposed establishment of a Conasauga River National Wildlife Refuge (NWR). State government and public outreach is necessary before a final decision will be made about the refuge (Robin Goodloe, USFWS, personal communication).

The Conasauga River can be classified into an upper and lower section because of varying topography, ecological diversity, and land use (USFS 2000). For this project the upper Conasauga River (UCR) is the primary focus of concern, and the UCR is defined as the reach upstream of Dalton, Georgia extending north to the Georgia/Tennessee state line (Figure 1-1). Agriculture, notably row crop and livestock operations, is the primary land-use activity in the UCR (Sharpe and Nichols 2007; USFWS 2009), and agricultural acreage and the use of

agricultural products increased markedly over the period when fish and mussel species declines were observed (USFWS 2011; Georgia County Guide 2013). For example, Murray and Whitfield counties make up most of the upper Conasauga basin. From 1992 – 2007, Murray County farmland increased by 6,973 acres (21.2%) and Whitfield County farmland increased 4,059 acres (10.5%; Georgia County Guide 2013). The use of agricultural products, such as poultry litter to fertilize row crops and pastures, also increased along with use of Roundup-ready seed and Roundup™ herbicide (USFWS 2011). The Nature Conservancy (TNC) recently determined that agricultural drainage ditches, which move water from low-lying fields directly into creeks and rivers, were abundant throughout the UCR basin (USFWS 2009). These ditches also likely serve as conduits for pesticides and fertilizers to move from fields into aquatic systems (USFWS 2009).

A preliminary assessment of contaminants in the UCR basin performed in 2010 revealed high concentrations of hormones, notably estrogens, in UCR sediments (Lasier et al. 2012). Estrogen concentrations generally increased in a downstream direction throughout the basin, which suggests that inputs increased toward the lower end of the basin. Estriol concentrations ranged from below detectable levels to 136 µg/kg (dry wt) of sediment at the most downstream site. Estradiol concentrations ranged from below detectable levels to 126 µg/kg, and estrone concentration was as high as 3,200 µg/kg in Mill Creek (Lasier et al. 2012). Estrogens in agricultural areas often originate from animal byproducts (e.g., poultry litter, manure) in agricultural watersheds (Yonkos et al. 2010; Ciparis et al. 2012).

Rationale and Significance

Partitioning and bioavailability of estrogens in aquatic systems are not well characterized though estrogens are hydrophobic organic compounds with low volatility (Ying et al. 2002). The octanol/water partition coefficient (Log K_{ow}) provides information of lipophilicity of a chemical. The greater the K_{ow}, the more lipid soluble the compound: K_{ow} values greater than 4 suggest that the compound will accumulate in the lipids of biological organisms (USEPA 2012). Log K_{ow} values for natural and synthetic estrogens are estrone (E1) 3.43, estradiol (E2) 3.94, estriol (E3) 2.81, and 17α-ethynylestradiol (EE2) 4.15 (Ying et al. 2002). The soil sorption coefficient (Log K_{oc}) is a measure of the concentration of the target chemical that sorbs to the soil verses the concentration of the chemical in the water. The higher the Log K_{oc}, the higher the probability that the chemical will bind to the soil: values of 3.5 or greater suggest strong sorption (USEPA 2012). The Log K_{oc} for E1 is 3.69, E2 is 3.52, E3 is 3.29 and EE2 is 3.69 (Ying et al. 2002). These coefficients provide little clarity for how estrogen will partition in aquatic systems. Because estrogens are known to cause endocrine disruption in aquatic organisms (Desbrow et al. 1998), understanding their sources and bioavailability may contribute to understanding the decline of fishes in the UCR and other rivers.

Project Goal and Objectives

The goal of this study was to examine the sources and effects of elevated hormone levels in the UCR. Study objectives included (1) determining which UCR tributaries had the highest concentrations of estrogenic compounds; (2) assessing the bioavailability and bioactivity of estrogens in UCR water and sediment; (3) surveying UCR fish for incidence and severity of testicular oocytes (intersex); (4) determining if gonopodia of UCR Western Mosquitofish

(*Gambusia affinis*) were shorter than populations collected from sites located in the lower Conasauga River with little agriculture in the watershed and mainly an urban/suburban land-use. I hypothesized that (1) tributary sites with higher potency of estrogenic compounds in the water would be positively correlated with a higher production of vitellogenin in male fish; (2) tributary sites with higher potency of estrogenic compounds in the sediment would be positively correlated with increased production of vitellogenin in male fish; (3) incidence and severity of testicular oocytes (TO) would be higher at tributaries with higher estrogen activity; (4) gonopodia of Western Mosquitofish collected from a tributary of the UCR would be shorter relative to fish collected from sites in the lower Conasauga basin.

Findings from this study will aid in the management of the UCR and development of an aquatic species recovery plan by increasing the understanding of stressors and effects in the basin. Additionally, the results may foster identification of areas where agricultural best management practices (BMPs) can be implemented for the greatest effects to reduce estrogen loading.

Literature Review

Endocrine disruption

The endocrine system is comprised of glands located throughout the body that synthesize hormones and release them into the bloodstream (Norris 1997); it is critical for growth, development, behavior, reproduction, and homeostasis (Gross et al. 2003). Hormones travel to target cells in the body where they bind to a specific receptor molecule (Porterfield 1997), and the hormone-receptor complex then transmits a signal to the cell via secondary messenger chemicals or, in the case of steroid hormones, binds to DNA to influence protein transcription

and other cellular processes (Norris 1997). Endocrine-disrupting chemicals (EDCs) are exogenous compounds that enter the body and either mimic natural hormones or alter the synthesis or metabolism of a hormone (Kime 1998).

Exogenous compounds can interfere with hormonal processes in various ways; compounds can mimic hormones by binding and activating the receptor, block the hormone from binding to the receptor, or alter synthesis, transport, metabolism, or excretion of hormones (National Research Council 1999). For instance, exposure of Atlantic Croaker **(***Micropogonias undulatus***)** to 1 mg/kg body weight of a polychlorinated biphenyl (PCB) mixture (Aroclor 1254) interfered with the fish's hypothalamus-pituitary-gonadal axis, which controls reproductive processes in vertebrates, by disrupting the secretion of the luteinizing hormone (gonadotropin) from the pituitary gland (Khan and Thomas 1997, 2000). Since gonadotropins stimulate the gonads by binding to specific membrane receptors (Weltzien et al. 2004), this interference inhibited gonadal growth in the Atlantic Croaker (Khan and Thomas 1997, 2000).

Agrichemicals and animal byproducts, such as poultry litter and cattle manure, in water and sediment have been identified as EDCs for many aquatic species (Yonkos et al. 2010; Sellin Jeffries et al. 2011). Yonkos et al. (2010) examined the effects of runoff of poultry litter-associated contaminants (PLAC) from poultry waste applied as fertilizer to row crops and found PLAC exposure after 21 days induced Vtg production in male Fathead Minnows (*Pimephales promelas*) and had a feminizing effect on larval fish, either altering phenotypic sex or inducing development of feminine oviduct-like structures within reproductive tracts of immature males. Steroid hormones associated with the production of beef cattle have also been measured in riverbed sediments and reduced estrogen receptor α expression in female Fathead Minnows (Sellin Jefferies et al. 2011).

Endocrine disrupting chemicals have been linked to decreased reproductive capabilities through studies on individual fish reproductive organs, secondary sex characteristics, and mating behavior (Kime 1995; Seki et al. 2006; Salierno and Kane 2009). For example, estrogenic EDCs in the St. Lawrence River were associated with impaired male reproduction in the Spottail Shiner (*Notropis hudsonius)* as demonstrated by lower sperm concentrations and decreased mobility, TO, and delayed spermatogenesis in fish with high hepatic VTG mRNA levels (Aravindakshan et al. 2004). Furthermore, male Fathead Minnows have demonstrated decreased competitive behavior associated with reproduction after being exposed to estrogenic EDCs (Martinović et al. 2007).

Environmental Estrogens

Environmental estrogens are a subset of EDCs that exist in the environment and possess estrogenic activity in organisms (Stancel et al. 1995; Fernandez et al. 2007). They include natural hormones, synthetic hormones, and a number of organic compounds, such as pesticides, industrial byproducts, household chemicals, and pharmaceuticals (Kolpin et al. 2002). Natural estrogens (i.e., E1, E2, and E3) are sex steroid hormones that are secreted by the adrenal cortex, testis, ovary and placenta in humans and other vertebrates (Raven and Johnson 1999). Synthetic estrogens (e.g., EE2) are used in birth-control pills and estrogen replacement therapy among other uses. Many organic compounds (e.g., organochlorine pesticides and industrial pollutants, organophosphate pesticides, herbicides, and fungicides) have a molecular structure with a similar shape to those of natural estrogens and result in several compounds that are able to bind with estrogen receptors and thereby disrupt the endocrine system (Fry 1995; Norris 2007; Miller and Spoolman 2012).

Environmental estrogens enter aquatic environments in a variety of ways (Leet et al. 2011). Sex steroid hormones are excreted naturally via urine and feces by humans and other vertebrates, and these sex steroids enter the environment through sewage discharge and animal waste disposal (Ying et al. 2002). As a result, estrogenic activity has routinely been detected in effluents from sewage treatment plants and in surface waters that receive treated municipal effluent (Desbrow et al. 1998; Ternes et al. 1999; Williams et al. 2003). In addition, pesticides, herbicides, fungicides, and animal byproducts, such as poultry litter and cattle manure, are often applied to row crops and pasture land. These agricultural products can be transported into aquatic systems, often by precipitation (Yonkos et al. 2010) and have been measured in surface waters (Kolok et al. 2007) and riverbed sediment (Pereira et al. 1996; Peck et al. 2004; Hela et al. 2005; Kolok et al. 2007; Sellin Jeffries et al. 2011). For example, as density of animal feeding operations increased in agricultural watersheds, there was a proportional increase in compounds that can cause endocrine disruption in aquatic organisms (Ciparis et al. 2012).

In male fish, a common biomarker of exposure to estrogens and estrogen mimics is the production of vitellogenin (Vtg), an egg yolk protein precursor synthesized in the liver (Sumpter and Jobling 1995; Jobling et al. 1996; Donohoe et al. 1996; Tyler et al. 1999; Filby et al. 2007). Typically Vtg is synthesized by females during oocyte maturation and is released into the bloodstream where it binds with calcium ions and is sequestered into growing oocytes (Norris 1997). Males and females contain the Vtg gene, which is sensitive to estrogens and estrogen-mimicking compounds, but males typically lack the estrogen stimulus to trigger Vtg transcription (Ding 2005). Estrogen-induced Vtg mRNA synthesis in fish has been well documented (Jobling and Tyler 2003), and male fish have been shown to produce Vtg when exposed to E2 (Hemmer et al. 2002). Vtg concentrations can decrease in male fish previously exposed to estrogen

compounds if they undergo a period of depuration (Folmar et al. 2001; Liney et al. 2005), and studies have also shown male fish can likely acclimate, as demonstrated by a decrease in Vtg, to estrogens when chronically exposed (Nash et al. 2004). However, clearance of Vtg from fish plasma once induced with high levels can take as long as five months after the estrogenic cue is removed (Elliot et al. 1979), and effects of prolonged Vtg synthesis can include metabolic stress that leads to kidney and liver damage and necrosis (Herman and Kincaid 1988) and calcium losses from skeleton and scales (Carragher and Sumpter 1999).

Male fish exposed to exogenous estrogens can also develop oocytes (Nash et al. 2004) and an ovarian cavity (Jobling et al. 1998; Nolan et al. 2001; Brion et al. 2004; Nash et al. 2004) in their testicular tissue. The presence of oocytes in the testicular tissues of gonochoristic fish species is referred to as TO, or intersex (Jobling et al. 1998). Testicular oocytes are used as a signature effect of estrogen exposure (see Bahamonde et al. 2013 for a review) and can be used to gauge the reproductive health of fish (Jobling et al. 2002); a decrease in testis size, sperm mobility, and spermatogenesis in male fishes can result in diminished reproductive fitness (Bayley et al. 2002; Jobling et al. 2002; Aravindakshan et al. 2004; Jensen et al. 2004; Toft and Guillette 2005; Blazer et al. 2012). Exposure to estrogens has also resulted in altered secondary sex characteristics (Miles-Richardson et al. 1999) and mating behaviors in male fishes (Gray et al. 1999) as well as female-skewed sex ratios (Brion et al. 2004; Vajda et al. 2008).

Several field studies have reported TO in wild fish populations. Jobling et al. (1998) examined the incidence and severity of TO in Roach (*Rutilus rutilus)* collected upstream and downstream of wastewater treatment plant (WWTP) effluent on eight British rivers and found the proportion and severity of TO in males at downstream sites to be higher. Intersex frequency in Roach was also correlated with exposure to domestic sewage effluent in Danish streams

(Bjerregaard et al. 2006). Hinck et al. (2009) examined occurrence of intersex in male and female freshwater fishes collected from nine United States (U.S.) river basins and observed intersex in 3% of the fish collected (n = 3110), specifically in 4 of the 16 species sampled - Largemouth Bass (*Micropterus salmoides*), Smallmouth Bass (*M. dolomieui*), Common Carp (*Cyprinus carpio*), and Channel Catfish (*Ictalurus punctatus*). Hinck et al. (2009) found the greatest incidence of TO in the southeastern U.S., and Blazer et al. (2007) also reported high rates of intersex in Centrarchids, specifically Smallmouth Bass. That study collected Smallmouth Bass from sites in the Potomac River and nearby drainages and found a high prevalence of male Smallmouth Bass with TO (45% - 77%) at sites with watersheds dominated by agriculture, primarily poultry rearing and poultry processing.

Exposure to estrogen compounds during the period of sexual maturation can also have effects on the reproductive morphology of male Mosquitofish (Doyle and Lim 2002). Mosquitofish (western, *Gambusia affinis,* and eastern, *G. holbrooki*) are sexually dimorphic live-bearing fishes, and males have an elongated, modified anal fin (gonopodium) used to transfer sperm during copulation (Angus et al. 2002). Doyle and Lim (2002) exposed juvenile males to a range of E2 concentrations during the period of sexual maturation and measured significantly shorter gonopodium lengths on fish exposed to the highest concentrations (100 and 500 ng/L E2). They also observed a dose-dependent decrease in the percentage of fish with hooks on the gonopodium tip, a structure used as a holdfast during copulation, and a decrease in sexual activity.

Estrogens and other hormones are among the most biologically active compounds on the planet, and concentrations of estrogens as low as 1.0 ng/L of estradiol led to the induction of vitellogenin in male Trout (Hansen et al. 1998). Exposing breeding populations of Zebrafish

(*Danio rerio*) to low concentrations (5 ng/L) of a synthetic estrogen (17α-ethynylestradiol) reduced fecundity and caused complete population failure with no fertilization, suggesting population-level effects for aquatic species are probable (Nash et al. 2004). In a rare whole-lake exposure study, Kidd et al. (2007) found that chronic exposure to environmentally-relevant concentrations of 5-6 ng EE2/L led to total reproductive failure and near extirpation of the population of Fathead Minnows. Kidd et al. (2007) documented reproductive effects including feminization of males through the production of Vtg, TO, decreased average gonadosomatic index (GSI) in males, and altered oogenesis in female fish.

In summary, environmental estrogens occur in the aquatic environment as a result of a variety of anthropogenic activities. They have been shown to cause endocrine disruption in fish, ranging from subtle changes in physiology and reproductive behavior to permanently altered sexual differentiation and impaired fertility. By further investigating environmental estrogens, and the roles they are playing in aquatic systems, we may contribute to understanding the effects they are having on fish populations.

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Table 1-1. Federal (F) and state (Georgia, GA and Tenneessee, TN) protected species (endangered, E and threatened, T) in the Conasauga River. State status is only reported for species that are not protected by federal law.

Family Genus species	Common Name	Status
Cyprinidae *Cyprinella caerulea*	Blue Shiner	F-T
Percidae *Percina antesella*	Amber Darter	F-E
Percidae *Percina jenkinsi*	Conasauga Logperch	F-E
Acipenseridae *Acipenser fulvescens*	Lake Sturgeon	TN-E
Cyprinidae *Macrhybopsis* sp. cf. *M. aestivalis*	Coosa Chub	GA-E
Cyprinidae *Notropis asperifrons*	Burrhead Shiner	GA-T
Ictaluridae *Noturus munitus*	Frecklebelly Madtom	GA-E/TN-T
Percidae *Etheostoma brevirostrum*	Holiday Darter	GA-E/TN-T
Percidae *Etheostoma ditrema*	Coldwater Darter	GA-E/TN-T
Percidae *Etheostoma trisella*	Trispot Darter	GA-E
Percidae *Percina lenticula*	Freckled Darter	GA-E
Percidae *Percina kusha*	Bridled Darter	GA-E

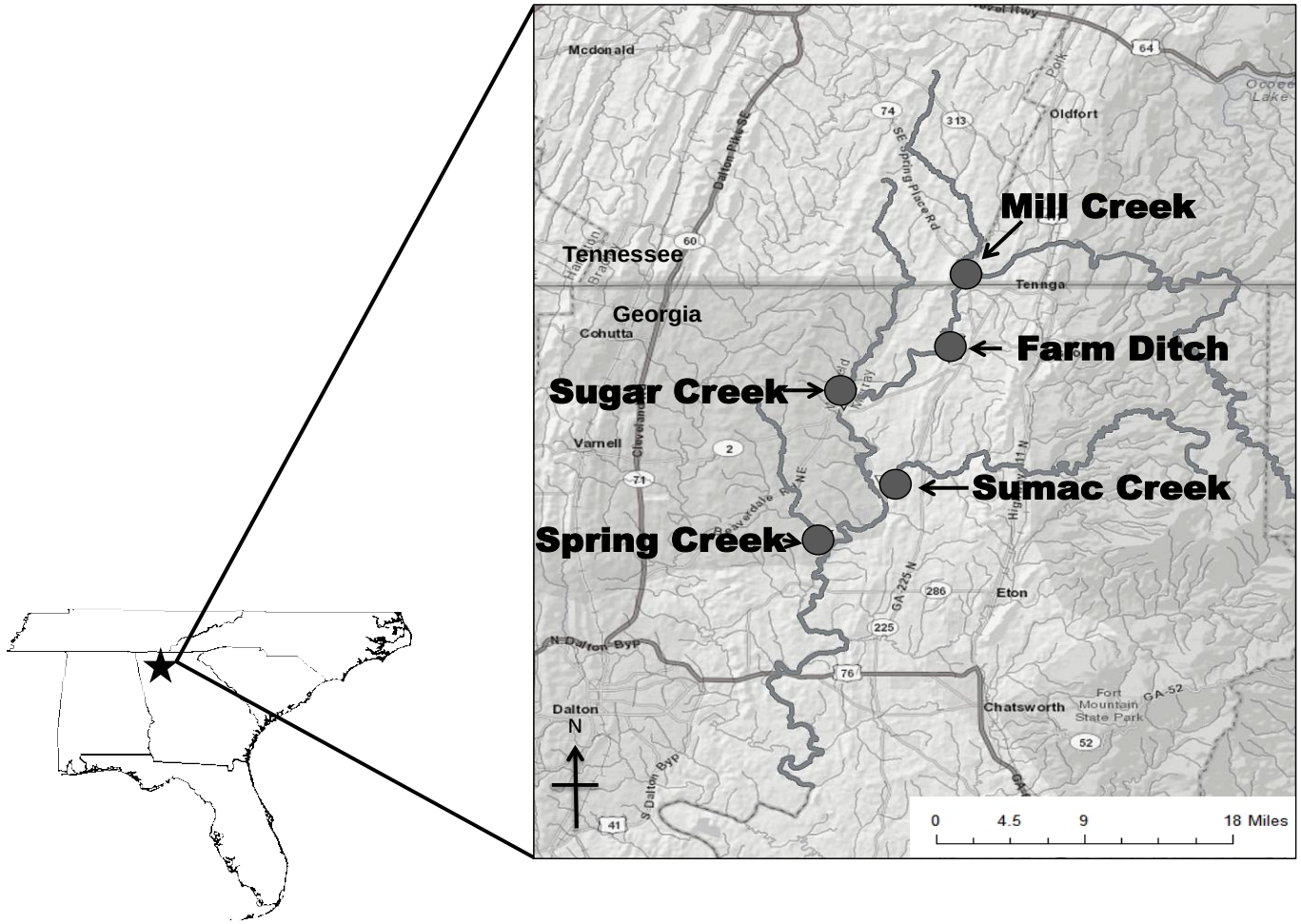

Figure 1-1. The upper Conasauga River is in the Coosa River Basin and extends from the Georgia/Tennessee border downstream to Dalton, Georgia. The study evaluated effects environmental estrogens were having on male fish in the river and identified tributaries with the highest estrogen input. Study tributaries were Mill Creek, a private ditch draining a dairy farm and row crop field ("Farm Ditch"), Sugar Creek, Sumac Creek, and Spring Creek

CHAPTER 2

AN INTEGRATED ASSESSMENT OF ENVIRONMENTAL ESTROGENS IN THE UPPER CONASAUGA RIVER, GA, USA[1]

[1]Jacobs, W.J., P.J. Lasier, S.M. Hassan, R.B. Bringolf. To be submitted to *Environmental Toxicology and Chemistry*.

Abstract

The current study evaluated the effects of estrogens on fishes in the upper Conasauga River (UCR) and identified tributaries with the highest estrogen inputs. Estrogen concentrations were higher in sediment than surface water. However, vitellogenin, a biomarker of estrogen exposure, was higher in male fish exposed to UCR water than those exposed to UCR sediments, which suggests estrogens in the water were more bioavailable than those in the sediment. In a survey of wild adult fishes from the UCR, 13 (7.5%) of the 174 male fishes (27 species) collected had testicular oocytes. Additionally, gonopodia of Western Mosquitofish collected from a UCR farm drainage ditch were shorter compared to mosquitofish from other areas of the basin with different land uses. Larval fish exposed to sediment from Mill Creek and Sugar Creek had lower growth compared to controls. Results indicate estrogen compounds in the UCR may be linked to declines in fish populations.

Introduction

Environmental estrogens are a growing concern in aquatic environments (Leet et al. 2011) and include natural hormones, synthetic hormones, and a number of organic compounds such as pesticides, industrial byproducts, household chemicals, and pharmaceuticals (see Mills and Chichester 2005 for a review). Environmental estrogens can act as endocrine-disrupting chemicals (EDCs) for aquatic species (Yonkos et al. 2010; Sellin Jeffries et al. 2011; Ciparis et al. 2012) and have been linked to decreased reproductive capabilities in fishes through changes in reproductive organ structure, secondary sex characteristics, and mating behaviors (Kime 1995; Van der Oost et al. 2003; Seki et al. 2006; Salierno and Kane 2009). Exposure to environmental estrogens has been shown to feminize male fish (Desbrow et al. 1998; Routledge et al. 1998),

and the most common biomarker of feminization is the production of vitellogenin (Vtg), a protein typically produced by females during oocyte maturation (Tyler et al. 1999; Filby et al. 2007). Since the process of vitellogenesis is highly controlled by estradiol (Sumpter and Jobling 1995), Vtg production in male fish exposed to estrogenic point-source pollution, such as wastewater treatment effluent (Desbrow et al. 1998; Vajda et al. 2011), has been used to identify estrogenic potency of wastewater and surface water (Bringolf and Summerfelt 2003; Jobling et al. 2002).

Testicular oocytes (TO; intersex), the presence of immature egg cells in testicular tissues of fishes that are normally gonochoristic (Baldigo et al. 2006; see Bahamonde et al. 2013 for a review), have also been used as an indicator of feminization and to gauge the reproductive health of a fish (Blazer et al. 2007). Laboratory studies have induced TO in fish by exposing them to the pharmaceutical estrogen 17α-ethynylestradiol (EE2) from hatch to sexual maturity (Yalcin et al. 2002; Balch et al. 2004). Hirakawa et al. (2012) induced TO in adult Japanese Medaka (*Oryzias latipes*) exposed to 100 ng/L EE2 for 4 weeks as well as Japanese Medaka exposed to 800 ng estradiol benzoate for 3 weeks and 20 ng/L EE2 for 6 weeks. Field studies examining the effects of different anthropogenic sources of estrogenic pollutants in freshwater systems, such as agricultural and sewage and industrial effluent, have also reported TO in wild fish (Bjerregaarda et al. 2006; Blazer et al. 2007; Hinck et al. 2009; Marentette et al. 2009; Prado et al. 2011; Tetreault et al. 2011; Blazer et al. 2012). Although Vtg and TO are both effects of estrogen exposure, a relationship between the two endpoints has not been fully established, which suggests that the pathways involved in TO and Vtg induction in male fish are not entirely correlated (Bahamonde et al. 2013).

Additional effects of estrogen exposure have been documented in laboratory studies with fish. For example, studies have reported a reduction in secondary sex characteristics and reproductive behaviors in male fish (Miles-Richardson et al. 1999; Coleman et al. 2009) as well as shortened gonopodia on Mosquitofish (*Gambusia*; Doyle and Lim 2002; Angus et al. 2005). Angus et al. (2005) exposed Western Mosquitofish (*G. affinis)* to different concentrations of EE2 (0.1, 1.0, 2.5, 5.0, 7.5 and 10.0 µg/g) in their food for 150 days and found groups exposed to higher concentrations had higher proportions of fish that failed to complete gonopodial development, increased levels of Vtg, shorter gonopodia, and reduced spermatophore counts. Estrogen exposure can also decrease growth rates and condition factor in early life stages of fish (Brion et al. 2004).

The upper Conasauga River (UCR), located in the Coosa River Basin in northwest Georgia, is home to a variety of rare fishes and mussels but many of the species are declining. Six UCR fish species are presumed to be extirpated (Walters 1997), three are federally protected, nine are state protected in Georgia and/or Tennessee (Skelton and Albanese 2006), and of the 37 native freshwater mussel species, only 19 remain (Novak et al. 2004). The UCR watershed is heavily influenced by agricultural activities (Sharpe and Nichols 2007; USFWS 2009) and during the period of fish and mussel declines, the agricultural acreage in the basin has increased (Georgia County Guide 2013). Murray and Whitfield counties make up most of the UCR basin, and from 1992 – 2007, Murray County farmland increased by 6,973 acres (21.2%) and Whitfield County farmland increased by 4,059 acres (10.5%; Georgia County Guide 2013). Application of poultry waste as fertilizer for row crops and pasture is common practice on farmland in these counties (Katie Owens, TNC, personal communication). Poultry excrete mostly 17β-estradiol, estrone, estriol, and their respective sulfated and glucuronidated counterparts (Hanselman et al.

2003), which can be transported into the aquatic system in runoff (Yonkos et al. 2010). Hormones have been documented in surface water (Kolok et al. 2007) and riverbed sediment (Pereira et al. 1996; Peck et al. 2004; Hela et al. 2005; Kolok et al. 2007) in agricultural areas. A preliminary UCR contaminant assessment in 2010 revealed elevated estrogen concentrations in UCR sediment, with concentrations generally increasing in a downstream direction (Lasier et al. 2012). These findings demonstrate the need to investigate the connection between agriculture, environmental estrogens, and the declines in aquatic biota in the UCR.

The goal of this study was to determine the potential connection between environmental estrogens and declines in fish populations in the UCR. The primary objectives were to identify UCR tributaries with the greatest concentrations and bioactivity of estrogens in sediment and water, and survey the incidence and severity of reproductive anomalies of fishes in the UCR.

Methods

Study Site

For the purposes of this study, the UCR is defined as extending from the Georgia/Tennessee state line south to Dalton, Georgia (Figure 2-1). Study sites were the five major tributaries in the UCR including (from upstream to downstream) Mill Creek, a private ditch draining a dairy farm and row crop field ("Farm Ditch"), Sugar Creek, Sumac Creek, and Spring Creek (Figure 2-1). At the time of sampling, land cover for watersheds of all of these tributaries was dominated by agricultural and forest (Figure 2-2). Percent agriculture ranged from 18% for the Sumac Creek watershed to 49% for the Farm Ditch watershed. Farm Ditch had the lowest percentage of forest land (35%), while the rest of the watersheds contained between 40 to 50% forest (MRLC 2006).

Estrogenic potency of UCR water: caged fish exposures

In October 2012, 10 sexually-mature male Fathead Minnows (*Pimephales promelas*) (Aquatic BioSystems, Fort Collins, Colorado) were transported to each of the five UCR study tributaries and placed in minnow cages (Frabill Deluxe Minnow Trap, 17 mm x 13 mm). The fish were acclimated to the river water for one hour, and two cages, each containing five fish, were haphazardly placed within each tributary for anchoring purposes (> 5 meters apart) for 10 days. Cages were anchored to structure in the water column or to iron stakes mounted in the sediment. Cages deployed within a tributary were not considered replicates as different locations likely resulted in different exposure conditions. Temperature during deployment was taken with a Hach® HQ40d portable meter. Fish were not fed during the deployment period but they may have obtained natural food items from the water moving through the cage. Ten male fish served as controls and were transported to the UCR sites and back to the University of Georgia (UGA) Aquatic Science Laboratory where they were held in dechlorinated tap water for the 10-d exposure period.

Following the 10-d exposure, the fish were recovered from the cages and transported alive in their respective tributary water to the UGA Aquatic Science Laboratory. At the lab, fish were euthanized with an overdose of buffered MS-222, weighed to the nearest 0.01 g, measured to the nearest mm, and dissected to confirm sex. Gonads were weighed to the nearest 0.001 g and gonadosomatic index (GSI) was calculated by multiplying the quotient of gonad weight divided by body weight by 100. Fulton's condition factor (K) was calculated for each fish (Murphy and Willis 1996), and whole fish were stored at -80 ^{o}C until Vtg analysis could be conducted.

For Vtg analysis, fish were pooled by sex (confirmed by dissection) from each cage and homogenized. Whole-body vitellogenin was quantified with a commercial enzyme-linked immunosorbent assay (ELISA; Cayman Chemical, Ann Arbor, Michigan) validated for Fathead Minnow Vtg, following the protocol provided by the manufacturer. The sandwich ELISA used a capture antibody of monoclonal anti-carp Vtg antibody and a detecting antibody of polyclonal anti-carp Vtg antibody.

Estrogenic potency of UCR water: POCIS

Polar Organic Chemical Integrative Samplers (POCIS; Environmental Sampling Technologies, St. Joseph, Missouri), passive sampling devices that accumulate organic chemicals from the water, were deployed from June 28 to August 1, 2012 in each of the five study tributaries. The POCIS sampler, specific for measuring pharmaceuticals and hormones, consisted of three OASIS HLB membranes mounted in a single canister (15-cm high x 16-cm wide). Prior to use, canisters were cleaned by soaking in a hot solution of non-perfumed detergent (Alconox, Inc., White Plains, New York), then washed with pesticide grade acetone (Fisher Scientific, Pittsburgh, Pennsylvania) before being rinsed thoroughly with hexane (Fisher Scientific, Pittsburgh, Pennsylvania). Membranes were stored at -20 ^{o}C until deployment and were transported to the field sites in their original packaging on ice. Samplers were anchored to structure in the water column and deployed for 34 days before membranes were recollected, disassembled, wrapped in methanol-rinsed aluminum foil and transported on ice to the UGA Aquatic Science Lab. The Mill Creek POCIS sampler was unable to be recovered; therefore, another sampler was deployed in Mill Creek from October 19 to November 30, 2012. Temperature during Mill Creek-POCIS deployment ranged from 6.0 ^{o}C to 15.5 ^{o}C. All

membranes were stored after deployment at -20 ^{o}C until they could be shipped to Environmental Sampling Technologies Lab (EST Lab, St. Joseph, Missouri) for hormone extraction.

Briefly, each membrane was extracted individually with 40 mL methanol (pesticide grade, Fisher Scientific, Pittsburgh, Pennsylvania). The samples were evaporated under ultra-high purity nitrogen to a volume of 0.5 mL, filtered through glass fiber paper (Grade G-6, Fisher Scientific, Pittsburgh, Pennsylvania) then quantitatively transferred to 2-mL amber ampules with methanol as the transfer solvent. Ampules were chilled in dry ice, flame sealed, and shipped to the UGA Environmental Analysis Lab where they were analyzed by gas chromatography-mass spectroscopy (GC-MS) for the three natural estrogens – estrone (E1), estradiol (E2), and estriol (E3). After extraction and analysis, hormone concentrations were back-calculated to account for river flow during the 34 days (42 days for Mill Creek POCIS) deployment period. The time-weighted average concentration of target compounds in the water was calculated as:

$$C_w = \mathrm{N} / (R_s \; x \; t)$$

Where C_w is the chemical concentration (ng/L); N is the mass (ng) of the chemical accumulated by the sampler; R_s is the sampling rate (L/day); and t is the exposure time (days). Following back-calculation, the total estrogenic equivalency (EEq) of each membrane was determined by normalizing the concentrations of the three natural estrogens based on their binding affinity for the estrogen receptor relative to E2 (Coldham et al. 1997). To determine if a relationship existed between estrogens in the water and land cover, two correlation analyses were preformed: one with POCIS EEqs and percent agricultural land for the tributary watersheds and another with POCIS EEqs and percent forest land for the tributary watersheds.

Field and laboratory blanks accounted for quality assurance and quality control. A field blank (membrane) was transported to each site and the lid of the container was opened at each

site allowing exposure to the surround air. Field blank membranes were then wrapped in methanol-rinsed aluminum foil and transported to the UGA Aquatic Science Lab on ice. EST Lab manufactured the laboratory blank, which consisted of 2.0 ml of methanol in an amber ampule, when POCIS membranes were extracted.

Estrogenic potency of UCR sediments

To determine estrogenic potency of the UCR basin sediments, sexually mature male Fathead Minnows (Aquatic BioSystems, Fort Collins, Colorado) were exposed to UCR sediment for 21 days. In June 2012, surface sediment (~3.5 L) was collected from each of the five study tributaries and exposures began on July 3, 2012. Controls consisted of commercial sand that was washed and amended to 0.3% - 0.6% organic content with sediment from the Florida Everglades. Sediment was homogenized by mixing the sample for 1 minute then 800-mL of sediment was added to each 19-L aquarium maintained in a water bath. Sediment was then overlaid with 15 L of aerated decholorinated tap water, five adult male Fathead Minnows were added, and treatments were replicated four times. Overlying water was renewed 50% daily, and fish were fed 1.5-mm fish pellets (Zeigler Bros., Inc, Gardners, Pennsylvania) once daily (3% of average body weight). Temperature, D.O., and pH were measured daily with Hach® HQ40d portable meter on one of the four replicates, which were cycled throughout the exposure. Alkalinity, hardness, and total ammonia nitrogen were measured with LaMotte Titration Kits on days 0, 7, 14, and 21 on one of the four replicates. On day 21, fish were euthanized with an overdose of buffered MS-222, weighed to the nearest 0.01 g, measured to the nearest mm, dissected to confirm sex, and gonads were weighed to the nearest 0.001 g. Fulton's condition factor and GSI were calculated for each fish as previously described. Fish were stored at -80 ^{o}C until Vtg

analysis could be conducted. For Vtg analysis, fish were thawed at room temperature, pooled by sex from each replicate and whole body Vtg was quantified with a commercially available ELISA kit as previously described for male Fathead Minnows deployed in cages.

Sediment hormone concentrations were analyzed for the adult male Fathead Minnow assay. Hormones were extracted from sediment by sonicating a 10-g sediment sample overlaid with 15 mL dichloromethane (DCM; Fisher Scientific). Samples were then centrifuged at 4000 rotations per minute for 15 minutes and solvent was decanted into 50-mL amber centrifuge vials. This process was repeated three times for each sample, but samples were sonicated with 10 mL DCM the second and third time. Liquid phases from each extraction were combined into a single extract, which was evaporated to dryness under liquid nitrogen, then resuspended to 3 mL with HPLC-grade acetone (Fisher Scientific). Extracts were analyzed by GC-MS for the three natural estrogens – E1, E2, and E3, which were then converted to EEq values. Two correlation analyses were performed: one with EEq values and percent agricultural land and another with EEq values and percent forested land for each of the study tributary's watershed. For quality assurance and quality control purposes, one sample was duplicated and another spiked with 100 µg/kg of E1, E2, and E3. Particle-size distributions of sediment samples were determined using the micropipette method of Miller and Miller (1987) for all three sediment tests.

Survey of testicular oocytes

Fishes were collected in May and June 2012 from the study area to assess the incidence and severity of TO in UCR fishes. Prior to sampling, the UCR was divided into six reaches to reflect the input of the various tributaries (see Figure 2-1): Reach 1 was from the GA/TN state line (approximately 4.0 km upstream of Mill Creek) downstream to, but not including, Mill

Creek; Reach 2 included Mill Creek downstream to, but not including, Farm Ditch; Reach 3 was Farm Ditch downstream to, but not including, Sugar Creek; Reach 4 was Sugar Creek downstream to, but not including, Sumac Creek; Reach 5 was Sumac Creek downstream to, but not including, Spring Creek; Reach 6 was only within Spring Creek. Capture techniques included kick seines, hoop net, trotline, and hook and line. Fishes were identified to species, euthanized with an overdose of buffered MS-222 and kept on ice during transport back to the UGA Aquatic Science Laboratory, where they were weighed to the nearest 0.1 g, measured to the nearest mm, and gonads dissected and weighed to the nearest 0.001 g. Gonads were preserved in buffered formalin, and gonad weight was used to determine GSI.

The formalin-fixed gonads were processed for histopathological analysis by the Veterinary Diagnostic Laboratory at the UGA. Briefly, gonads were embedded in paraffin wax, thin sectioned (5 µm) and stained with Hematoxin and Eosin for histological analysis of the gonad structure. The gonads were sectioned transversely to maximize the surface area examined (Kellock et al. in press). Intersex was determined based on the presence of oocytes in testicular tissue. If oocytes were present, TO was scored from 1-4 for severity based on a modified version of Blazer et al. (2007). Briefly, a severity rank of 1 was for only one oocyte present in the entire gonad section examined; rank 2 was more than one oocyte in the gonad, but oocytes were isolated from one another, or not in the same microscopic field of view (4x objective); rank 3 was at least one TO cluster, defined as more than one but less than five oocytes adjacent to or closely associated with each other in the gonad; rank 4 was two or more TO clusters in the same microscopic field of view (4x objective). For quality assurance and quality control purposes, a second reader evaluated a subset of 15 (8.6%) haphazardly chosen slides. Results between the two readers were compared for agreement of presence of oocytes.

Gonopodium lengths

Western Mosquitofish were captured by seine and dip net from Farm Ditch in May 2012 but were not captured at the other UCR study sites. Morphological features, such as smaller body size and presence of a gonopodium (anal fin extension), were used to identify males. Fish were euthanized with an overdose of buffered MS-222, transported on ice to the UGA Aquatic Science Laboratory and preserved in buffered formalin. Measurements included body length (nearest 0.1 mm) and gonopodium length (anal fin ray 4; nearest 0.1 mm; Image Pro 7.0). A ratio of gonopodium length to body length was then calculated for each fish. Similar measurements were taken on Western Mosquitofish collected from two sites in the Coahulla Creek watershed located in the lower Conasauga River. These two collections were borrowed from the UGA Natural History Museum. Although comparisons to fish collected from a pristine site would have been preferred, the Coahulla Creek collections met the requirements of being from the Coosa River basin from a watershed with differing land use. Coahulla Creek (Whitfield County, Georgia) is in an urban/suburban area with very little agriculture, whereas Farm Ditch drains a row crop field and dairy operation. Measurements of Mosquitofish collected from Farm Ditch were compared to measurements for Mosquitofish collected from the Coahulla Creek sites.

Sediment toxicity

Two assays were performed to test general toxicity of UCR sediment to larval fishes: an early-life stage (ELS) exposure with Fathead Minnows and an ELS exposure with Tricolor Shiners (*Cyprinella trichroistia*). Surface sediments (~ 0.5 L) were collected November 30, 2012 from the five study tributaries, and the 10-day sediment ELS exposure with Fathead Minnows

began on December 4, 2012. Sediments were transported on ice to the UGA Aquatic Science Laboratory in acid-rinsed glass containers and stored in a refrigerator between 2 and 4 ^{o}C prior to start of the exposure. Controls consisted of commercial sand that was washed and amended to 0.3% - 0.6% organic content by mixing with sediment from the Florida Everglades. Sediments were homogenized by thoroughly mixing for 1 minute before adding 100 mL of sediment to 300-mL beakers. Sediment was overlaid with 175-mL moderately-hard reconstituted water with a target hardness measurement between 80 to 100 mg/L as $CaCO_3$ (USEPA 2002). Each treatment was replicated four times, and 10 larval (< 24-h old) Fathead Minnows were stocked in each beaker. Water was renewed approximately 70% every six hours, and fish were fed brine shrimp (*Artemia salina)* twice daily *ad libitum*. Water chemistry variables were measured on days 1, 7, and 10 during the exposure in a 50-mL composite sample from each replicate in a treatment. On day 10, survival was assessed by movement and response to tactile stimulus before fish were euthanized with an overdose of buffered MS-222, measured to the nearest 0.01 mm (Image Pro 7.0), and dried in the oven at 60 ^{o}C for 24 hours before being weighed to the nearest 0.01 mg.

The ELS sediment exposure with Tricolor Shiners was preformed from August 16 to 26, 2013. Methods were similar to those used for the Fathead Minnow ELS exposure. Surface sediment was collected May 17, 2013. Controls consisted of beakers with water only and beakers with commercial sand. Sediment was overlaid with decholorinated tap water, and each treatment was replicated five times. Seven 3- to 5-day old Tricolor Shiners were stocked in each beaker. Fry were obtained by spawning adults collected from Shoal Creek in the Etowah River Basin, Georgia (Burkhead and Jelks 2001) and combining fish from four broods (>50 fry each) to ensure genetic diversity. Water in the beakers was renewed 100% every six hours, and fish were fed brine shrimp (*Artemia salina)* and rotifers (*Brachionus plicatilis) ad libitum* twice daily.

Composite water samples were taken from each replicate and water quality variables were measured on days 1, 6, and 10. Endpoints included survival, length, and dry weight.

Statistical analysis

Data for mortality of sediment-exposed fry, GSI for adult male Fathead Minnows exposed to sediment, and Vtg of sediment-exposed Fathead Minnows were analyzed by a non-parametric Kruskal-Wallis test at $\alpha \leq .05$. Single-factor analysis of variance (ANOVA; SAS Institute) was used to test for differences among sites for POCIS EEq values, condition factor, GSI of caged male Fathead Minnows, and gonopodium length of Mosquitofish. In sediment tests with fry, ANOVA was used to compare means for length and weight of treatment fish to the means of the control fry. When the ANOVA indicated significant differences existed, treatment means were compared to the control with a Dunnett's post-hoc test. Significance was determined at $\alpha \leq .05$ for all tests.

Results

Estrogenic potency of water: caged fish exposures

Following the 21-d exposure of caged male Fathead Minnows in UCR tributaries, whole body Vtg of males ranged from 0.02 µg/g in Spring Creek to 3.50 µg/g in Sugar Creek (Table 2-1, Figure 2-3). Within sites, Vtg induction varied from 0.22 µg/g to 3.50 µg/g between the two cages deployed in Sugar Creek and 0.03 µg/g to 1.56 µg/g in Sumac Creek (Table 2-1, Figure 2-3). Condition factor (± standard deviation (SD)) ranged from 1.08 (± 0.08) for fish in Sumac Creek to 1.21 (± 0.11) for those deployed in Farm Ditch (Table 2-1). Significant differences were not detected among sites for condition factor (ANOVA, $F = 1.73$, df = 5, 59, $p = 0.1431$).

Average GSI (± SD) ranged from 0.95 (± 0.43) for Spring Creek-deployed fish to 1.74 (± 0.44) for Mill Creek-deployed fish (Table 2-1) but means values were not significantly different among sites for GSI (ANOVA, $F = 1.01$, df = 5, 59, $p = 0.4192$). Temperature during deployment ranged from 10.5 ºC to 15.5 ºC, dissolved oxygen ranged from 8.1 mg/L to 8.7 mg/L, and pH from 7.1 to 7.6.

Estrogenic potency of water: POCIS

All three natural estrogens – E1, E2, and E3 – were detected in the POCIS extracts from all study tributaries, with the exception of E3 in Farm Ditch and Sugar Creek. The concentration of E1 (± SD) ranged from 1.80 ng/L (± 0.34) in Spring Creek to 15.39 ng/L (± 5.7) in Mill Creek; E2 ranged from 1.40 ng/L (± 0.2) in Spring Creek to 14.84 ng/L (± 6.0) in Mill Creek; and E3 ranged from 0.00 ng/L (± 0.0) in Farm Ditch and Sugar Creek to 7.26 ng/L (± 3.4) in Mill Creek. EEq values ranged from 1.60 ng E2/L (± 0.14) in Spring Creek to 16.36 ng E2/L (± 5.82) in Mill Creek (Figure 2-4). EEqs differed among tributaries (ANOVA, $F = 11.97$, df = 4,14, $p = 0.0008$) but the relationship of EEqs to watershed land cover was very weak; $r^2 < 0.12$ for POCIS EEqs vs. percent agriculture and percent forest land. Temperature during deployment ranged from 24.0 ºC to 31.0 ºC.

Estrogenic potency of UCR sediments

Average Vtg concentrations (± SD) of adult male Fathead Minnows ranged from 0.00 (± 0.00) µg/g for fish exposed to Spring Creek and Mill Creek sediment to 0.38 (± 0.59) µg/g for fish exposed to Sugar Creek sediment (Table 2-2, Figure 2-3), but Vtg concentrations of fish exposed to UCR tributary sediments were not significantly different from the controls (Kruskal-

Wallis, $\chi^2 = 8.0601$, df = 5, 23, $p = 0.1530$). Condition factor (± SD) varied from 0.99 (± 0.08) in controls to 1.10 (± 0.07) for Farm Ditch (Table 2-2), and fish exposed to Farm Ditch sediment had a higher average condition factor compared to fish exposed to control sediment (ANOVA, $F = 3.18$, df = 5, 103, $p = 0.0105$). Average GSI (± SD) ranged from 0.63 (± 0.30) for Sumac Creek-exposed fish to 1.03 (± 0.61) for Mill Creek-exposed fish (Table 2-2), but GSI did not differ between treatment groups and controls (Kruskal-Wallis, $\chi^2 = 7.5484$, df = 5, 103, $p = 0.1829$). Heater malfunction caused temperature to range from 22.7 ^{o}C to 26.5 ^{o}C, D.O. ranged from 5.58 to 8.11 mg/L, pH ranged from 6.8 to 8.0, alkalinity ranged from 12.0 to 40.0 mg/L $CaCO_3$, hardness from 16.0 to 52.0 mg/L $CaCO_3$, and total ammonia nitrogen from 0.0 to 1.5 mg/L.

Sediment analysis revealed that only E1 and E2 were present in UCR sediments used for adult male Fathead Minnow exposures. Concentrations of E1 were generally highest of the three estrogens and ranged from 25.5 µg/kg in Mill Creek to 193.0 µg/kg in Sugar Creek. The E2 concentrations ranged from 1.0 µg/kg in Mill Creek to 7.7 µg/kg in Sugar Creek. EEq was highest in Sugar Creek sediment at 26.2 ng E2/L and lowest in Mill Creek sediment at 3.5 ng E2/L (Figure 2-4). Recovery of spiked samples ranged from 68.9% to 104.0% (n = 4). The relationship to watershed land cover was very weak; $r^2 < 0.07$ for POCIS EEqs vs. percent agriculture and percent forest land. For all three sediment tests, total organic material ranged from 1.2% to 4.1%, sand from 37.9% to 85.4%, silt from 9.8% to 44.9%, and clay from 3.5% to 17.2% (Appendix A).

Survey of testicular oocytes

Fish species captured and their relative proportions of TO varied by reach, but of the 174 male fishes collected from the UCR, 13 (7.5%) had ooctyes in their testicular tissue (Table 2-3). Ten of the 13 intersex males (76.9%) were from the family Centrarchidae, two (15.4%) from family Percidae, and one (7.7%) from family Cyprinidae (Table 2-3). The most common severity level observed was Level 2, which was assigned to seven fishes. The rate of TO was greatest in Reach 4 (13.6%, 3 intersex males out of 22 male fishes collected), 10.5% (2 of 19) at Reach 6, 9.4% (3 of 32) at Reach 3, 6.3% (4 of 68) at Reach 1, 4.4% (1 of 23) at Reach 2, and 0.0% (0 of 14) at Reach 5. The two independent slide readers agreed on 93.0% (14 of 15) of slides for determination of presence of oocytes in the testicular tissue.

Gonopodium length

The average ratio (± SD) of gonopodium length to body length of Western Mosquitofish (n = 45) collected from Farm Ditch was 0.25 (± 0.02), and average ratios for the two collections of Mosquitofish made from the Coahulla Creek watershed in the lower Conasauga River were 0.28 (± 0.03; n = 9) and 0.28 (± 0.01; n = 28). Gonopodium size (relative to body length) of Mosquitofish from Farm Ditch was significantly shorter than both of the sites in Coahulla Creek (ANOVA, $F = 17.73$, df = 2, 81, $p = <0.0001$).

Sediment toxicity

The average dry weight (± SD) for Fathead Minnow fry at the end of the 10-day exposure ranged from 0.60 mg (± 0.16) for fry exposed to Mill Creek sediment to 0.80 mg (± 0.08) for fry exposed to Farm Ditch sediment (Figure 2-5). The dry weight of fry exposed to Mill Creek,

Sugar Creek, Sumac Creek, and Spring Creek sediment were significantly lower from the control (ANOVA, $F = 5.88$, df = 5, 23, $p = 0.0022$). Mean survival (± SD) of Fathead Minnow fry at the end of the exposure ranged from 45.0% (0.41) for Sumac Creek to 85.0% (0.13) for Farm Ditch (Figure 2- 5). Survival of fish exposed to UCR sediments did not differ significantly from the control (Kruskal-Wallis, $\chi^2 = 7.7162$, df = 5, 23, $p = 0.5$). The mean length (± SD) of Fathead Minnow fry ranged from 7.8 mm (± 1.0) for fry exposed to Sugar Creek sediment to 8.3 mm (± 1.1) for fry exposed to Farm Creek sediment, and length of fry exposed to Sugar Creek was significantly lower than fry exposed to control sediment (ANOVA, $F = 2.3714$, df = 5, 169, $p =$ 0. 0481). Temperature ranged from 22.5 ºC to 23.4 ºC, D.O. ranged from 38 to 97% saturation, pH ranged from 7.6 to 7.9, alkalinity from 66.0 to 84.0 mg/L as $CaCO_3$, and hardness from 78.0 to 86.0 mg/L as $CaCO_3$. Total ammonia nitrogen was 0.0 to 2.7 mg/L, and conductivity was 381-436 µS/cm.

Average mass (± SD) of Tricolor Shiner fry at the end of the 10-day exposure ranged from 0.53 mg (± 0.05) for fry exposed to Mill Creek sediment to 0.62 mg (± 0.04) for fry exposed to Sumac Creek sediment (Figure 2-5). A post-hoc multiple comparison test revealed the dry weights of fry exposed to Mill Creek, Farm Ditch, and Sugar Creek sediment were significantly lower than the water-only control (ANOVA, $F = 4.86$, df = 6, 34, $p = 0.0016$). Mean survival (± SD) of Tricolor Shiners fry at the end of the exposure ranged from 88.6% (± 0.12) for Spring Creek to 97.2% (± 0.06) for Farm Ditch (Figure 2-5). Survival of fish exposed to treatment sediments did not differ significantly from the water-only control (Kruskal-Wallis, $\chi^2 = 5.3553$, df = 6, 34, $p = 0.4991$). The average length (± SD) of Tricolor Shiners ranged from 7.19 mm (± 0.28) for fry exposed to Mill Creek sediment to 7.45 mm (± 0.49) for fry exposed to Farm Ditch sediment. Differences in fry length of treatment-exposed fish were not different than

the control (ANOVA, $F = 1.01$, df = 6, 180, $p = 0.4230$). Temperature ranged from 21.1°C to 22.2°C, D.O. ranged from 59% to 92% saturation, pH ranged from 7.3 to 7.9, alkalinity from 26.0 to 36.0 mg/L $CaCO_3$, and hardness from 40.0 to 50.0 mg/L $CaCO_3$. Total ammonia nitrogen was 0.0 to 0.7 mg/L, and conductivity was 140 to 157 µS/cm.

Discussion

Results of this study indicated estrogens were present in UCR surface water and sediment, and the concentrations were sufficient to cause endocrine disruption in fishes. Feminization of male fish in the UCR was evident from elevated Vtg production in caged Fathead Minnows, TO in multiple species, and shortened gonopodia of Western Mosquitofish. Decreased weights of larval fishes exposed to UCR sediments are consistent with the other findings but growth is a holistic endpoint and may have been influenced by a variety of factors in addition to estrogens. Additionally, estrogens or their effects or both were measured in each of the UCR tributaries; however, some tributaries appeared to have greater estrogen activity than others, and activity varied temporally and spatially.

Estrogens appear to be accumulating in the UCR sediment because concentrations were 200x to 13,000x greater in sediment than in surface water, but this accumulation does not necessarily equate to bioavailability. Instead, Vtg concentrations in male fish exposed to UCR water were overall higher than concentrations in male fish exposed to UCR sediment (Figure 2-3), so despite lower concentrations in water, estrogen-active compounds appear to be more bioavailable in surface water than sediment. Interestingly, the tributaries with the highest sediment estrogen values were also the tributaries and reaches of river with the highest rates of TO.

Based on levels of estrogens measured in UCR surface water, effects on fish populations (Kidd et al. 2007) and feminization of aquatic reptiles and amphibians (Palmer and Palmer 1995) may be expected. Kidd et al. (2007) dosed an entire lake with 5-6 ng/L of the synthetic estrogen ethynylestradiol and observed a population collapse in Fathead Minnows within 3 years. Prior to reproductive failure and the population collapse, male Fathead Minnows had high Vtg levels, retarded gonad development rates, and TO (Kidd et al. 2007). Total estrogen activity (EEq) measured in UCR surface water was similar to the concentrations in Kidd et al. (2007) and suggests that reproduction may be impaired for at least for some fish species in the UCR.

Surface water estrogen concentrations were also similar to those measured in water downstream of dairy operations and cattle grazing rangeland (Kolodziej and Seklak 2004; Kolodziej and Seklak 2007) and wastewater treatment discharge (Desbrow et al. 1998; Kolodziej and Seklak 2003; Joss et al. 2004; Kolok et al. 2007). Kolodziej et al. (2007) quantified estrogens in creeks in central California affected by grazing livestock and detected estrone as high as 38.0 ng/L and E2 concentrations up to 1.7 ng/L. Although concentrations of E1 measured in UCR surface water were lower than those measured by Kolodziej et al. (2007), E2 concentration in Mill Creek was approximately 8x higher than those measured by Kolodziej et al. (2007). Furthermore, Desbrow et al. (1998) measured estrogen compounds from seven wastewater treatment plant effluents discharging in British river systems and found both E1 and E2 in all seven effluents. Of the 21 concentrations of E1 measured throughout a 1-year period, 76% (16 of 21) were in the E1 range measured in the UCR in the present study, and 86% (18 of 21) were in the E2 range measured in the UCR (Desbrow et al. 1998). Interestingly, Mill Creek POCIS measured the highest EEq value of all the tributaries but had the lowest sediment EEq. However, the temporal variation of land-use activities should be considered when interpreting these results

because the sampler was unable to be recovered when first deployed during summer 2012 and was re-deployed in fall 2012.

Estrogen concentrations measured in UCR sediment were comparable to estrogens levels measured in riverbed sediment downstream of wastewater treatment plant effluent (William et al. 2003) and in activated and digested sewage sludge (Ternes et al. 2002). However, UCR sediment concentrations for E1 were between 20x to 200x higher and E2 concentrations 4x to 35x higher than levels measured in riverbed sediment from streams in the Potomac basin affected by wastewater treatment plant effluent, septic discharges, and agricultural sources (Kolpin et al. 2013). Research on estrogens affected by agricultural runoff has focused on quantifying surface water concentrations, and studies reporting estrogen concentrations for riverbed sediment, especially in agricultural watersheds, are scarce. Additionally, E1 was the highest estrogen concentration in all UCR tributaries, which is consistent with other studies (López de Alda et al. 2002; Kolpin et al. 2013) and likely a result of E2 oxidation to E1 (Ternes and Mueller 1999).

Sugar Creek had the highest levels of E1 and E2 in sediment, which was not surprising given that Sugar Creek had the highest percent of fine particle fraction (clay, 17.2%) relative to the other study tributaries, and sorption affinity of estrogens is correlated with mineral particle size (Casey et al. 2003; Duong et al. 2010) and clay minerals (Casey et al. 2003). Fine particle fractions such as clay are a more effective sorbent (50-90% higher in K_{oc}) than larger fractions (Karickhoff et al. 1979) because fine particle fractions have higher surface areas and provide more binding sites for hormones (Yamamoto et al. 2003). The relatively high sorption affinity of estrogens to small particles also explains the low bioavailability of estrogens in UCR sediment. All UCR study tributaries generally had a low percentage of fine particle fraction (clay, 6% to 17%) relative to larger particle fractions, such as sand (38% to 80%) and silt (15% to 45%).

Duong et al. (2010) reported that smaller fractions of sediment increased Vtg concentrations in male Japenese medaka (*Oryzias latipes*) after a 7-day exposure, while larger particle fractions showed no effects on Vtg induction in fish exposed to the same amount of E2. The accumulation of estrogens in UCR sediment is consistent with Jurgens et al. (1999), who investigated partitioning of estrogens in aquatic systems and found that between 13% and 92% of the estrogens entering a river system would end up in the bed-sediment compartment with the majority of sorption occurring within the first 24 hours of contact. Furthermore, López de Alda et al. (2002) analyzed the fate of estriol (E3) in the aquatic environment and determined the compound would have a general tendency to accumulate in sediments.

Fry were sensitive to the UCR sediments as demonstrated by decreased growth in Fathead Minnows and Tricolor Shiners (Figure 2-5). However as previously mentioned, growth is a holistic endpoint and may have been influenced by a variety of factors in addition to estrogens. Studies have shown reduced growth and condition factor in early life stages of Zebrafish exposed to waterborne E2 (Brion et al. 2004), but other studies did not report reductions in growth or condition factor after exposing juvenile Fathead Minnows to waterborne 17α-ethynylestradiol (Panter et al. 2002). Sediments from the UCR may contain a variety of agrochemicals including pesticides, nutrients, and hormones and specific causes of reduced growth in UCR-exposed fry remain unknown and a priority research need.

Hormone concentrations in the water and sediment for the five study tributaries were not related with general land cover classifications such as forest or agricultural land; therefore, hormones levels in UCR tributaries may be linked to specific land use activities and may vary seasonally. Possible sources include, but are not limited to, use of estrogen-active fertilizers such as poultry litter and manure (Shore et al. 1995; Nichols et al. 1997; Yonkos et al. 2010) applied

to pastures and row crops, livestock in or near streams (Kolodziej and Sedlak 2007), or septic tank discharge (Pal et al. 2010). Land cover analyses did not account for specific factors such as stream buffer width, streams in or adjacent to pastures, number of drainage ditches from row crop fields to streams, so a more detailed land use analysis may provide additional insight into specific sources of estrogens.

Sugar Creek had the highest effects with elevated estrogen concentrations for both water and sediment (Table 2-4). Consistent with the estrogen concentration data, Sugar Creek also generally demonstrated the greatest effects in fish of all study tributaries (Table 2-4). General land-use patterns in the watershed of this creek did not differ markedly from the other watersheds, and reasons for the higher estrogen concentrations are unknown. However, in addition to the aforementioned factors, timing of poultry litter or manure (fertilizers) applications may have influenced the chemical and biological results in this study. Additionally, sampling events were limited in the present study for estrogen concentrations and biological effects, which may vary temporally with changes in factors such as land-use activities, chemical degradation, temperature, and tributary size.

Vtg concentrations for caged male Fathead Minnows in the UCR were only 3x to 5x greater than Vtg levels of control male Fathead Minnows maintained in the lab. This result suggests that Vtg induction in caged fish (and sediment-exposed fish) was relatively low overall. Furthermore, Vtg concentrations in caged male Fathead Minnows in the UCR were 10,000x to 12,000x lower than Vtg concentrations measured in estrogen-exposed male Fathead Minnows in the Kidd et al. (2007) study. The estrogen exposure level used by Kidd et al. (2007) was similar to EEq measured in UCR tributary water samples, but differences in duration of exposure may

explain the difference in Vtg levels between the two studies. Caged fish were deployed in the UCR for only 10 days, whereas Kidd et al. (2007) conducted a 3-year (full lifecycle) exposure.

Despite the high surface water EEq value measured in Mill Creek relative to the other tributaries, caged fish deployed in Mill Creek did not induce as elevated Vtg levels as fish deployed at other sites. Mill Creek POCIS measured estrogen concentrations in the water at the time the fish were deployed in October 2013, whereas samplers at the other sites were deployed in summer 2012 and fish deployed in October 2012. Temporal variation of estrogens concentration in the water because of land use activities or rain events could account for the apparent discrepancy between Vtg and EEq levels among the tributaries.

Induction of Vtg varied by >115% between the two cages within some UCR streams, such as Farm Ditch, Sugar Creek, and Sumac Creek. The two cages within a stream were never placed within < 5 meters from one another, therefore estrogen inputs and other conditions may have varied spatially. Conversely, trends in UCR sediment-exposed fish Vtg concentrations were generally consistent with estrogen concentrations measured in the sediment. For example, the highest EEq and Vtg were measured in Sugar Creek. However, Spring Creek was an exception with an elevated estrogen concentration but Vtg was not measured in male fish.

Fathead Minnows are widely used to test estrogenic potency of surface water, but other species in the UCR and elsewhere may be more sensitive to estrogen-active compounds; as such, our results should be interpreted with some caution. For example, Rankouhi et al. (2004) found Carp (*Cyprinus carpio*) and Rainbow Trout (*Oncoryhynchus mykiss*) to be more sensitive to E2 than Bream (*Abramis brama***)**, which indicates possible differences in E2 binding affinity for the estrogen receptor or differences in metabolic rate or both among species. Studies examining Vtg induction in male fish from exogenous steroids in agricultural watersheds are rare, but research

has shown demasculinization in male fish exposed to hormones in agriculturally-affected waterways (Orlando et al. 2004; Kolok et al. 2007).

Testicular oocytes in male fishes, currently believed to be a signature effect of estrogen exposure, were found throughout the UCR. The overall rate of TO for the UCR (7.5%) was similar to the overall rate (3%) observed by Hinck et al. (2009), who surveyed multiple fish species. In the UCR, the highest prevalence of TO was found in the Centrarchidae family (10 of the 64 centrarchids collected), consistent with reports from other intersex surveys (Hinck et al. 2009; see Bahamonde et al. 2013). However, the rate of TO for centrarchids in the UCR was generally lower compared to Iwanowicz et al. (2009) who reported a prevalence of 82% - 100% intersex in smallmouth bass collected both upstream and downstream of WWTP effluent and Kellock et al. (in press) who reported 48% TO in black basses (*Centrarchidae*) in Georgia. The reaches with the highest rates of TO (reach 4, Sugar Creek, and reach 6, Spring Creek) were also the reaches with the highest sediment EEq values, which raises the question of possible exposure and effects to developing eggs incubating on the sediment and newly hatched fry. Additionally, Mill Creek (reach 2), which had one of the lowest testicular rates (4.4%), had the highest surface water EEq (16.4 ng E2/L).

Harris et al. (2011) examined reproductive success of male fish with TO and found that although most intersex fish were able to participate in spawning, there was a negative relationship between intersex severity index and reproductive success. The most prevalent TO severity level of UCR male fish examined was 2 (54%) and 1 (31%), which suggests TO will not severely affect reproductive performance; however, sample sizes for several of the UCR tributaries were small (e.g., only 14 fish were examined from reach 5 (Sumac Creek)).

Angus et al. (2005) reported that shorter gonopodia in Mosquitofish are a biomarker for estrogen exposure. The smaller gonopodium to body length ratios in Farm Ditch when compared to Mosquitofish collected from Coahulla Creek suggest hormonally-active compounds draining from the adjacent row crop field or dairy farm may have altered the sexual development of fish living there**.** Proper morphologic development of sex organs is necessary for correct function (Collier 1936), and male Mosquitofish exposed to water contaminated by agrichemicals have demonstrated decreased sperm count and sexual behavior (Toft and Guillette 2005). Additionally, Coahulla Creek is located in the lower Conasauga River and has an urban/suburban watershed with very little agriculture. Unfortunately Mosquitofish were not collected at any of the other UCR tributaries and therefore could not be compared. Angus et al. (2002) investigated a population of Western Mosquitofish collected below a major WWTP in Alabama for evidence of adverse effects of estrogen exposure, (i.e., comparison of gonopodium length of effluent-exposed fish to control populations). Although the study did not find differences between the effluent exposed and control group, it served as a good comparison as fish were collected in the Southeastern U.S. Mean gonopodium lengths reported by Angus et al. (2002) were generally similar to those collected from Farm Ditch with one exception: longer fish, specifically 31.7 mm to 36.0 mm in length, collected from Farm Ditch had a shorter mean gonodpodium lengths (7.7 ± SD =0.5) than Angus et al. (2002) control population (8.27 ± 0.42) and effluent-exposed fish (8.22 ± 0.08). Mean gonopodium length of Mosquitofish collected from Coahulla Creek in the lower Conasauga River were between 0.50 mm to 0.80 mm longer than those of effluent-exposed fish (Angus et al. 2002). These comparisons suggest that larger fish in Farm Ditch might be most affected by contaminants entering the system via field drainage.

The present study was limited to a single surface water sample (composite) for hormone analysis. Multiple water samples collected throughout the year would helped elucidate the seasonal fluctuation of estrogens in the water column, where estrogens appear to be most bioavailable to fishes. Additionally, a larger sample size of fishes for analysis of TO would have been preferable, specifically containing more of the larger, longer-lived species, which were largely absent in the present study. For example, White Suckers (family Catostomidae; *Catostomus commersonii)*, occur in the Conasauga River and have been reported with TO downstream of wastewater treatment effluents with estrogenic activity (Woodling et al. 2006; Vajda et al. 2008). Only two fishes from the family Catostomidae, male Golden Redhorse (*Maxostoma erythrurum)*, were collected in the present study and neither exhibited TO.

The effects of elevated hormones in the sediment on benthic and nest-building fishes are unknown. Studies have shown poultry waste containing natural sex hormones can affect sex ratios of wild fish (Yonkos et al. 2010), and life-cycle exposures to low levels (<5 ng/L) of synthetic estrogen have resulted in reproductive anomalies such as skewed sex ratios, decreased egg fertilization, and demasculinization (Parrott and Blunt 2005; Länge et al. 2001). However, data are still largely absent for the effects of sediment-bound hormones on developing eggs and larvae as well as effects on benthic invertebrates such as freshwater mussels, oligochaete worms, amphipods.

In conclusion, results from this study demonstrate estrogens are present in water and sediment samples of the UCR basin and estrogens may be linked to the declines of fishes in the basin. These findings can serve to inform natural resource managers of tributaries where agricultural best management practices (BMPs) would be best served. Agricultural BMPs, often used to safeguard against nutrient loading in aquatic systems include, but are not limited to,

fencing to control the access of livestock to streams, grass waterways and field borders (Nichols et al. 1998), developing a management plan to apply nutrients and fertilizers to fields at rates necessary to achieve realistic crop yields and, if possible, avoiding fertilizer application before a rain event (EPA 1993). By implementing BMPs in strategic locations, levels of estrogen, nutrients and other agricultural chemicals in the UCR (and similar rivers) may be reduced to enhance stream protection and the opportunity for recovery of aquatic life.

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Table 2-1. Fulton's condition factor (K), gonadosomatic index (GSI), and vitellogenin (Vtg; µg Vtg /g fish wet weight) for adult male Fathead Minnows (*Pimephales promelas*) deployed in cages in upper Conasauga River study tributaries for 10 days. Two cages, each containing five fish, were placed in separate locations (A and B) in each of the tributaries, and thus not considered replicates. Standard deviation is noted in parenthesis. There were no differences (ANOVA) in K (F = 1.73, df = 5, 59, p = 0.1431) or GSI (F = 1.01, df = 5, 59, p = 0.4193) among sites. Significance was determined at α= 0.05.

Cage Site	K	GSI	Vtg
Control - A	1.18 (0.11)	1.40 (0.78)	0.72
Control - B	1.19 (0.10)	1.29 (0.49)	0.07
Mill Creek - A	1.17 (0.13)	1.62 (0.44)	0.69
Mill Creek - B	1.05 (0.17)	1.74 (0.44)	0.40
Farm Ditch - A	1.21 (0.11)	1.41 (0.36)	1.91
Farm Ditch - B	1.14 (0.06)	1.53 (0.45)	0.75
Sugar Creek - A	1.19 (0.04)	1.54 (0.46)	3.50
Sugar Creek - B	1.20 (0.15)	1.35 (0.40)	0.22
Sumac Creek - A	1.08 (0.08)	1.05 (0.49)	0.03
Sumac Creek - B	1.09 (0.09)	1.37 (0.62)	1.56
Spring Creek - A	1.11 (0.06)	0.95 (0.43)	0.02
Spring Creek B	1.17 (0.06)	1.64 (0.66)	0.05

Table 2-2. Mean Fulton's condition factor (K), gonadosomatic index (GSI), and vitellogenin (Vtg; µg/g fish wet weight) for adult male Fathead Minnows (*Pimephales promelas*) exposed to sediment collected from upper Conasauga River study tributaries. Standard deviation is noted in parenthesis. Single-factor analysis of variance (ANOVA) followed by Dunnett's post-hoc test was used to test for differences between sites and control for K (F = 3.18, df = 5, 103, p = 0.0105). No differences existed among sites for GSI (χ^2 = 7.5484, df = 5, 103, p = 0.1829) or Vtg (χ^2 = 8.0601, df = 5, 23, p = 0.1530). Significant differences from control fish are noted with an asterisk. Significant differences (α= 0.05) from control fish are noted with an asterisk.

Site	K	GSI	Vtg (µg/g)
Control	0.99 (0.08)	0.90 (0.31)	0.05 (0.04)
Mill Creek	1.02 (0.07)	1.03 (0.61)	0.00 (0.00)
Farm Ditch	1.10 (0.07)*	0.77 (0.38)	0.19 (0.37)
Sugar Creek	1.02 (0.11)	0.85 (0.32)	0.38 (0.59)
Sumac Creek	1.07 (0.11)	0.63 (0.30)	0.10 (0.18)
Spring Creek	1.06 (0.09)	0.72 (0.30)	0.00 (0.00)

Table 2-3. The occurrence of testicular oocytes (TO) in male fish by species in the upper Conasauga River[a]. Severity of TO was scored from 1-4: 1, only one oocyte was present in the entire gonad section; 2, more than one oocyte but oocytes were isolated from one another, or not in the same microscopic field of view (4x objective); 3, at least one TO cluster, defined as more than one but less than five closely related oocytes in the gonad; 4, two or more TO clusters in the same microscopic field of view (4x objective).

Family *Genus species*	Common name	Severity	% with TO
Centrarchidae *Micropterus coosae*	Redeye Bass	2	7 (1/13)
Centrarchidae *Lepomis auritus*	Redbreast	1, 2, 2, 4	31 (4/13)
Cyprinidae *Notropis stilbius*	Silverstripe Shiner	3	7 (1/13)
Centrarchidae *Micropterus salmoides*	Largemouth Bass	1	7 (1/13)
Centrarchidae *Lepomis megalotis*	Longear	2, 2	15 (2/13)
Percidae *Etheostoma stigmaeum*	Speckled Darter	1	7 (1/13)
Centrarchidae *Pomoxis nigromaculatus*	Black Crappie	2	7 (1/13)
Centrarchidae *Lepomis punctatus*	Spotted Sunfish	2	7 (1/13)
Percidae *Percina nigrofasciata*	Blackbanded Darter	1	7 (1/13)

[a]Testicular oocytes were not observed in Golden Redhorse (*Moxostoma erythrurum*), Shadow Bass (*Ambloplites ariommus*), Green Sunfish (*Lepomis cyanellus*), Warmouth (*Lepomis gulosus*), Bluegill (*Lepomis macrochirus*), Alabama Bass (*Micropterus henshalli*), Alabama Shiner (*Cyprinella callistia*), Largescale Stoneroller (*Campostoma oligolepis*), Tricolor shiner (*Cyprinella trichroistia*), Blacktail Shiner (*Cyprinella venusta*), Striped Shiner (*Luxilus chrysocephalus*), Burrhead Shiner (*Notropis asperifrons*), Rainbow Shiner (*Notropis chrosomus*), Riffle Minnow (*Phenacobius catostomus*), Coosa Darter (*Etheostoma coosae*), Greenbreast Darter (*Etheostoma jordani*), Mobile Logperch (*Percina kathae*), and Bronze Darter (*Percina palmaris*)

Table 2-4. Matrix ranking the concentrations of vitellogenin induced in caged Fathead Minnows (*Pimpephales promelas*) deployed in study tributaries in the upper Conasauga River (UCR), vitellogenin induced in Fathead Minnows exposed in the lab to UCR sediment, estrogen equivalent (EEq) values calculated from the three natural estrogens – estrone, estradiol, and estriol – measured in UCR water and sediment, and the rate of testicular oocytes (TO) in fishes collected in or downstream of each UCR study tributary. Rankings were summed to provide an indication of where the greatest effects occurred in the UCR. A 1 was assigned to the tributary with the lowest data point in the set, and a 5 was assigned to the tributary with the highest data point in the set.

	Vitellogenin		EEq			
Tributary	Water*	Sediment	Water	Sediment	TO Survey	Sum
Mill Creek	2	2	5	1	2	12
Farm Ditch	4	4	3	3	3	17
Sugar Creek	5	5	4	5	5	24
Sumac Creek	3	3	2	2	1	11
Spring Creek	1	1	1	4	4**	11

*The data set of vitellogenin induction in caged Fathead Minnows deployed in each tributary had two concentrations since the two cages deployed in each tributary were not considered replicates. The higher of the two concentrations was used.

**Fishes were not collected downstream of Spring Creek, only in Spring Creek.

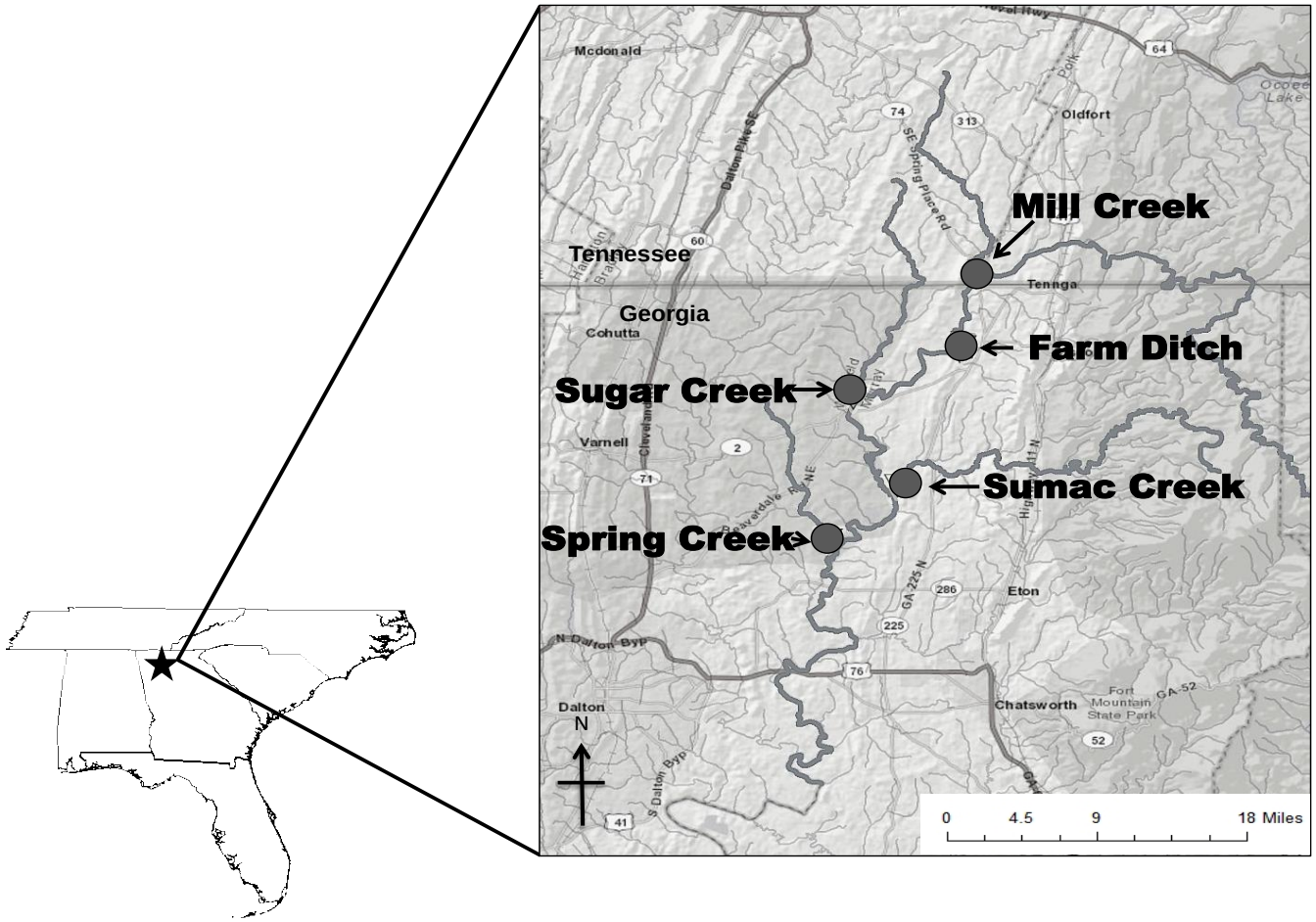

Figure 2-1. The upper Conasauga River is in the Coosa River Basin and extends from the Georgia/Tennessee border downstream to Dalton, Georgia. The study evaluated effects environmental estrogens were having on male fish in the river and identified tributaries with the highest estrogen input. Study tributaries were Mill Creek, a private ditch draining a dairy farm and row crop field ("Farm Ditch"), Sugar Creek, Sumac Creek, and Spring Creek.

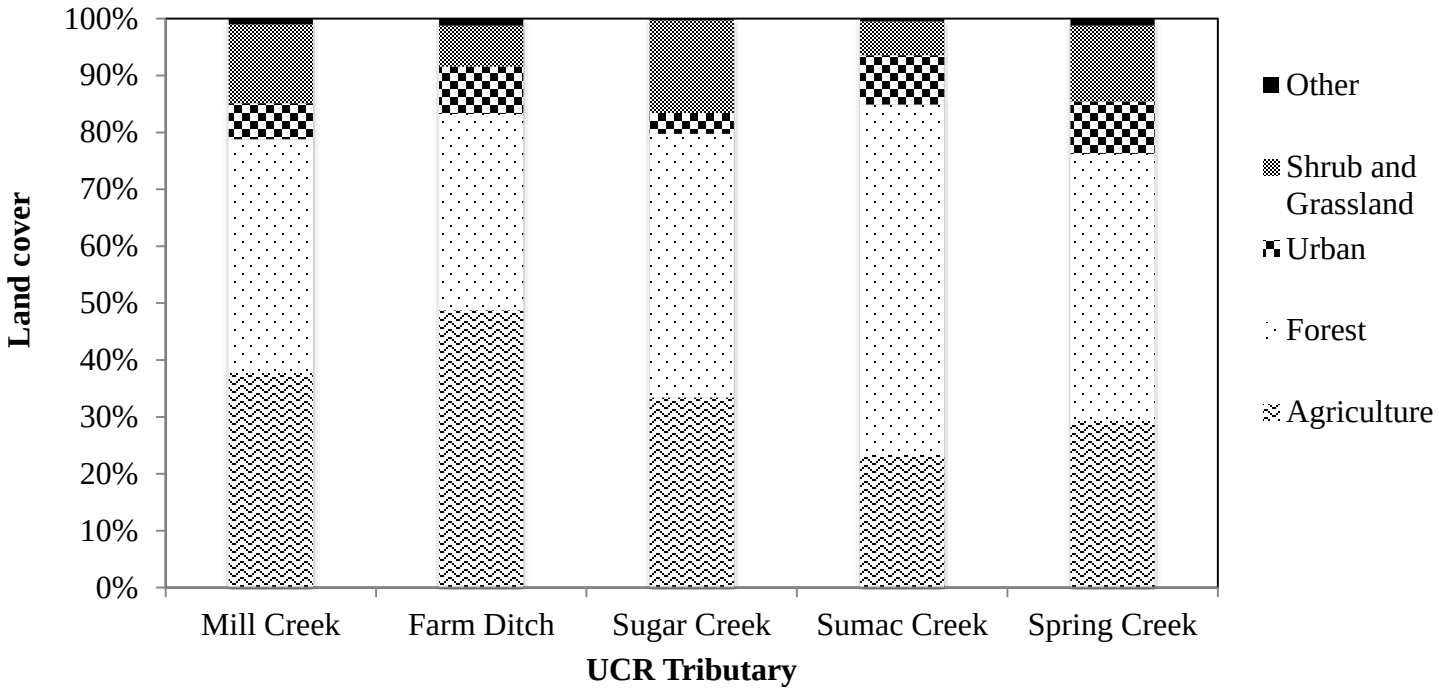

Figure 2-2. Percent land cover for watersheds of study tributaries in the upper Conasauga River (UCR). Other encompasses wetland, water, and barren land. Land cover was used to determine if a relationship existed between estrogen present in UCR and land activity. Source of land cover data was the Multi-Resolution Land Characteristics Consortium's National Land Cover Database 2006.

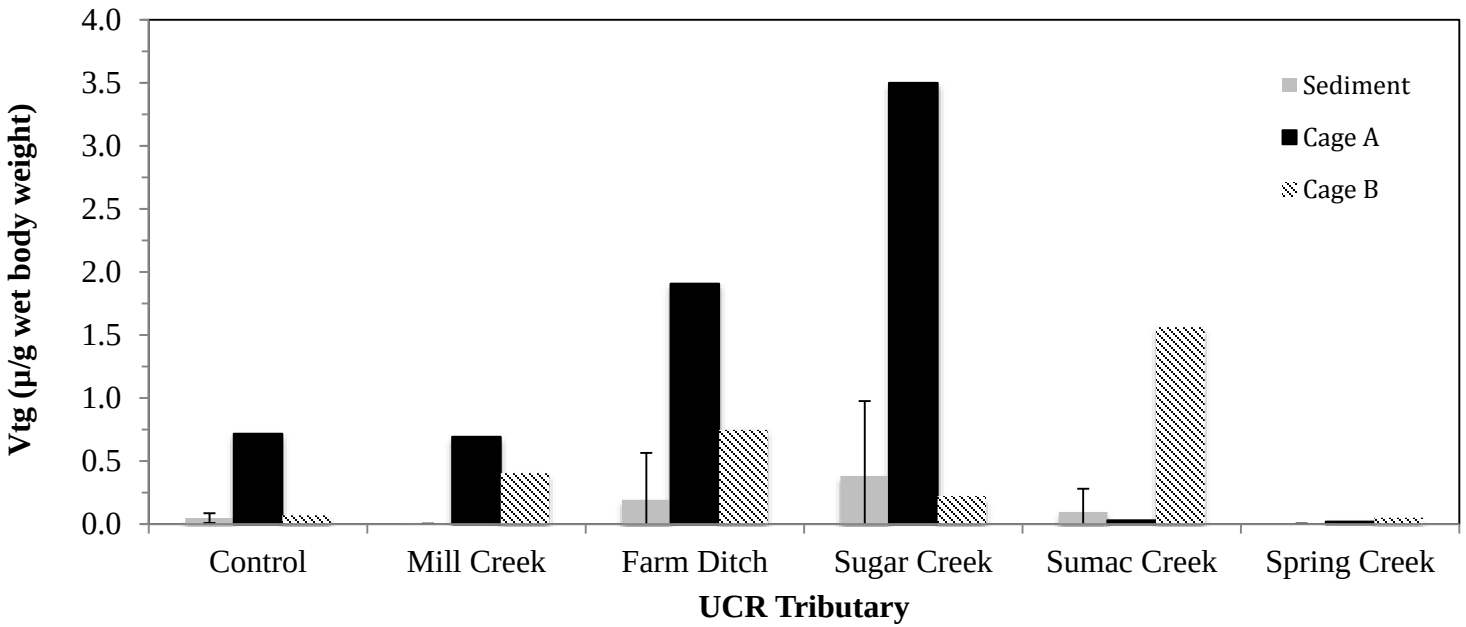

Figure 2-3. Vitellogenin (Vtg; µg / g fish wet weight) induction in adult male Fathead Minnows (*Pimephales promelas*) exposed to upper Conasauga River (UCR) tributary sediment and deployed in cages in each of the study tributaries. Minnow cages were placed in separate locations in tributaries, and thus not considered replicates. All Fathead Minnows in a cage were homogenized into a single sample for Vtg analysis so statistical comparisons were not possible for caged fish. Vtg induced in sediment-exposed fish were not statistically different from the control (Kruskal-Wallis, $\chi^2 = 8.0601$, df = 5, 23, $p = 0.1530$). Error bars represent standard deviation.

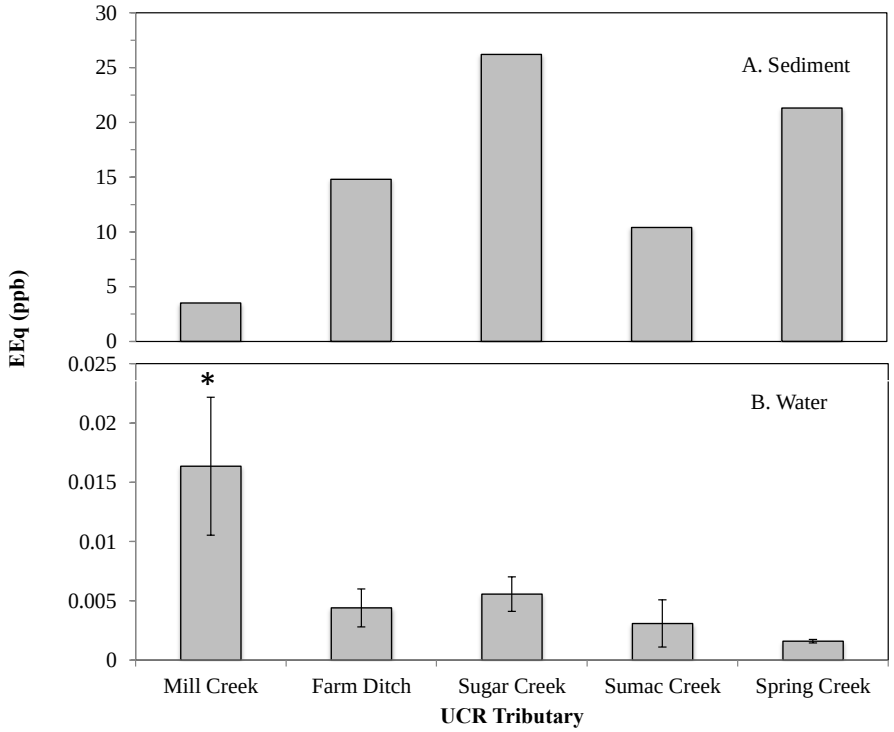

Figure 2-4. Estradiol estrogenic equivalencies (EEq) for the three natural estrogens (estrone, estradiol, estriol) (A) detected in sediment samples collected from study tributaries June 2012 and used for a sediment exposure with adult male Fathead Minnows and (B) in surface water by Polar Organic Chemical Integrative Samplers (POCIS) deployed in UCR study tributaries in July 2012. Differences in POCIS EEq were tested with a single-factor analysis of variance (ANOVA) followed by a Dunnett's post-hoc test. An asterisk indicates Mill Creek POCIS EEq was significantly different from Farm Ditch, Sugar Creek, Sumac Creek, and Spring Creek EEqs (ANOVA, $F = 11.97$, df = 4,14, $p = 0.0008$). Error bars represent standard deviation.

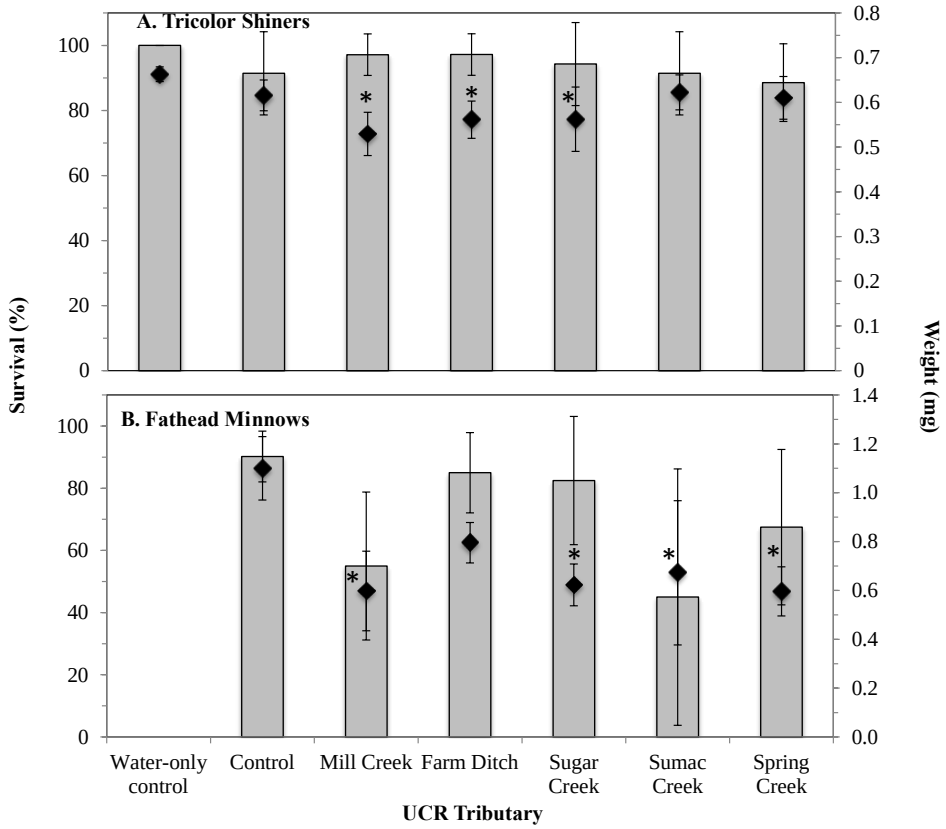

Figure 2-5. Mean survival (%, bars) and mean dry weights (mg; ◆) of (A) Tricolor Shiner (*Cyprinella trichroistia*) and (B) Fathead Minnow fry after a 10-day exposure to upper Conasauga River (UCR) tributary sediments. Tricolor Shiner fry were stocked in experimental beakers between three and 5-days post-hatch, and Fathead Minnow fry less than 24-hours post-hatch. Commercial sand was used as control sediment in the Tricolor Shiner exposure, and commercial sand washed and amended to 0.3% - 0.6% organic content was used as the control sediment in the Fathead Minnow exposure. Differences in survival among treatments were tested with a non-parametric Kruskal-Wallis test, and differences in weight were tested with a single-factor analysis of variance (ANOVA; $p <$

0.05) followed by Dunnett's post-hoc test. Survival of Tricolor Shiner fry was not significantly different from the water-only control (χ^2 = 5.3553, df = 6, 34, p = 0.4991), and survival of treatment-exposed Fathead Minnow was not significantly different from the control (χ^2 = 7. 7162, df = 5, 239, p = 0.5). Weights of Tricolor Shiner fry exposed to Mill Creek, Farm Ditch, and Sugar Creek sediment were significantly different from the water-only control (F = 4.86, df = 6, 34, p = 0.0016), and weights of Fathead Minnow fry exposed to Mill Creek, Sugar Creek, Sumac Creek, and Spring Creek sediment were significantly different from the control (F = 5.88, df = 5, 23, p = 0.0022). Asterisks denote significant differences from the control, and error bars indicate standard deviation. Significance was determined at α= 0.05.

Appendix A

Particle-size distributions – percent total organic material (TOM), percent sand, percent silt, and percent clay – from UCR-sediment exposures: an early-life stage (ELS) exposure using <24 hour post-hatch Fathead Minnows (*Pimephales promelas)*, ELS exposure using three to five day old Tricolor Shiners (*Cyprinella trichroistia*), and an adult exposure using sexually mature male Fathead Minnows. Sediment samples were collected November 2012 (ELS fathead minnow exposure), May 2013 (ELS Tricolor Shiner exposure), and June 2012 (adult fathead minnow exposure). Particle-size distributions were determined using the micropipette method of Miller and Miller (1987).

Sediment Trial	TOM (%)	Sand (%)	Silt (%)	Clay (%)
ELS Fathead Minnow				
Control *	0.3	99.3	0.7	0.0
Mill Creek	2.2	85.4	9.8	4.8
Farm Ditch	2.9	45.3	43.3	11.4
Sugar Creek	1.8	38.8	44.9	16.3
Sumac Creek	1.2	80.8	14.2	4.9
Spring Creek	2.8	56.2	34.0	9.8
ELS Tricolor Shiner				
Control**	0.4	94.6	4.8	0.6
Mill Creek	4.1	71.8	22.9	5.3
Farm Ditch	1.4	64.9	22.9	12.2
Sugar Creek	3.0	50.7	41.7	7.7
Sumac Creek	1.9	77.2	19.3	3.5
Spring Creek	2.0	79.3	16.6	4.1
Adult Fathead Minnow				
Control*	0.6	99.4	0.7	0.0
Mill Creek	3.1	64.0	26.7	9.3
Farm Ditch	3.9	46.9	37.0	16.1
Sugar Creek	3.0	37.9	44.9	17.2
Sumac Creek	2.1	80.0	14.6	5.5

Spring Creek	2.8	56.4	33.7	9.8

*Commercial sand washed and amended to 0.3% - 0.6% organic content; **Commercial sand

Appendix B

Description of attempted Tricolor Shiner (Cyprinella trichroistia) egg production experiment

Concentrations of estrogens as low as 1.0 ng/L have been shown to cause endocrine disruption in male fish (Hansen et al. 1998[1]). Endocrine disrupting chemicals (EDCs), such as estrogen, have been linked to decreased reproductive capabilities through studies on individual fish's reproductive organs, secondary sex characteristics, and mating behavior (Kime 1995[2]; Taylor and Harrison 1999[3]; Van der Oost et al. 2003[4]). Findings from other studies also have shown that population-level effects for aquatic species are likely when exposed to low concentrations of estrogenic endocrine disruptors (Nash et al. 2004[5]). Kidd et al. (2007)[6] found that chronic exposure to environmentally relevant concentrations of 5-6 ng/L 17α-ethynylestradiol (EE2), a synthetic estrogen used in birth-control pills, led to near extinction of an experimental population of Fathead Minnows (*Pimephales promelas*). With the exception of this whole-lake exposure study, few studies have linked environmental estrogens and reproduction failure in fishes with population declines. The objective of this experiment was to determine the reproductive effects of estrogen exposure on Tricolor Shiners (*Cyprinella*

[1] Hansen P.D., H. Dizer, B. Hock, A. Marx, J. Sherry, M. McMaster and C. Blaise. 1998. Vitellogenin—a biomarker for endocrine disruptors. Trends Analytical Chemistry17: 448- 451.

[2] Kime, D. E. 1995. The effects of pollution on reproduction in fish. Reviews in Fish Biology and Fisheries. 5:52-96.

[3] Taylor, M.R. and P. T. C. Harrison. 1999. Ecological effects of endocrine disruption: current evidence and research priorities. Chemosphere. 39: 1237-1248.

[4] Van Der Oost, R., J. Beyer and N. P. E. Vermeulen. 2003. Fish bioaccumulation and biomarkers in environmental risk assessment: a review. Journal of Environmental Toxicology and Pharmacology. 13: 57-149.

[5] Nash, J. P., D. E. Kime, L. T. M. Van der Ven, P. W. Wester, F. Brion, G. Maack and P. Stahlschmidt-Allner, and C. R. Tyler. 2004. Long-term exposure to environmental concentrations of the pharmaceutical ethynylestradiol causes reproductive failure in fish. Environmental Health Perspectives 112:1725–1733.

[6] Kidd, K. A., P. J. Blanchfield, K. H. Mills, V. P. Palace, R. E. Evans. J. M. Lazorchak and R. W. Flick. 2007. Collapse of a fish population after exposure to a synthetic estrogen. PNAS 104: 8897–8901.

trichroistia), a Coosa River endemic whose numbers are declining in the Conasauga River system (Burkhead and Jelks 2001)[7].

To determine the reproductive effects of estrogen exposure on native fish, I attempted to expose Tricolor Shiners to 17β□estradiol (E2) in the laboratory for 21 days. Sexually mature Tricolor Shiners were seined on July 2, 2013 from Shoal Creek (34º42'89.9"N, 84º13'81.5"W) in the Etowah River Basin (Georgia). Only fish exhibiting secondary sexual characteristics (i.e., males with tubercles and nuptial coloration and females with swollen abdomens and visible ovipositors) were retained. Approximately 200 fish were transported back to the University of Georgia (UGA) Aquatic Science lab in aerated coolers, separated by sex, and held in four 76-L tanks with dechlorinated tap water at about 20.0 ºC and fed flake food. Following a two-week acclimation period, groups of one male and two females were stocked into 24 38-L glass aquaria. Each aquarium contained 32-L dechlorinated tap water, aeration, a Mini Jet Aquarium Pump (Marineland, Blacksburg, Virginia) for current, and a spawning tower, which consisted of a stack of five ceramic tiles (51 mm x 51 mm) on a clay flowerpot (Burkhead and Jelks 2001). Aquaria were kept between 19.0 – 21.0 ºC. A 50% water change was performed every 48 hours to keep total ammonia nitrogen levels below 1 mg/L. Fish continued to be fed flake food, and spawning towers were checked daily for eggs. A tank was considered reproductively active after one healthy spawning event (at least 30 fertilized embryos). Once six tanks produced a healthy spawn within seven-day period of one another, the tanks were assigned a phase number and one of six treatments (control, solvent control (10 µl/L methanol), 5, 20, 50, 100 ng/L E2. A working solution of 10 mg E2/ L was prepared using methanol as the solvent (pesticide grade, Fisher Scientific, Pittsburgh, Pennsylvania), and tanks were spiked with their respective treatment on

[7] Burkhead, N. M. and H. L. Jelks. 2001. Effects of suspended sediment on the reproductive success of the tricolor shiner, a Crevice-Spawning Minnow. Transactions of the American Fisheries Society 130: 959–968.

day 0, and 50% estrogen additions were made every other day after a water change. Phase 1 began on July 25, 2013, but was discontinued on July 30, 2013 because of high female mortality by male aggression in both Phase 1 tanks and non-phase tanks.

During the five day exposure period, spawning towers were monitored daily for total egg production. If eggs were present, they were counted, moved to a beaker containing clean declorinated tap water, and aerated so they were suspended in the water column. A 50% water change was preformed daily and debris and fungus were removed. Hatched larvae were fed rotifers (*Brachionus plicatilis)*. Temperature, dissolved oxygen (D.O.), and pH were measured daily on Phase 1 tanks, and three non-phase tanks, and alkalinity, hardness, and total ammonia nitrogen were measured on day 0. Temperature ranged from 22.0 ^{o}C to 23.2 ^{o}C, D.O. ranged from 8.2 to 8.3 mg/L, pH ranged from 6.7 to 7.0, alkalinity from 24.0 to 28.0 mg/L $CaCO_3$, hardness was 40.0 mg/L $CaCO_3$ for all tanks, and ammonia ranged from 0.0 µg/L to 0.2 µg/L. Endpoints included total egg production, number of fertilized eggs, percent of embryos developing to the 'eyed' stage, percent hatch, and survival of fry to 7-day post-hatch. Exposure concentrations were intended to be validated by composite water samples from each treatment at least twice during the study by gas chromatography–mass spectrometry at the UGA Environmental Analysis Laboratory. Data from this experiment was going to be analyzed to determine the lowest observed effects concentration (LOEC) for reproductive effects of estradiol on Tricolor Shiner reproduction.

Only two of the six tanks (5 ng/L and 20 ng/L) in Phase 1 spawned during the 5-day exposure period. Fish exposed to 5 ng/L produced 89 eggs on day 5, and fish exposed to 20 ng/L produced 95 eggs on day 2 and 45 eggs on day 3 of the exposure. The fish exposed to 5 ng/L spawned three times in the pre-exposure period with egg counts ranging from 65 to 70 eggs. The

fish exposed to 20 ng/L spawned twice in the pre-exposure period, and egg counts ranged from 35 to 38 eggs.

The limited data collected from the 5-day exposure was not analyzed statistically. The experiment was ultimately discontinued because of female mortality by male aggression. Male Tricolor Shiners are known to demonstrate aggressive reproductive behaviors such as butting, nipping, and chasing intruders away from the nest site (Stephens and Mayden 1998)[8], and these behaviors could have been pronounced because of tank size, misidentifying immature or small males as females. Tanks were changed from 19-L to 38-L aquaria to elevate aggression by overcrowding. This change did appear initially to help, but the aggressive behavior and resulting mortality eventually returned.

[8] Stephens C.M. and R. L. Mayden. 1998. Description of antagonistic and courtship behaviors of the Tricolor Shiner, *Cyprinella trichroistia*, and the Tallapoosa shiner, *Cyprinella gibbsi*, with recommendations for the conservation of the Blue Shiner, *Cyprinella caerulea*. The Journal of the Elisha Mitchell Scientific Society. 114: 209-214.