FATHEAD MINNOW AND BLUEGILL SUNFISH LIFE-STAGE RESPONSES TO 17β-ESTRADIOL EXPOSURE IN OUTDOOR MESOCOSMS

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ABSTRACT

Developmental and reproductive effects of 17β-estradiol (E2) exposure on two generations of fathead minnows and one generation of bluegill sunfish were assessed. Fish were exposed to E2 for 6 continuous weeks in outdoor mesocosms simulating natural lake environments. First generation fish were exposed while sexually mature. Second generation fathead minnows were exposed either during early development, sexual maturity, or both stages. Multiple endpoints were measured to assess effects of E2 exposure on fecundity and fish health and development. Plasma vitellogenin concentrations were highly variable in all fish. Differences in egg production timing for both species indicate differences in fecundity between females exposed to E2 and controls. First generation fathead minnows exposed to E2 had lower body condition factors and reduced secondary sexual characteristic expression by males. Only a difference in relative liver weight was observed in second-generation fathead minnows. First generation bluegill males exposed to E2 had significantly smaller testes compared to controls. Although fish response was highly variable, results indicate that exposure to E2 at environmentally relevant concentrations affect fathead minnow and bluegill sunfish health and development,

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which may have implications for the health and sustainability of fish populations. Furthermore, exposure timing and environmental factors affect fish response to E2 exposure.

KEY TERMS: endocrine disruption; mesocosm; multi-generational exposures; estrogen; fish; lakes

INTRODUCTION

The presence of endocrine active compounds (EACs) in aquatic environments and the consequential effects on fish have been well-documented (Jobling *et al*., 1998; Kolpin *et al*., 2002; Lee *et al*., 2008, 2010; Hinck *et al*., 2009; Writer *et al*., 2010). Specifically, estrogenic hormones can demasculinize male fish (Vajda *et al*., 2008; Sowers *et al*., 2009). Commonly detected estrogens include 17β-estradiol (E2) (biogenic), estrone (E1) (degradation product of E2), and 17α-ethinylestradiol (EE2) (synthetic estrogen and the active ingredient in many birth control pharmaceuticals). Estrogen sources vary but may include livestock operations, wastewater effluent, and wildlife (vertebrate) excretia (Lee *et al*., 2011; Wise *et al*., 2011). Little is understood about the mechanisms and environmental factors affecting fish response to exogenous estrogenic compound exposure beyond the organismal level. Furthermore, whether cumulative, generational effects threaten population sustainability is unclear.

Responses in fish after exposure to estrogenic compounds have been well documented for several model species including fathead minnow (*Pimephales promelas*), Japanese medaka (*Oryzias latipes*), and zebrafish (*Danio rerio*) (Jukosky *et al*., 2008; Vajda *et al*., 2008; Coe *et al*., 2010; Lange *et al*., 2011). Observed responses in males include increased plasma vitellogenin (VTG) (egg yolk pre-cursor protein) concentrations (Folmar *et al*., 2000; Panter *et*

al., 2000; Jukosky *et al*., 2008), decreased prominence of secondary sexual characteristics (Parrot and Blunt, 2005; Sowers *et al*., 2009), and decreased nest holding ability (Hyndman *et al*., 2010). However, results of short, continuous exposures do not easily translate to natural environments where wild fish often are exposed to lower concentrations for longer periods of time or exposed intermittently. Additionally, information is lacking for other important freshwater fish species (e.g. bluegill sunfish, walleye, bass, etc.) that often have strong economic and recreational value for local communities.

Limited research suggests that EACs may have important implications for wild fish populations. A fathead minnow population crashed in response to longer term (three consecutive summers) exposure to low levels of EE2 (Kidd *et al.,* 2007). Evidence of the failing population became apparent only 1 year after addition of the hormone. Similar results were observed in response to life-long exposures of zebrafish at similar levels of EE2 used in Kidd *et al.* (2007) (Nash *et al.*, 2004). Although the mechanisms affecting wild fish at the population level are not well understood, several studies indicate that reproductive disruption may be the main cause. However, exactly how reproduction is affected is still unclear. Fish surveys conducted by Kidd *et al*. (2007) revealed an aging fathead minnow population with minimal juvenile recruitment beginning just 1 year after EE2 addition. Nash *et al*. (2004) identified reduced fecundity and lack of fertilization success in the second generation as potential mechanisms for population decline in zebrafish exposed to EE2. Consequences of population crashes in forage fish, such as fathead minnows, extend beyond the loss of a particular species. Declines of higher trophic species dependent on them for forage may also result (Palace *et al*., 2009).

Some intersex fish were observed after long-term exposure to EE2 (Nash *et al.*, 2004; Kidd *et al.*, 2007). Intersex fish (defined as the presence of ova-testes in males) have been identified nationwide with a high prevalence in bass species (Hinck *et al.*, 2009). Fish determined to be intersex have been correlated with decreased sperm motility and reproductive success in roach (*Rutilus rutilus*) (Jobling, *et al.*, 2002). Intersex has even been observed in wild fish collected at sites relatively unaffected by EACs (Jobling *et al.*, 1998; Lee *et al.*, 2010) indicating that intersex naturally occurs indiscriminate of EACs or other known pollutants. However, if a relatively higher than natural prevalence of intersex occurs in wild populations, the result may be reproductive failure causing local extinction in extreme cases.

Large-scale population-level investigations encounter difficulties in assessing wild fish response to EAC exposure. In a statewide survey of Minnesota lakes, endpoints evaluated as evidence of endocrine disruption were highly variable and did not follow patterns of EAC detection, composition, or concentration (Writer *et al.*, 2010). Fish exhibiting some of the greatest responses were collected from lakes believed to be relatively unaffected by anthropogenic influences. In addition, biogenic hormones (E2, estrone, androstenedione) were the most frequently detected estrogenic compounds in water (Writer *et al.*, 2010). Unknown factors, such as duration and timing of exposure, as well as comprehensive knowledge of bioavailable compounds, contribute to the difficulty of making such assessments for wild fishes. For example, wastewater effluent can induce VTG in males (Barber *et al.*, 2007) and reduce egg production in females (Thorpe *et al.*, 2009); however, wastewater effluent also has been shown to exhibit temporal variation (Martinović *et al.*, 2008). Hyndman *et al.* (2010) reported the importance of exposure timing in relation to sampling for the detection of biomarker expressions

89 in fathead minnows when elevated VTG levels were observed in fish exposed to E2 for at least 7
90 days prior to analysis.
91
92 This study was conducted to evaluate multigenerational effects of E2, a biogenic estrogen, on
93 two ecologically and economically important fish species, fathead minnow (*Pimephales*
94 *promelas*) and bluegill sunfish (*Lepomis macrochirus*). Fish were exposed to environmentally
95 relevant concentrations of E2 for 6 continuous weeks during two sensitive life stages, the early
96 developmental stage and sexual maturity. The biogenic origin, prevalence in aquatic
97 environments, and well-understood mode-of-action at the organismal level made E2 a model
98 estrogen for fish exposure. Exposures were conducted in mesocosms that simulated natural lake
99 ecosystems with established plankton communities and trophic dynamics. Our main objectives
100 were to assess: (1) developmental effects of E2 exposure on juvenile fish and (2) reproductive
101 effects of E2 exposure on juvenile and adult fish.
102
103 METHODS
104 *Experimental Design and Setup*
105 Fathead minnow and bluegill sunfish were exposed to either a control or an E2 solution (30 ng/l,
106 nominal) for 6 weeks during two sensitive life stages. Bluegill response was only obtained for
107 the first generation because of a high larval mortality rate in the second generation. The target
108 concentration was selected to reflect environmental concentrations (Kolpin *et al*., 2002).
109 Although exposure studies often use higher concentrations than those used in this study, those
110 concentrations mostly reflect extreme scenarios (e.g. direct wastewater effluent discharge,

111 agricultural runoff) and may not reflect exposure in environments that are not directly affected
112 by point sources.
113
114 Fish were randomly taken from a brood stock (parental generation – F0) reared at the U.S.
115 Geological Survey Upper Midwest Environmental Sciences Center (UMESC) in La Crosse, WI.
116 Juvenile (F1 generation) exposures began when fry were approximately 48 hours post-hatch.
117 Upon completion of the juvenile exposure, fry were transferred indoors and overwintered at
118 approximately 14°C until sexual maturity. The following spring, sexually mature F1 adults were
119 divided into four treatment groups for an additional exposure to E2: never exposed (0/0),
120 exposed only as juveniles (30/0), exposed only as adults (0/30), and exposed as both juveniles
121 and adults (30/30) (Figure 1).
122
123 The E2 mesocosm exposures were conducted at UMESC. Mesocosm tanks were 1.1 m^3
124 containers (Rubbermaid Commercial Products, Winchester, VA). Replicate tanks were grouped
125 in blocks of three. Three blocks were placed into each of three 40.5-m^2 concrete enclosures
126 (Figure 2) that were flooded to buffer diurnal temperature cycling in the mesocosms. Treatments
127 were randomly assigned to mesocosms and enclosures using SAS (version 9.2) statistical
128 software to reduce the effects of environmental covariates. Source water obtained from a 617-m^3
129 fish culture pond was continuously fed to headboxes and mixed with a control or E2 solution.
130 Each tank was equipped with a submersible pump and biofilter to provide continuous circulation
131 and promote denitrification. Fathead minnow tanks were stocked with 10 adult females, 10 adult
132 males, and 10 spawning tiles (sections of 10-cm polyvinyl chloride pipe cut in half) or
133 approximately 100 fry. Adult bluegill tanks were stocked with four females, two males, and two

spawning nests (shallow bowls containing artificial substrate). Daily maintenance of the mesocosms included measuring flow rates of E2 and control solutions to headboxes, measuring flow rates of mixed solutions from headboxes to mesocosms, clearing line blockages, checking biofilter and air pump operation, and removing eggs for enumeration. Water temperature, pH, and dissolved oxygen in each mesocosm were recorded daily. Adult fish were fed a commercial fish feed (Silver Cup, Skretting USA, Tooele, UT) while F1 fish were fed a non-soy based diet (Otohime Larval Feeds, Aquatic Eco-Systems Inc., Apopka, FL). Females and males were kept separate before placement into mesocosms to prevent spawning prior to exposure. All fish were presumed to be in similar stages of spawn upon initiation of E2 exposure.

Chemical Preparation

Chemical solutions were prepared by mixing 13.5 mg E2 (Sigma-Aldrich Co., St. Louis, MO) in 120 ml of reagent-grade ethanol (EMD Chemicals, Darmstadt, Germany) and storing the mixture in a 2.0-ml charge at 4°C. Control solutions consisted of a 2.0-ml charge of reagent-grade ethanol (EMD Chemicals). Approximately every 4 days, one E2 and one control 2.0-ml charge were each diluted with 10 l of deionized water in a glass carboy. Control and E2 solutions were then continuously pumped with a peristaltic pump to the appropriate headbox where solutions were mixed with the source water. The control or E2 solution was then gravity-fed from the headboxes to individual tanks with black Teflon® tubing, providing approximately 20% volume exchange per day.

Water Sampling and Analysis

Aqueous E2 concentrations were monitored in individual mesocosms and the source pond twice weekly during Year 1 (2010) and weekly during Year 2 (2011). Efforts to obtain samples representative of the entire water column were made by collecting integrated grab samples.

Samples were stored at 0°C until E2 analyses were conducted. Unfiltered water samples were analyzed for E2 concentration in duplicate using magnetic particle competitive Enzyme Linked Immunosorbent Assay (ELISA) (Abraxis, Warminster, PA). The quantitation range of the ELISA ranged from 2.5 to 25 ng/l. Samples were allowed to come to room temperature and then analyzed according to the manufacturer's instructions. One laboratory blank and one replicate were also analyzed with every assay.

Biological Endpoints

Multiple endpoints were measured to achieve a broad understanding of the effects of exposure across multiple biological levels of organization. Biological analyses were conducted at the St. Cloud State University Aquatic Toxicology Laboratory in St. Cloud, MN. Adult fish were anesthetized with 200 mg/l MS222 at the end of each 6-week exposure. Weight (g) and total length (mm) of each fish were measured. All adult fish were assessed for biological endpoints, with the exception of the females from the F1 fathead minnows. The egg yolk pre-cursor protein VTG was measured in fish plasma as an indicator of a physiological response in male fish to the presence of E2. Histological analyses of livers (the primary detoxifying organ) and reproductive organs of male and female fish were conducted to examine anatomical changes that may be related to E2 exposure or may explain changes in reproductive fitness.

Several indices were calculated for each fish, including body condition factor (BCF) [(total mass, g)/(total length, mm)^3 x 100,000)] to gauge the relative metabolic health of fish (Fulton 1904), gonadosomatic index (GSI) [(testes mass, g/fish mass, g) x 100] and hepatosomatic index (HSI) [(liver mass, g/ fish mass, g) x 100] (Shappell *et al.*, 2010). The Secondary Sexual Characteristics Index (SSCI) was used to quantify the presence and prominence of secondary sex

characteristics in male fathead minnows. Secondary sex characteristics are regulated mostly by androgen production and serve as an anatomical manifestation of male fish reproductive maturity. The sum of individual scores (scale of 0 to 3; 0 representing absent characteristics and 3 representing prominently visible characteristics) for tubercle prominence, dorsal pad thickness, and color was used to calculate a common score to compare across treatments (Shappell *et al.*, 2010).

Histological analyses were conducted following standardized procedures as outlined by U.S. Environmental Protection Agency (2008). After fish were euthanized with an overdose of anesthetic, gonad and liver tissues were excised, weighed, and placed in a tissue cassette. Tissue cassettes were stored in 10% buffered formalin (50:1 volume/tissue ratio) until processing. Tissues were dehydrated and paraffin embedded using a Leica automated tissue processor TP 1050 (Leica, Wetzlar, Germany) and Thermo Scientific Microm EC 350-1 embedding station (Waltham, MA). Paraffin embedded tissues were sectioned at 5 µm tissue thickness using a Reichert-Jung cassette microtome (Leica, Wetzlar, Germany). Tissue sections were stained with a Leica Autostainer XL (Leica, Wetzlar, Germany) using a standard hematoxylin and eosin staining protocol (Gabe, 1976; Carson, 1997), similar to methods used in other histopathological studies (Kidd *et al*., 2007; Vajda *et al*., 2008; Barber *et al*., 2011). Gonad tissues were microscopically analyzed for sex and graded for development stage on a scale of 0 to 5 (undeveloped to post spawn). Liver tissues were graded for severity of hepatocyte vacuolization on a scale of 0 to 4 (no vacuolization visible to >50% vacuolization).

Blood was drawn from the caudal vasculature, stored on ice in heparinized hematocrit vials, and centrifuged (5,000 x g for 5 min) for plasma separation. Plasma samples were stored at -80°C until analyzed for VTG using polyclonal ELISA techniques. An antibody-capture competitive ELISA incorporating a species-specific anti-vitellogenin antibody and purified vitellogenin as standard was used to measure plasma VTG (Shappell *et al*., 2010). The ELISA for both species was similar but used species-specific anti-fathead minnow/anti-sunfish VTG antibody and purified fathead minnow/sunfish VTG. Microtiter plate wells were coated with 600 ng of species-specific VTG in carbonate coating buffer (pH 9.6). A pre-competition step was performed with the antibody (1:20,000 final dilution) and standard VTG, sample plasma or control plasma in 1% albumin from bovine serum/phosphate buffer saline (BSA/PBS) (pH 7.5). After incubation, this mixture was loaded into the wells and incubated at room temperature for 1 hour, followed by secondary antibody (anti-rabbit IgG-HRP, Sigma, St. Louis, MO) incubation at a dilution of 1:10,000. The substrate tetramethylbenzidine (TMB) was added and incubated for 16 minutes at room temperature and color development measured at 620 nm on a Thermo (Waltham, MA) Multiscan plate reader. Standard curves were generated using the accompanying software. The standard curve plots were generated using at least seven standard concentrations ranging from 0.075 to 4.8 µg/ml. Coefficient of determination (r-squared) values for standard curves were greater than 0.97, with most equal to 0.99. The minimum detection limit using this standard curve was 3.75 µg/ml.

Female fecundity was tracked throughout the mesocosm exposures in both species as an indicator of reproductive fitness with direct implications for population sustainability. Tiles or nests with eggs were removed from the mesocosms daily and replaced with new spawning

substrate. Eggs were counted, resulting in total number of eggs produced per tank per day. An average cumulative number of eggs per day was calculated for each tank and those daily averages were compared on a weekly basis to compare rates of egg production among treatments.

Statistical Analysis

All statistical analyses were completed using TIBCO Spotfire S+, version 8.1. Due to the lack of normality, presence of outliers, and censored values in most of the datasets, non-parametric Wilcoxon and Kruskal-Wallis Rank Sum tests were used to compare differences among treatments. Multiple comparisons were conducted using a Bonferroni correction, when appropriate.

RESULTS

Environmental Conditions and Fish Survival

Environmental conditions changed over the course of the summer and differed considerably between the first and second years of study. These differences aided in reaching the experimental goal of establishing mesocosms of different estrogenicity, similar to environmental variation. Because wild fry will likely not experience identical environmental conditions as parent fish, it was preferred that this study did not attempt to maintain the exact same experimental conditions between years or exposures. Average monthly air temperatures during May through September ranged from 16 to 24°C in 2010 and 14 to 26°C in 2011 (National Weather Service, 2008. Accessed March, 2012. http://www.nws.noaa.gov/climate/index.php?wfo=arx). Precipitation was 30.5 cm above normal in 2010 and relatively normal in 2011. Environmental conditions within the mesocosms were

generally stable. Mean dissolved oxygen ranged from 8 to 10 mg/l. Mean water temperatures in the mesocosms ranged from 21 to 24°C. Mean pH ranged from 8.7 to 9.6.

Adult survival within the mesocosms was >80 and 100% for fathead minnows and bluegills, respectively. Larval bluegill survivorship was extremely low (not quantified). Overwinter survival of F1 fathead minnow fry during 2010 resulted in few mortalities, with >95% of juveniles surviving. Unfortunately, overwinter survival of bluegill fry was almost 0% and as a result, F1 bluegill exposures were not feasible.

Aqueous E2 Concentrations

Analysis of source pond water indicated that E2 was present in the source water. Median concentrations in the ponds were 3.55 and 3.79 ng/l in 2010 and 2011, respectively. These observed concentrations are consistent with previous studies of reference lakes that contained estrogenicity from unknown sources (Writer *et al*., 2010) and demonstrate the ubiquitous presence of estrogenic EACs in the environment.

Median E2 concentrations within the mesocosms for each exposure were two to three-fold higher in the E2 treatment mesocosms than in the controls and approximately half the target concentration of 30 ng/L (Table 1). Concentrations in the E2 treatments fluctuated throughout the exposure periods as a result of the contribution of E2 from source water ponds, fluctuation of environmental conditions, and biological growth within the mesocosms. Concentrations of E2 in the control mesocosms were generally less than 10 ng/l. Despite E2 detections in the control mesocosms, significant differences in E2 concentrations were observed between controls and E2

269 treatments ($p<0.01$) for all experiments. Median E2 concentrations were typically higher in adult
270 treatments compared to fry, indicating that adult females may be an important source of E2.
271 *F0 Fathead Minnow Response*
272 BCF was significantly higher in control fish of both sexes ($p<0.01$). On average, fish exposed to
273 E2 were slightly longer and weighed proportionally less compared to controls (differences not
274 significant). Males exposed to E2 had significantly higher HSI compared to controls (1.55 and
275 1.35, respectively; $p<0.01$), indicating that their livers were more compromised. The SSCI was
276 significantly lower in E2 treated males compared to controls ($p<0.01$) (Table 2). Males exposed
277 to E2 generally had thinner dorsal pads and lighter color banding. Tubercles were not visible in
278 approximately 80% of all males, regardless of treatment, indicating limited reproductive
279 opportunities for a subset of males in each mesocosm.
280
281 Livers from E2 treated males were slightly more compromised compared to controls. More E2
282 treated males had severe liver vacuolization compared to controls ($p<0.05$). Proteinaceous fluid
283 (likely indicative of the presence of VTG) was observed in approximately 50% more males
284 exposed to E2 compared to controls. Greater than one-half of the ovary and testes samples were
285 in early sexual maturity stages in both treatments. Plasma VTG concentrations ranged from
286 below detection limit to 1,600 µg/ml. Males generally had lower concentrations compared to
287 females, although differences were not statistically significant. Control fish had significantly
288 higher plasma VTG concentrations compared to those exposed to E2 in both sexes ($p<0.01$).
289
290 Daily average cumulative egg counts increased throughout the exposure period for F0 fathead
291 minnows (Figure 3a). The number of spawn events was variable and likely represents variation

among tanks and/or individuals. Females in control treatments ceased egg production after 3 weeks of exposure. It appears that E2 females continued producing eggs later into the experiment compared to controls; however, the spawning events in the latter part of the experiment were from one tank. Unfortunately, it is unknown if this is the result of one 'late bloomer' in that tank or if all the females in that tank continued laying eggs. Although there appear to be trends in egg production, no differences were significant in total egg production, eggs per female, cumulative weekly egg counts, or number of spawn events between treatments.

F1 Fathead Minnow

Males exposed to E2 during both life stages had significantly lower HSI compared to those only exposed as adults ($p<0.01$). Furthermore, fish exposed as juveniles, regardless of adult exposure, generally had lower HSI compared to fish not exposed during the juvenile life stage. No other statistically significant differences were observed for the other endpoints assessed (Table 2). Contrary to what was observed in the F0 generation, no statistically significant differences were observed in SSCI among treatments (medians ranged 5-6).

No differences in testes developmental stage or liver health among treatments were significant. Greater than 20% of the testes samples within all treatments were in later development stages. Regardless of juvenile exposure, earlier developmental stages were observed in testes of males exposed to E2 as adults. Generally, few fish had severe liver damage (<10%). Proteinaceous fluid was observed in 15% of males in all treatments, except for those from the 30/30 treatment, where proteinaceous fluid was observed in 20% of samples.

Plasma VTG concentrations in F1 fathead minnow males ranged from below detection limit to greater than 4,000 µg/ml. The highest median VTG concentration was observed in fish exposed

only as juveniles. Similar to observations from the F0 generation, VTG concentrations were generally lower in E2 treated fish compared to controls, although differences were not statistically significant.

Daily average cumulative egg counts increased throughout the exposure for all treatments and no differences were significant in total eggs, eggs per female, or number of spawn events among treatments (Figure 3b). However, some differences were observed when average cumulative eggs were compared by week. During weeks two and three, females exposed to E2 only as juveniles produced significantly more eggs compared to those exposed during both life stages ($p<0.01$). Additionally, females exposed only as juveniles produced significantly more eggs compared to those exposed only during the adult life stage during weeks three and five ($p<0.01$). Overall, females exposed as juveniles, regardless of adult treatment, produced more eggs compared to those exposed as adults or never exposed.

F0 Bluegills

No significant differences were observed in BCF or HSI for either sex (Table 2). Males exposed to E2 had significantly lower GSI compared to controls ($p<0.05$), indicating relatively smaller testes in relation to fish size. A similar response was observed in females, although not at a significant level.

Histology results revealed no statistical difference in gonad developmental stage or liver health between treatments for either sex. However, a slight shift in testicular developmental stage between treatments was observed. Males exposed to E2 had the greatest percentage of testes in early development stages. Slightly less than 10% of E2 exposed males and 15% of controls had

testes in later development stages. All development stages were present in females of both treatments.

Plasma VTG concentrations were highly variable; similar to what was observed in both fathead minnow generations. Concentrations ranged from below detection limit to greater than 6,000 µg/ml. Plasma VTG was generally higher in both E2 exposed males and females compared to controls, although differences were not statistically significant. Plasma VTG was not elevated in males compared to females for either treatment.

All female bluegills started producing eggs approximately 10 days into the exposure. Daily average cumulative egg counts exhibited more of a step trend compared to fathead minnows (Figure 3c), reflecting fewer mated pairs and nests per tank. Females exposed to E2 started spawning 3 days after controls, and produced fewer numbers of eggs. During weeks four, five, and six, female bluegills exposed to E2 produced significantly less eggs compared to control females ($p<0.01$).

DISCUSSION

Some statistically significant differences were observed in HSI and GSI between treatments in both species. Similar results have been reported for sheepshead minnows (*Cyprinodon variegatus*) exposed to E2 over multiple generations (Cripe *et al*., 2009). Although few statistically significant anatomical differences were observed in F1 fathead minnows in this study, some general patterns emerged. For example, regardless of adult exposure, F1 fathead males exposed to E2 as juveniles generally had higher BCF and lower HSI compared to those not exposed to E2 as juveniles. Exposure to estrogenic compounds during juvenile stages affects

362 growth in fish to a greater degree compared to unexposed fish. For example, growth was
363 enhanced by E2 in rare minnows (*Gobiocypris rarus*) (Liao *et al*., 2009) and decreased by EE2
364 in fathead minnows (Länge et al., 2001) exposed as juveniles. Results from this study indicate
365 that exposure to estrogens during early developmental life stages also may suppress anatomical
366 development in male fathead minnows. As shorter and smaller males, they could be more
367 susceptible to predation or other stressors and be reproductively outcompeted by larger males.
368
369 Although not much is known regarding the effects of endocrine disruptors on bluegills, some
370 studies have shown diazinon (an organophosphorus pesticide believed to affect the reproductive
371 system) to reduce plasma E2 concentrations in females (Maxwell and Dutta, 2005) and reduce
372 fertility in both females and males (Dutta and Meijer, 2003). Results from our study indicate
373 gonadal development may be impeded by E2 exposure, resulting in smaller gonads, especially in
374 males that are not accustomed to metabolizing relatively greater concentrations of E2.
375 Additionally, because the endocrine system is being thrown off balance by excess amounts of
376 one hormone or compound, it may suppress production of other hormones (e.g. growth hormone)
377 in an effort to balance itself out.
378
379 Observed differences in SSCI in the fathead minnow F0 generation indicate that adult males
380 exposed to elevated E2 concentrations may be at a reproductive disadvantage compared to those
381 not exposed. Sowers *et al*. (2009) observed decreased expression of secondary sexual
382 characteristics in first generation males exposed to wastewater effluent; however second
383 generation males exposed to the same effluent exhibited increased expression of those
384 characteristics. A similar generational difference was not observed in this study. In fact, no clear

pattern was observed for expression of secondary sexual characteristics within the F1 generation. Wastewater often is composed of chemicals of varying estrogenic potency, producing a more estrogenic solution than E2. Additionally, mixtures of estrogenic compounds have been observed to elicit greater responses in fathead minnows compared to exposure to the individual estrogenic compounds in the mixture (Brian *et al*., 2007). The differing estrogenic effects of wastewater (or chemical mixtures) compared to one compound is one explanation for the varying results found in the literature. That being said, the importance of learning how fish are affected by exposure to single compounds (e.g. E2) is still important because not all exposure environments in the wild are situated within the context of wastewater effluent and the complicated mixtures it introduces into the environment. Often times in the natural environment where no direct wastewater effluent is present, such as the littoral zone in lakes, the most commonly detected hormones (and often endocrine disruptors) are the natural hormones such as E2 and plant sterols (Writer *et al.*, 2010).

Plasma VTG concentrations were highly variable in both species and all treatments, highlighting the natural variability of this biomarker among individuals. Jobling *et al*. (1998) and Nichols *et al*. (1999) also reported higher VTG concentrations in control fish compared to those exposed to EACs, similar to what was observed in both fathead minnow generations in this study. Additionally, large ranges of VTG concentrations have been observed in wild-caught fish, sometimes spanning two orders of magnitude, in both females and males (Lee *et al.*, 2010; Writer *et al.* 2010). These results highlight the need to interpret VTG data cautiously and use the information in conjunction with other endpoints. Increased VTG concentration is often reported in response to EAC exposure. However, increased VTG typically corresponds to fish exposed to

EACs at higher concentrations than observed in this study, more potent compounds, or mixtures of compounds, such as those found in wastewater effluent (Parrot and Blunt, 2005; Vajda *et al*., 2008; Thorpe *et al*., 2009). As highlighted in Mills and Chichester (2005), fish responses observed in the laboratory often do not transfer directly to those observed in the environment.

Little documentation exists on the effects of EAC exposure on bluegills and results from this study indicate that exposure to environmentally relevant concentrations of E2 alone does not consistently induce VTG production in males. As a result of the presence of E2 in control treatments the desired difference of absolutely no exposure in controls was not achieved. However, these same gradients may be present in natural lake settings where fish that are frequenting deeper waters of the lake may be slightly exposed to E2 from sources such as other vertebrates. The results presented here indicate that in similar scenarios where it might be expected that the littoral zone presents a relatively increased exposure over other micro-habitats in a lake, there is not enough of a difference in exposure to elicit significant increases in VTG concentration in males.

The results of this study also indicate that measuring plasma VTG concentration at the end of the experiment was not useful for characterizing differences in fecundity. Plasma VTG in female bluegill sunfish is known to vary as a function of serum E2 concentrations in controlled settings (Cheek *et al*., 2004) and has been shown to exhibit complex dynamics over the spawning cycle in wild long-ear sunfish populations (Fentress *et al*., 2006). Plasma VTG concentrations measured at the end of our experiment are characteristic of over-wintering fish (Cheek *et al*., 2004), indicating that the bluegills in the mesocosms may have reached the end of their summer

reproductive period. Although measuring plasma VTG throughout the spawning period may have better facilitated comparisons between control and treatment groups, the effects that may have occurred as a result of stress from additional handling may have presented confounding factors

Short-term effects of E2 exposure on spawning and egg production were observed throughout the E2 exposures. Relative to controls, a small delay in bluegill spawning and a lower initial rate of egg production in E2 exposed females of both species was observed. Elevated E2 concentrations may delay spawning through interaction with other growth processes as seen in the BCF index for F0 fathead minnows or through changes to behavioral cues associated with spawning (not assessed in this study). One consequence of delayed spawning in the wild could be lower survivability of young-of-the-year fish. Juveniles spawned later in the growing season will be smaller than their earlier hatched counterparts resulting in greater susceptibility to predation or other environmental stressors (Divino and Tonn, 2007) and reduced ability to compete for resources (Kaemingk *et al*., 2012).

Juvenile exposure to E2 may affect development to a greater extent compared to adult exposure. This may have important consequences because many juvenile fish inhabit the littoral area of lakes where we might expect concentrations of E2 and other contaminants to be higher compared to the pelagic zone. Both F1 fathead minnow treatments that included juvenile exposure produced higher numbers of eggs compared to control and adult-only exposures. Previous studies have observed greater egg production in F0 fish compared to subsequent generations. Increased egg production is often a response to exposure of more potent estrogens or exposure at

454 a much higher concentration than used in this study (Cripe *et al*., 2009). However, exposure to
455 estrogenic EACs does not always elicit changes in fecundity (Brian *et al*., 2007; Cripe *et al*.,
456 2009; Coe *et al*., 2010). Variability in egg production among individuals presents a confounding
457 factor for determining whether observed differences are a result of natural variability or exposure
458 to EACs. This study attempted to minimize the natural variability in egg production by having
459 more than one female per mesocosm; however, quite a bit of variability was still observed.
460 Embryo viability or fertilization success may provide better evidence of how fecundity is
461 affected by E2 and other EACs (Parrott and Blunt, 2005; Coe *et al*., 2010). Additionally,
462 exposure timing may explain some of the conflicting results in the literature. For example,
463 exposure during different phases of spawn may affect fecundity differently. When females are in
464 the beginning of the spawn cycle and plasma E2 levels are high, exogenous E2 sources may not
465 have a significant effect on egg production. However, at the end of the spawn cycle when
466 plasma E2 levels are reduced, exogenous E2 sources may ramp VTG production back up and egg
467 provisioning continues to occur (Giesy *et al*., 2000). While an attempt was made to keep all fish
468 in a pre-spawn state prior to exposure, the possibility of individual variability was still very
469 likely due to natural variability.
470
471 While some differences were observed between the responses of the two fish species exposed in
472 this study, some similarities in fish response to environmentally relevant concentrations of E2
473 were observed between the two species. Exposure to E2 by itself did not produce exaggerated
474 responses that would indicate populations would be negatively affected by reproductive failure;
475 however, application of a population model to the fecundity data obtained from this study may
476 provide more clarity with regards to the long-term health of the population Although responses

to low levels of E2 were not dramatic in either species in this study, exposure to similar concentrations of E2 in combination with other compounds may exhibit a cumulative effect on development and reproduction (Thorpe *et al*., 2001; Brian *et al*., 2007). Additionally, fate and transport of E2 may have been a confounding factor in this study. Fate and transport within the mesocosms was not assessed, so it is not entirely known how well mixed the mesocosms were and if the fish actually received a continuous dose of E2 throughout the entire exposure period. In addition, large amounts of algal biomass accumulated in many of the tanks. Uptake of E2 by algae may have effectively lowered the dose that fish were exposed to by making less E2 bioavailable to the fish. Measuring transport and bioavailability of E2 within the mesocosms may have aided in interpretation of the biological data obtained from this study. However, these factors are often unknown or difficult to quantify in the environment when field studies are conducted. Determining wild fish response to exposure of EACs is equally as complicated. Results from this study indicate that exposure to relatively low concentrations of E2 may elicit responses in fathead minnow and bluegill sunfish. Although responses may be muted compared to those observed during controlled laboratory exposures, the observed responses more closely mimic the varied responses observed in the environment and may have important implications for the future sustainability of fish populations.

CONCLUSIONS

Effects of exposure to E2 were measured in fathead minnows and bluegills using a mesocosm approach to simulate a lake environment complete with trophic dynamics. Fish were exposed during two sensitive life stages to evaluate fish response and effects on population sustainability. Concentrations of E2 fluctuated greatly in the mesocosms; likely a result of varying rates of biological uptake by planktonic organisms and fish. Although concentrations in the tanks often

were less than the target concentration, fluctuating E2 concentrations may have more closely simulated a natural lake environment because fish are mobile in the wild and most likely intermittently exposed to estrogens and other endocrine active compounds.

Given the observed E2 concentrations in both control and E2 treatments and the variability in plasma VTG concentrations, plasma VTG was not a sensitive biomarker for assessing effects of E2 exposure on either species in this study. Furthermore, VTG concentrations did not correlate well with any other endpoint. The most prominent differences between treatments were observed in male F0 fathead minnow SSCI and male bluegill GSI. Additionally, several differences were observed between two generations of fathead minnows exposed to E2 during different life stages, indicating cumulative effects may be important in determining population level effects of exposure to E2 and potentially, other EACs.

The results from this study indicate that results from controlled laboratory experiments do not correspond well with fish response to E2 exposure in complex aquatic systems, where other environmental factors can greatly affect observed developmental and reproductive responses. Endocrine disruptors often occur in complex mixtures of compounds in aquatic systems and perhaps the cumulative effects of multiple compounds are greater than those elicited by E2 alone. Even though E2 is not the most potent estrogen found in aquatic environments, the biogenic origin makes E2 a frequently detected estrogen. Results from this study indicate that effects of E2 on fish appear to be largely affected by exposure timing, with respect to both life stage and in relation to sampling events, and other environmental factors. Fathead minnow and

522 bluegill sunfish response to E2 exposure may have important implications for population
523 sustainability.

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TABLE 1. Statistical Summary of Aqueous 17β-estradiol (E2) Concentrations (ng/l) Within Mesocosms For Each 6-Week Exposure.

Species	Generation	Life Stage	Exposure Period	Control		E2	
				Median	IQR	Median	IQR
Fathead Minnow	F0	Adult	2010/08/02 – 2010/09/12	5.76	3.13	14.6	12.58
	F1	Fry	2010/08/16 – 2010/09/30	3.68	1.69	7.81	15.23
	F1	Adult	2011/07/22 – 2011/09/02	7.7	5.7	13.93	9.41
Bluegill	F0	Adult	2011/06/07 – 2011/07/19	9.41	9.0	18.75	E20.47*

Notes: E = estimated value; IQR = interquartile range. *IQR was calculated using an estimated value for third quartile extrapolated above the highest standard value used for the calibration curve.

TABLE 2. Median Values of Biological Condition Factor (BCF) (%), Gonadosomatic Index (GSI) (%), Hepatosomatic Index (HSI) (%), Plasma Vitellogenin (VTG) (µg/ml), and Secondary Sexual Characteristics Index (SSCI) for Adult Fish Analyzed After Exposure to a Control or 17β-estradiol (E2) (30 ng/l, nominal) Solution for 6 Weeks.

Species	Generation	Treatment	BCF		GSI		HSI		VTG		SSCI
			Female	Male	Female	Male	Female	Male	Female	Male	Male
Fathead Minnow	F0	Control	0.999	1.10	2.15	0.761	1.43	1.35	749	452	3
		E2	0.925**	1.07**	2.19	0.802	1.36	1.55**	424*	256**	2**
	F1	0/0	-	1.16	-	1.15	-	1.28	-	627	5
		30/0	-	1.21	-	1.10	-	1.13	-	752	6
		0/30	-	1.16	-	1.18	-	1.42**	-	241	6
		30/30	-	1.19	-	1.21	-	1.14	-	497	5
Bluegill Sunfish	F0	Control	2.01	2.54	7.50	1.46	1.53	1.01	665	975	-
		E2	2.04	2.55	5.88	0.970*	1.37	1.04	1,330	1,260	-

Notes: F0 = parental generation; F1 = second generation; *significantly different from controls within same generation and sex ($p<0.05$); **significantly different from controls within same generation and sex ($p<0.01$); Dashes indicate no data was collected. 0/0 = no exposure during either juvenile or adult life stages; 30/0 = exposure only during juvenile life stage; 0/30 = exposure only during adult life stage; 30/30 = exposure during both juvenile and adult life stages.

FIGURE 1. Flowchart Showing Experimental Design of Fathead Minnow and Bluegill 17β-Estradiol (E2) Exposures.

FIGURE 2. Photograph Showing One Enclosure Containing Mesocosms Equipped For Bluegill Exposure. Artificial bluegill nests can be seen in the mesocosms.

FIGURE 3. Time Series of Daily Average Cumulative Egg Counts Among Replicates of Individual Treatments For (a) F0 Fathead Minnow, (b) F1 Fathead Minnow (0/0 = control; 30/0 = juvenile exposure; 0/30 = adult exposure; 30/30 = juvenile and adult exposure) and (c) F0 Bluegill Exposed to Either a Control or 17β-Estradiol (E2) (30 ng/l, nominal) Solution. *significant difference between treatments ($p<0.01$); [a]significant difference between 30/0 and 30/30 treatments ($p<0.01$); [b]significant difference between 30/0 and 0/30 ($p<0.01$).