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Gonadal Development in Smallmouth Bass (*Micropterus Dolomieu*) Reared in the Absence and Presence of 17-α-Ethinylestradiol

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Abstract: Testicular oocytes in wild adult bass (*Micropterus* spp.) are considered a potential indication of exposure to estrogenic compounds in municipal, agricultural, or industrial wastewater. However, our ability to interpret links between testicular oocyte occurrence in wild fish species and environmental pollutants is limited by our understanding of normal and abnormal gonadal development. We previously reported low-to-moderate testicular oocyte prevalence (7%–38%) among adult male bass collected from Minnesota waters with no known sources of estrogenic compounds. In the present study, two experiments were conducted in which smallmouth bass (*Micropterus dolomieu*) fry were exposed to control water or 17-α-ethinylestradiol (EE2) during gonadal differentiation, then reared in clean water for an additional period. Histological samples were evaluated at several time points during the exposure and grow-out periods, and the sequence and timing of gonadal development in the presence of estrogen were compared with that of control fish. Testicular oocytes were not observed in any control or EE2-exposed fish. Among groups exposed to 1.2 or 5.1 ng/L EE2 in Experiment 1 or 3.0 ng/L EE2 in Experiment 2, ovaries were observed in 100% of fish up to 90 days after exposure ceased, and approximately half of those ovaries had abnormal characteristics, suggesting that they likely developed in sex-reversed males. Groups exposed to 0.1, 0.4, or 1.0 ng/L in Experiment 2 developed histologically normal ovaries and testes in proportions not significantly different from 1:1. These findings suggest that, while presumably able to cause sex reversal, juvenile exposure to EE2 may not be a unique cause of testicular oocytes in wild bass, although the long-term outcomes of exposure are unknown. *Environ Toxicol Chem* 2022;00:1–13. © 2022 SETAC. This article has been contributed to by U.S. Government employees and their work is in the public domain in the USA.

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INTRODUCTION

Gonadal intersex in the form of testicular oocytes has been reported in populations of black basses (Family: Centrarchidae, *Micropterus spp.*) from many locations in North America, and is often assumed to imply exposure to anthropogenic estrogenic compounds (i.e., estrogen receptor agonists, including natural estrogens, synthetic estrogens, and estrogen mimics). This is because (1) estrogenic compounds can cause gonadal intersex in fish in laboratory studies (Andersen et al., 2003; Balch et al., 2004; Depiereux et al., 2014; Diniz et al., 2005; Gimeno et al., 1998; Gray & Metcalfe, 1997; Koger et al., 2000; Lange et al., 2001; Metcalfe et al., 2001; Seki et al., 2002), and (2) intersex is causally linked to estrogenic wastewater treatment plant (WWTP) effluent in some wild fish populations, most notably roach (*Rutilus rutilus*) in the United Kingdom (Jobling, Beresford, et al., 2002; Jobling, Coey, et al., 2002; Jobling et al., 1998, 2006) and darters in Ontario (Tetreault et al., 2011). Testicular oocyte prevalence in some wild smallmouth bass (*Micropterus dolomieu*) populations has been reported to be as high as 100% near suspected estrogenic sources including municipal and industrial WWTPs and agricultural activities (Blazer et al., 2007, 2012; Hinck et al., 2009;

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Iwanowicz et al., 2009; Abdel-Moneim et al., 2017). However, testicular oocyte prevalence ranging from 7% to 60% has also been observed in populations with no known sources of estrogenic compounds, including those sampled from a rural, forested river upstream from WWTP (Blazer et al., 2012), in US National Wildlife Refuges with no nearby WWTP or paper mills (Iwanowicz et al., 2016), and in Minnesota lakes within watersheds with very little human activity (Kadlec, 2017; Kadlec et al., 2017). These findings imply that smallmouth bass can develop testicular oocytes in the absence of estrogenic compounds; alternatively, estrogenicity at some locations was higher than expected based on land use, and within the range of gonadal sensitivity. The likelihood of spontaneous testicular oocyte formation during normal development is unknown in smallmouth bass. Likewise, to our knowledge, the effects of exogenous estrogens and the details of normal gonadal development have not yet been examined in smallmouth bass under controlled laboratory conditions. These are crucial knowledge gaps that hinder our understanding of bass testicular oocytes in relation to established biomarkers of estrogenic exposure in other wild populations.

Depending on the exposure timing and concentration, effects of the potent synthetic estrogen 17-α-ethinylestradiol (EE2) on fish testicular development can range from partial feminization (which may manifest as testicular oocytes or mixed ovarian/testicular tissue) to complete male-to-female sex reversal. 17-α-Ethinylestradiol-induced testicular oocytes have been observed in fathead minnows (*Pimephales promelas*; Lange et al., 2001) and medaka (*Oryzias latipes*; Balch et al., 2004), with lowest-observed effect concentrations (LOECs) of 4 and 2 ng/L EE2, respectively. In this concentration range, EE2 has also caused sex reversal in fish exposed during early life stages, with LOECs for significantly female-biased sex ratios as low as 0.2 ng/L in Chinese rare minnow (*Gobiocypris rarus*; Zha et al., 2008) and 4 ng/L in fathead minnow (Lange et al., 2001). In addition to threshold effects, gradients of testicular feminization have also been observed to occur in a monotonic dose-dependent manner, with testicular oocytes induced by low concentrations of EE2 exposure and full sex reversal resulting from high concentrations. Oocytes were observed in the testes of 22% and 64% of juvenile Chinese rare minnows exposed to 0.2 and 1 ng/L EE2, respectively, and 100% of fish exposed to 4 ng/L EE2 developed ovaries (Zha et al., 2008), indicating a pattern of increased severity of feminization with increasing concentration. Similarly, the percentage of fathead minnows and rainbow trout (*Oncorhynchus mykiss*) with either intersex gonads or ovaries was positively correlated to EE2 exposure concentration (Depiereux et al., 2014; Lange et al., 2001).

At a minimum, a basic understanding of the effects of estrogenic compounds on developing gonadal tissue of smallmouth bass is needed to determine if a causal relationship exists between estrogenic exposure and testicular oocytes in wild smallmouth bass. This can best be accomplished in a controlled laboratory setting by comparing the sequence and relative timing of gonadal development of fish exposed to a known estrogen with those of fish reared in the absence of estrogen. With this aim, we conducted two separate experiments using wild-collected, early life stage smallmouth bass. We selected EE2 as a model estrogen receptor agonist because of its high potency and large body of reproductive effects literature. In Experiment 1, bass were reared in either a low (1.2 ng/L) or high (5.1 ng/L) concentration EE2 solution for 90 days, followed by 90 days in clean water, or reared in clean water for 180 days. In Experiment 2, bass were exposed to a range of EE2 concentrations (0.1, 0.4, 1.0, or 3.0 ng/L) for 100 days, followed by 20 days in clean water, or reared in clean water for 120 days. Histological evaluations were conducted at multiple time points during the exposure periods and post-exposure grow-out periods of both experiments. The goals of Experiment 1 were to thoroughly characterize ovarian and testicular development in the absence and presence of EE2, to identify life stages at which abnormalities such as testicular oocytes may be diagnosed, and to inform the selection of exposure concentrations for Experiment 2. The goals of Experiment 2 were to evaluate dose-dependent effects of EE2 exposure on gonadal development, including the identification of threshold concentrations below which only normal ovaries and testes develop.

METHODS

Test organisms

Pike Lake (14.5 km W of Grand Marais, Cook County, MN) was selected from among the many remote lakes in northeastern Minnesota for the collection of wild smallmouth bass fry for both experiments (2013 and 2015, respectively). This lake has good water clarity and readily accessible smallmouth bass nests. It also has a low likelihood of fish exposure to estrogenic compounds, due to the absence of WWTPs and agricultural activities, minimal human development along the lakeshore, and a low human population in the immediate watershed (<1 person/km^2; Kadlec et al., 2017). A relatively low prevalence of testicular oocytes in mature male bass collected from Pike Lake (8%; $n = 24$) was previously reported compared with other lakes in the region with similar land uses (range: 7% to 38%; Kadlec et al., 2017). For the present studies, fish collections were performed in late June during the peak of spawning season (surface water temperature 21 °C) by divers with 1½″ diameter syringes designed for collecting live aquatic organisms (Florida Keys Watersports). For Experiment 1, smallmouth bass yolk-sac fry were collected from four nests with actively guarding males and large clutches (several thousand). For Experiment 2, yolk-sac fry were collected from two nests. Fry were placed in 1 L Nalgene bottles with native lake water and headspaces were filled with pure oxygen. Bottles were tightly packed into an insulated container to limit temperature fluctuations and turbulence during transport. During the 4 h that elapsed between collecting fry and transferring them to exposure tanks in the laboratory, dissolved oxygen was intermittently monitored to ensure that it remained above 60% saturation (e.g., 5.1 mg/L at 21 °C and 700 ft elevation).

Exposure system

The experiments were conducted in flow-through exposure chambers consisting of preassembled glass aquaria (16 L in Experiment 1 and 7 L in Experiment 2) with silicone-sealed joints, clear acrylic covers, and stainless-steel outflow standpipes with temperature controlled by a circulating water bath surrounding the aquaria. Control and dilution water was Lake Superior water that was degassed, filtered, and treated with ultraviolet light for disinfection. The solutions of EE2 exposure were prepared by diluting concentrated EE2 stock solutions using small-displacement volume ceramic piston pumps (Fluid Metering Inc., Syosset, NY). Target concentrations prior to final stock solution and dilution pump speed measurements were 2 and 10 ng/L in Experiment 1 and 0.1, 0.3, 1.0, and 3.0 ng/L in Experiment 2. Peristaltic pumps delivered a constant flow of either EE2 solution or control water to each exposure chamber via Teflon tubing.

Stock bottles of EE2 were prepared by a shell-coating method, where 100 µL of 100% ethanol EE2 superstock (Sigma-Aldrich; 733.4 mg/L in Experiment 1 and 205.2 mg/L in Experiment 2) was added to a 1 L solvent-cleaned glass flask. The flasks were rotated while filtered air was introduced to evaporate the solvent, evenly coating the inside of the flasks with EE2. The shell-coated flasks were then covered with aluminum foil and stored at 4 °C. As needed, each flask was filled with 1 L of deionized water and stirred for 24 h prior to rinsing the contents into a glass carboy with 18 L of Lake Superior water, resulting in 19 L of EE2 stock (nominally 3860 ng/L in Experiment 1 and 1088 ng/L in Experiment 2).

Analytical chemistry

During the exposure phase of Experiment 1, EE2 concentrations were analyzed using enzyme-linked immunosorbent assays (ELISA). Samples were collected from at least one tank per treatment (plus duplicates) before and after each sterilization procedure (described in the section *Experimental procedures*), approximately every 2 weeks, and analyzed. Samples were also collected from six of the seven EE2 stock bottles that were prepared during the exposure phase. Samples were either analyzed immediately or held at 4 °C for no more than 24 h before analysis. Tank samples, blanks, and matrix spikes were concentrated within the range of analytical standards (50 to 3000 ng/L) using solid-phase extraction (SPE) columns (JT Baker, C18, 500 mg). Stock bottle samples were diluted 10× for analysis. Samples were measured with ethinylestradiol ELISA kits (Abraxis Ecologenia 96-well microplate), using the recommended procedures, with a stated method detection limit of 1 ng/L prior to concentration. Measured EE2 concentrations for both exposure treatments are reported as the mean of all samples analyzed.

In Experiment 2, exposure tank samples were analyzed using liquid chromatography tandem mass spectrometry (LC-MS). All solvents used were of high-performance LC-grade or better. Initial method development showed that EE2 concentrations in the two lowest treatments (nominally 0.1 and 0.3 ng/L) were too low to quantify. Water samples were collected for analysis approximately every 2–4 weeks, as well as before and after each sterilization procedure (described in the section *Experimental procedures*). Samples were either extracted immediately or held at 4 °C for no more than 24 h before extraction. Tank water samples, matrix spikes, and method blanks were concentrated with SPE cartridges (Strata X 60 mg; Phenomenex Inc.), and reconstituted in 75:25 water:methanol (v/v). In addition, at least one sample was collected from each EE2 stock bottle that was prepared. Stock bottle samples were diluted with methanol for a final concentration of 25% and analyzed without further dilution or concentration. Isotopically labeled EE2 (17-α-ethinylestradiol-2,4,16,16-d4 [EE2-d4], >98% purity; CDN Isotopes) was used as an internal standard by spiking all samples and standards with 10 ng of EE2-d4. Tank samples were spiked prior to SPE to account for potential analyte loss during sample preparation. All samples, blanks, and standards processed after test day 30 were also spiked with 10 ng of ^{13}C-labeled estradiol (17-β-estradiol-13,14,15,16,17,18-^{13}C6 [E2-^{13}C], >98% purity; Cambridge Isotope Laboratories) during extract reconstitution as a secondary internal standard. Samples were analyzed and quantified using an Agilent 6410 LC-MS system with atmospheric pressure photoionization (APPI) in positive ionization mode. A 500 µl injection volume was separated by gradient elution of methanol and water at 0.3 ml/min on a Phenomenex PFP (2.1 × 100 mm, 2.6 µm) column. Under these conditions, EE2 had a retention time of 12.0 min. Toluene was used as a dopant for APPI and was infused post column into the ion source at 0.018 ml/min. A loss of water was observed from EE2 and E2-^{13}C, the [M + H – H_2O] ion was used as the precursor ion. Two ion transitions were monitored for EE2, one each for the internal standards EE2-d4 and E2-^{13}C (Supporting Information, Table S1).

Based on the mean measured stock bottle concentrations (3686 and 1088 ng/L for Experiments 1 and 2, respectively) and dilution pump speeds, the expected EE2 exposure concentrations were 2.1 and 10.5 ng/L for Experiment 1, and 0.1, 0.4, 1.1, and 3.3 ng/L EE2 for Experiment 2 (Supporting Information, Tables S2 and S3).

Experimental procedures

Both experiments were initiated with fish that appeared to be less than 7 days post hatch, based on low mobility and the presence of yolk sacs, although slight differences in yolk-sac size, and therefore presumed age, were observed among the nest cohorts. To control for the potential differences in developmental stages, as well as to promote even growth rates and minimize aggression within each tank, the experimental design was blocked so that each treatment consisted of four (Experiment 1) or three (Experiment 2) replicate exposure chambers, with each chamber containing fish from a single nest cohort only. For example, replicate A of each treatment contained only fish collected from nest site 1. In Experiment 2, fry were only collected from two nest sites, so replicates A and C of each treatment were both assigned fish from the larger of the two nest sites, and replicate B of each treatment was assigned fish from the smaller of the two nest sites. Each

exposure chamber started out with 100 arbitrarily selected fish; as biomass increased, fish were removed to reduce loading and provide histological samples. On day 126 of Experiment 1, half of the fish from each tank were transferred into a second set of tanks (increasing the number of replicates from four to eight per treatment) to allow for continued growth without overcrowding.

Water flow rate was adjusted to meet target dissolved oxygen saturation and biomass loading rates, with a minimum of 12 volume exchanges per 24 h (i.e., 140 ml/min in Experiment 1 and 60 ml/min in Experiment 2), and increased as needed (up to 280 ml/min in Experiment 1 and 80 ml/min in Experiment 2). Maximum biomass loading target was 5 g/L of standing volume and 0.5 g/L of water flowing through per 24 h, as recommended by ASTM International guidelines for early life stage tests (ASTM International, 1992). Tanks were supplied with filtered air through disposable borosilicate glass Pasteur pipettes. Dissolved oxygen was measured in at least one tank per treatment daily (Hach HQ40D). If oxygen saturation fell below 60% in any tank, aeration and/or flow rate was increased until all tanks measured >60% oxygen saturation. Temperature was monitored in at least one tank per treatment daily and maintained at 22 °C (±1 °C) by adjusting the relative flow rates of heated and unheated incoming water. The value of pH was measured in at least one replicate chamber per treatment weekly. Hardness, alkalinity, and conductivity of incoming Lake Superior water measured 44 mg $CaCO_3$/L, 45 mg $CaCO_3$/L, and 102.6 μS, respectively, at the beginning of Experiment 1. Lake Superior water has little variability in these parameters, and was monitored frequently by other researchers in the facility during both experiments. Total ammonia was measured in each tank using an ion-specific electrode (Thermo Scientific Orion 4-star meter with 9512HPBNWP Ammonia probe) two or four times during Experiments 1 and 2, respectively, and did not exceed 0.5 mg/L as N in either experiment. Light was maintained at 16:8-h light:dark cycle throughout both experiments. Tanks were cleaned daily by scrubbing and siphoning debris and uneaten food. Every 2 weeks during the exposure period of both experiments, fish were transferred to clean tanks within the same system, and the remaining tanks, pumps, and lines were sterilized with a 5% solution of peracetic or acetic acid sterilant (Mar Cor Purification) to prevent a buildup of microbial biofilm that might reduce EE2 concentrations.

Feeding rates were calculated in both experiments based on the average fish weight among all exposure chambers. Average weight was determined weekly by either individual blotted wet weights or composite live weights of arbitrarily selected fish from each exposure chamber. Individual blotted wet weights were obtained from each individual fish collected for histological evaluation. Composite live weights were obtained from three to seven fish in at least one tank per treatment by netting fish into a tared beaker of Lake Superior water.

In Experiment 1, fish were fed live brine shrimp nauplii (*Artemia spp.*) two or three times daily until test day 21, when blackworms (*Lumbriculus* spp., California Blackworm Co.) were added to the diet. Between test days 21 and 89, the *Artemia* ration was gradually decreased and replaced with *Lumbriculus*, until the majority of fish in each tank were able to consume their daily ration in a single feeding of *Lumbriculus*. Between days 126 and 170, the *Lumbriculus* diet was supplemented with earthworms (*Eisenia fetida*; Uncle Jim's Worm Farm) due to a *Lumbriculus* supply shortage. Prior to day 15, ration was not fixed and fish were fed to excess. Beginning on day 15, delivered rations were estimated on the basis of a fish wet weight to food wet weight ratio. Delivered rations were initially high to encourage fast, even growth rates within tanks, and gradually decreased throughout the experiment from an average of 46% wet weight between days 15 and 40 to 24% between days 40 and 92 and 14% between days 92 and 179. Consumption rates were not estimated but were certainly lower than the delivered rations, while *Artemia* was provided, and while fish were transitioning to *Lumbriculus,* as indicated by uneaten food that was removed during cleaning. After fish had transitioned to a diet composed of only *Lumbriculus* (starting on day 90), leftover worms were rarely observed; therefore, consumption rates were similar to delivered rations.

Some adjustments were made to the feeding regime for Experiment 2 to increase statistical power with more fish per tank without exceeding biomass loading targets. Fish were fed *Artemia* at a target ration of 20% of wet weight, divided into two or three feedings per day until test day 51. Starting on day 52, 1 g *Lumbriculus* were provided to each tank, and the remainder of the 20% daily ration was made up with *Artemia*. The proportion of the daily ration that consisted of *Lumbriculus* was gradually increased until test day 67, when fish in all tanks were able to consume the total daily ration in a single feeding of *Lumbriculus*. Beginning on test day 67, the daily ration was readjusted weekly to maintain an overall average specific growth rate that was predicted to result in biomass loading rates below 5 g/L maximum at the end of the experiment. The actual daily ration was 20% of the wet weight on test day 67; it was decreasing gradually to 13% by test day 120.

In both experiments, survival rates were calculated for individual exposure chambers based on the number of fish found dead as well as unhealthy fish that were euthanized to prevent the spread of disease.

Histological analysis

Fish were removed from tanks at regular intervals throughout the exposure and non-exposure periods for histological analysis. To avoid unnecessary handling, whenever possible, these collections coincided with times when fish were moved into clean tanks. Those collected for histological analysis were randomly selected (Random.org) to avoid bias and immediately euthanized with 3 g/L MS-222 (Western Chemical, buffered to neutral pH with $NaHCO_3$). In Experiment 1, individuals ($n = 7–17$) were sampled from the control and low exposure treatment groups (1.2 ng/L EE2) for histological evaluation at six time points during the exposure period (test days 25, 32, 40, 49, 63, and 74), and at five time points after exposure ceased (test days 92, 120, 151, 165, and 180; Table 1). In addition, three fish were sampled from the high

TABLE 1: Number of individual fish per treatment evaluated during each sampling period in Experiment 1[a]

	Experimental treatment		
Test day	Control	1.2 ng/L	5.1 ng/L
25	12	12	–
32	12	12	–
40	12	9	–
49	12	12	–
63	12	12	–
74	12	12	–
92	12	12	–
120	12	12	–
151	17	12	–
165	7	8	3
180	10	12	–

[a]Treatment groups are identified by their mean measured exposure concentrations.

concentration treatment (5.1 ng/L) on test day 165 for preliminary analysis and method development. In Experiment 2, fish were removed from tanks throughout the exposure period, but were only evaluated histologically on test days 90 ($n = 18$–20) and 120 ($n = 10$–12; Table 2). Total lengths (~1 mm) and blotted wet weights (~0.001 g) were recorded for each fish. Heads and tails were removed, abdomens were slit ventrally, and abdominal cavities and gill cavities were perfused with either Bouin's fixative (Experiment 1) or 4% paraformaldehyde in phosphate-buffered saline (Sigma-Aldrich; Experiment 2). After 72 h, the samples were rinsed in phosphate-buffered saline and transferred to neutral-buffered formalin (Experiment 1) or 70% ethanol (Experiment 2) for storage until processing. Prior to histological processing, fish (up through test day 120 in Experiment 1, and at all sampling periods in Experiment 2) were trimmed down to the abdominal portion between the vent and posterior edge of the gill chamber; for the larger fish in the later samplings of Experiment 1 (test days 151, 165, and 180), gonads and attached dorsal peritonea were excised from the abdominal chamber. Tissues were processed using an automated tissue processor (Sakura Tissue Tek VIP). During processing, tissues were dehydrated with a graded series of ethanol, cleared (Clear Rite 3, Thermo Scientific), and infiltrated and embedded with paraffin (Paraplast Plus, Millipore-Sigma). A preliminary evaluation of both transverse and longitudinal gonad sections from day 165 of Experiment 1 showed that the anterior-most regions were not consistently representative of the most advanced stage of development; therefore, a sectioning protocol was developed that focused on transverse sections of the middle and posterior regions. Each trunk piece or gonad pair with its associated tissue was bisected transversely through the mid-posterior region and both halves were embedded, cut face down, in a single paraffin block. For each fish, six sections (4 μm thick) of fully faced, paired gonad cross-sections, spaced 50 or 100 μm apart, were collected, stained with hematoxylin and eosin, and evaluated by light microscopy (Axiovert 35, Carl Zeiss Microscopy). Experiment 1 samples were used to develop criteria for phenotypically characterizing the gonads as undifferentiated, presumptive ovary, definitive ovary, abnormal ovary, definitive testis, or intersex. The most advanced stages of germ cells and somatic structure among all the observed sections from each fish were reported. The largest-observed diameter of germ cells, blood vessels, and gonad cross sections were measured to the nearest micron using a color digital camera (SPOT™ Insight 4.0 CCD Diagnostic Instruments) calibrated with a stage micrometer. Measurements were reported as the range of maximum measurements observed in each treatment-age group.

TABLE 2: Number of individual fish per treatment evaluated during each sampling period in Experiment 2[a]

Test day	Control	0.1 ng/L	0.4 ng/L	1.0 ng/L	3.0 ng/L
90	18	20	18	19	20
120	11	11	10	10	12

[a]Treatment groups are identified by their nominal (0.1 and 0.4 ng/L) or mean measured (1.0 or 3.0 ng/L) exposure concentrations.

Statistical analysis

All statistical analyses were performed with R (R Core Team, 2015). Differences in mean wet weight and length between groups were considered significant at the level of alpha = 0.05. Wet weights were log-transformed to meet the assumption of normality. Dunnett's multiple comparison test was used to determine whether mean fish total length and mean wet weight differed between the treatments and the control in each age group. Chi-square (χ^2) analysis was used to detect significant deviations from the expected proportion of fish that developed normal ovaries (0.5) for age groups in which both ovarian and testicular differentiation was evident in controls (days 92, 120, 151, 165, and 180 of Experiment 1, and days 90 and 120 of Experiment 2).

Welch's *t*-test was used to compare mean measured EE2 concentrations in Experiment 1 before and after biweekly sterilization procedures to evaluate the stability of the toxicant in the system over time.

In Experiment 2, analytical chemistry data were processed using MassHunter Quantitative Analysis vB.06.00 (Agilent Technologies). Each sample was quantified with two methods, using either EE2-d4 or E2-^{13}C as internal standard. Linear calibration curves were developed based on the response ratios between EE2 analytical standards and either EE2-d4 or E2-^{13}C. A 1/*x* weighting was applied to both EE2-d4 and E2 calibration curves to increase fit at lower concentrations. The lower limit of quantification for the method, determined as the lowest calibration standard with accuracy ranging 80%–120% and signal-to-noise ratio ≥ 10, was 0.64 ng/L. The lower limit of detection, determined at a signal-to-noise ratio ≥3, was 0.3 ng/L.

RESULTS

Water quality measurements

Temperature and pH remained consistent in all treatments throughout both experiments. In Experiment 1, mean (SD) temperature was 22.3 (0.3) °C, and pH was 7.6 (0.2).

In Experiment 2, mean (SD) temperature was 21.9 (0.3), and pH was 7.1 (0.2). In Experiment 1, mean (SD) dissolved oxygen was 6.8 (0.8) mg/L, corresponding to 78% mean saturation. Days on which at least some tanks measured low dissolved oxygen (between 45% and 59%) corresponded with days on which biomass loading was high; for example, between test days 88 and 92 (fish removed for sampling on day 92) and between test days 104 and 120 (fish removed for sampling on day 120). In Experiment 2, mean (SD) dissolved oxygen was 7.1 (0.7) mg/L, corresponding to 83% mean saturation, and was not lower than 60% on any day. Total ammonia did not exceed 0.4 mg/L in either experiment, with means (SDs) of 0.12 (0.06) mg/L and 0.24 (0.07) mg/L in Experiments 1 and 2, respectively.

EE2 analysis

A summary of analytical chemistry results for Experiment 1 is provided in Supporting Information, Table S2. In Experiment 1, background EE2 detection in control tanks and incoming dilution water was below the method detection limit for unconcentrated samples (1 ng/L). The mean (SD) measured stock bottle concentration was 3698 (951) ng/L, which was 96% of nominal (3860 ng/L). Mean (SD) measured exposure concentrations of 1.2 (0.7) and 5.1 (2.0) ng/L were reported and used for evaluations. Reported concentrations of the SPE-extracted samples were not adjusted for spike recovery (which had means [SD] of 1.8 [1.0; $n = 5$] and 9.5 [3.9; $n = 6$] ng/L, respectively). There were no significant differences in measured EE2 concentrations from samples collected just prior to the system sterilization procedure compared with samples collected immediately after sterilization ($p < 0.05$), suggesting that no substantial loss of EE2 occurred in the system over time (such as might be expected if accrued microbial growth was metabolizing EE2).

A summary of analytical chemistry results for Experiment 2 is provided in Supporting Information, Table S3. In Experiment 2, EE2 was not detected in method blanks or control tank samples (method detection limit 0.3 ng/L). A paired *t*-test showed no difference between quantified concentrations in the stock solutions using EE2-d4 as internal standard and those using E2-^{13}C ($p = 0.25$). Measurements of stock solution mean EE2 concentrations using either quantification method were very close to nominal and were consistent over time: means (SD) were 1080 (55) and 1088 (49) ng/L EE2 among samples quantified with the EE2-d4 and E2-^{13}C internal standards, respectively. However, EE2 concentrations in exposure water differed consistently and substantially between the EE2-d4 and E2-^{13}C quantification methods, with EE2-d4 quantifications averaging 50% higher than E2-^{13}C quantifications. Quantifications based on the EE2-d4 internal standard also averaged 50% above nominal, whereas E2-^{13}C quantifications aligned with nominal concentrations. Matrix spikes showed the same pattern, with EE2-d4 quantifications averaging 48% higher than E2-^{13}C quantifications. Analyte recovery from matrix spikes quantified with d4 averaged 182%, compared with 125% among spikes quantified with E2-^{13}C. The difference between quantification method results was related to low apparent recovery of EE2-d4, with no corresponding loss of unlabeled EE2. However, E2-^{13}C did not show a similar reduction in response, suggesting EE2-d4 signal reduction independent of matrix effects. Procedural loss of EE2 during SPE was not indicated, because E2-^{13}C quantifications, which account for potential matrix effects but not SPE-related loss, were close to nominal. Differences between d4 and E2-^{13}C quantifications existed only for samples that were processed with SPE (i.e., exposure water and matrix spikes, but not stock solutions), suggesting the discrepancy was related to the SPE process. The mechanism of this discrepancy is unknown, but deuterium exchange is a possible explanation. Therefore, because the stock solution analyses were very close to nominal, and the d4-based quantifications were higher than nominal by similar margins for both exposure samples and matrix spikes, only E2-^{13}C-based quantifications were used to report measured exposure concentrations. 17-β-Estradiol-13,14,15,16,17,18-^{13}C6 was not added to samples collected before day 30, but d4-based quantifications for that period were similar to those after day 30; so E2-^{13}C-based quantifications should be representative of the entire exposure. Based on the final mean measured stock solution concentration of 1088 ng/L, the expected exposure concentrations were 0.1, 0.4, 1.1, and ng/L. Mean (SD) measured exposure concentrations of 1.0 (0.2) and 3.0 (0.8) ng/L were reported for the two highest exposure treatments and used for evaluations. Concentrations in the two lowest treatments were below the limit of quantitation (0.64 ng/L), but the similarity between expected and measured concentrations for the two higher treatments, combined with monitoring of diluter pump flows in the exposure system, provide confidence that those exposures were likely near their expected (nominal) values. Therefore, nominal exposure concentrations of 0.1 and 0.4 ng/L were reported for the two lowest treatments and used for evaluations. Duplicate samples from the same exposure chamber ($n = 7$) had a mean (SD) relative percent difference of 20 (16)%. Reported water concentrations were not corrected for recovery from matrix spikes.

Survival and growth

Average survival rates for both experiments are shown in Supporting Information, Figure S1. In Experiment 1, mean survival rate among control tanks was 90% over the entire experiment. Between test days 28 and 34, unexplained mortalities occurred in two of the four 5.1 ng/L EE2 exposure tanks (31% and 44% loss) and three of the four 1.2 ng/L EE2 exposure tanks (18%, 19%, and 29% loss). Mean survival rate in all other treatment and control tanks between test days 28 and 34 was 97%. Based on subjective observations, we believe the lower survival rates in some tanks were not exposure-related, but were caused by an undiagnosed disease that resolved spontaneously, and average tank survival after the mortality event ranged from 91% to 97%. In Experiment 2, mean survival rate among all exposure chambers was 95% (range: 88%–99%). The few mortalities that occurred were concentrated during the first 30 days of the experiment (Supporting Information, Figure S1).

Summaries of wet weight measurements for both Experiments 1 and 2 are provided in Supporting Information, Figure S2. In Experiment 1, mean wet weights and lengths among EE2-exposed groups were not significantly different than control groups at any time point except for test day 120, when mean wet weight in the 5.1 ng/L EE2 exposure group (6.2 g) was significantly lower ($p = 0.02$) than the control group (7.2 g). The lower weight in this treatment group may have been a result of non-specific systemic toxicity caused by EE2, because relatively poor feeding behavior was also observed in that group starting on day 82. No other significant weight reductions were detected in exposed fish. Poor feeding behavior was also observed in some fish among all tanks during the diet transition from *Artemia* to *Lumbriculus* (test days 18–47), which may explain the slightly decreased growth rate between test days 32 and 63. In Experiment 2, mean wet weights of EE2-exposed fish were not significantly different than control groups at most time points, and the few individual exceptions were not suggestive of an EE2-related effect. Growth rates were consistent with those observed in Experiment 1 (Supporting Information, Figure S2).

Histological results

Gonadal development in unexposed (control) fish. Normal gonadal development, as observed in Experiment 1, control fish between test days 25–180 is fully described by Kadlec (2017) and summarized in Supporting Information, Table S4. The proportion of gonadal phenotypes observed at each sampling period is summarized for control fish in Figure 1A. Representative images of control group ovarian and testicular development are pictured in Figures 2 and 3, respectively. Gonads were present as elongated paired organs, each connected to the dorsal peritoneum by a single gonadal mesentery (Supporting Information, Figure S3). The anterior-most tips of the gonads did not contain germ cells; only somatic cells and blood vessels were there. Undifferentiated gonads with no sex-specific characteristics (Figure 2A) were observed in all fish collected on test day 25 ($n = 12$), and in 45% of control fish collected on test days 32 through 74 ($n = 60$). Presumptive ovaries, identified by ovarian cavity somatic projections or fully enclosed ovarian cavities (Figure 2B and C) were observed in 47% of control fish on test days 32 through 49 ($n = 36$). Presumptive ovaries also had larger cross-sectional diameters and contained more germ cells than undifferentiated gonads. Some germ cells were first observed in an early stage of meiosis on test day 40 (Figure 2C). Definitive ovaries, characterized by the presence of primary oocytes (Figure 2D–F), were first identified in a few fish on test day 49, and were present in 47% of control fish collected on test days 49 through 180 ($n = 94$).

Testicular differentiation was first identified in fish collected on test day 92, based on the presence of small spermatic tubules (Figure 3A). The onset of spermatogenesis was apparent in male fish collected on test day 120, identifiable by the proliferation of premeiotic germ cells, and a single fish also contained meiotic germ cells and cysts of spermatozoa in the center region of the gonad (Figure 3B). Multiple stages of spermatogenesis, including low numbers of spermatozoa (Figure 3B), were observed in at least some male fish collected on test days 151, 165, and 180.

In Experiment 2, gonadal histology was only evaluated in fish collected on test days 90 and 120. The proportions of gonadal phenotypes observed in fish collected on test days 90 and 120 from each experimental treatment are summarized in Figure 4A and B, respectively. In control groups, ovarian differentiation was observed in 44% and 55% of fish collected on days 90 ($n = 18$) and 120 ($n = 11$), respectively, and the total proportion of fish with ovaries was not significantly different than 0.5 ($p = 0.45$). Among control fish collected on test day 90, all fish that did not have ovaries had undifferentiated gonads. On test day 120, all control fish that did not have ovaries had gonads identified as testes, based on the presence of fully formed spermatic tubules containing increased numbers of germ cells, but more advanced stages of spermatogenesis

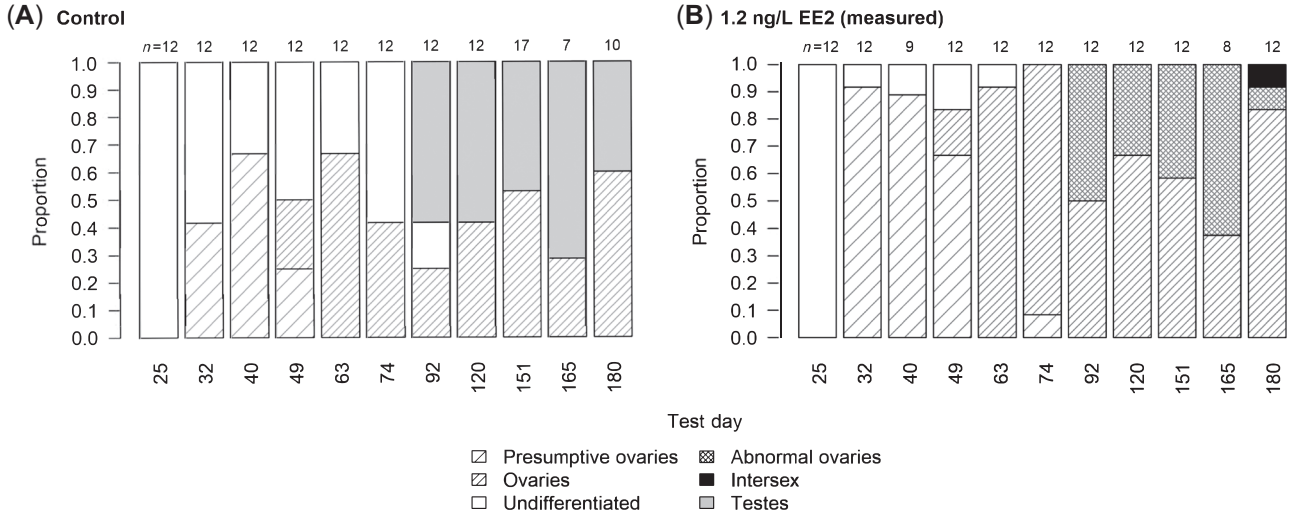

FIGURE 1: Proportions of gonadal phenotypes observed in (**A**) control, and (**B**) ethinylestradiol-exposed groups (1.2 ng/L) at each sampling period in Experiment 1.

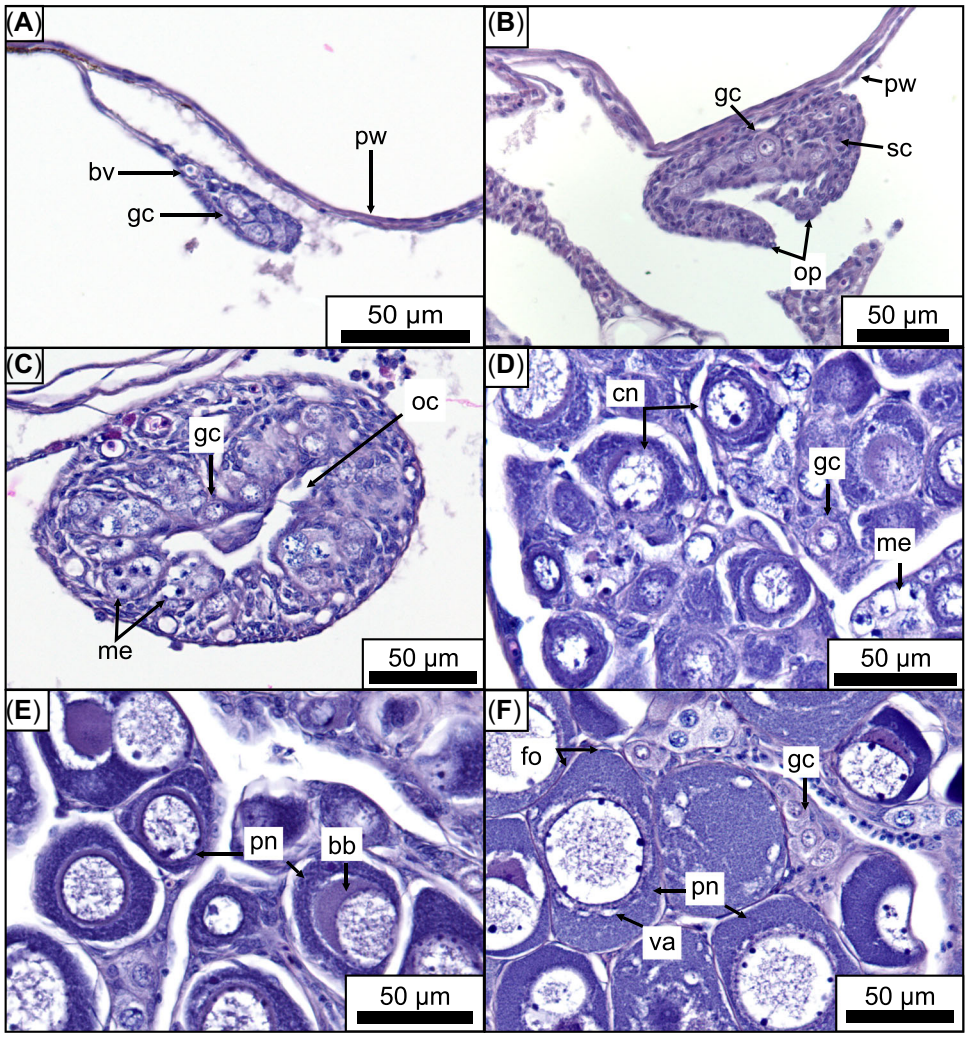

FIGURE 2: Undifferentiated gonads and ovarian differentiation in smallmouth bass reared in clean Lake Superior water from swim-up (test day 0) through 180 days in Experiment 1. (**A**) Day 25, undifferentiated gonad; (**B**) day 32, presumptive ovaries with ovarian cavity somatic projections; (**C**) day 40, presumptive ovary with patent ovarian cavity, germ cell proliferation and early meiosis; (**D**) day 63, ovary with chromatin nucleolar oocytes; (**E**) day 92, ovary with late perinucleolar oocytes; (**F**) day 180, ovary with late perinucleolar oocytes and early folliculogenesis. bb = balbiani body; bv = blood vessel; cn = chromatin nucleolar oocyte; fo = early follicle complex; gc = germ cell; me = meiotic germ cell cluster; oc = ovarian cavity; op = ovarian cavity somatic projection; pn = perinucleolar oocyte; pw = peritoneal wall; sc = somatic cell; va = vacuole.

were not present (similar to Figure 3A). No testicular oocytes or other abnormalities were found in control fish in either experiment.

Effects of EE2 exposure on gonadal development, Experiment 1. An initial evaluation of gonadal tissues from fish exposed to 1.2 ng/L ($n = 8$) and 5.1 ng/L ($n = 3$) EE2, and collected on day 165 of Experiment 1, indicated that all fish had developed ovaries, including presumed genetic males (a smallmouth bass genetic sex marker has not yet been developed at the time of publication). Because complete sex reversal represents the most extreme possible histologic endpoint of the present experimental design, only the tissues from the 1.2 ng/L EE2 exposure groups were evaluated and reported in the present study.

At test day 25, gonadal development of control and 1.2 ng/L EE2-exposed groups was indistinguishable, as all fish had small, undifferentiated gonads (Figure 2A). In fish sampled on test days 32–74, gonad phenotype in EE2-exposed fish was heavily biased toward ovarian characteristics (89%, $n = 57$; Figure 1B), compared to control fish (53%, $n = 60$), with definitive ovaries first detected in both groups on test day 49.

Testicular characteristics were detected in control groups starting at test day 92 ($n = 58$), whereas none of the EE2-exposed fish developed testes through test day 180. Conversely, in EE2-exposed fish at 92–180 days, 38% of fish ($n = 56$) had ovaries with fully developed ovarian cavities, but displayed abnormalities, including smaller cross-sectional diameters compared to age-matched controls (373–1780 vs. 624–2300 µm, respectively), increased pathologic findings (including mononuclear cellular infiltrate, granulomatous inflammation, and fibrotic cells and fibers), and very few oocytes found mainly in the center region (Figure 5A–C). The observation of normal testicular development in control fish and abnormal ovary

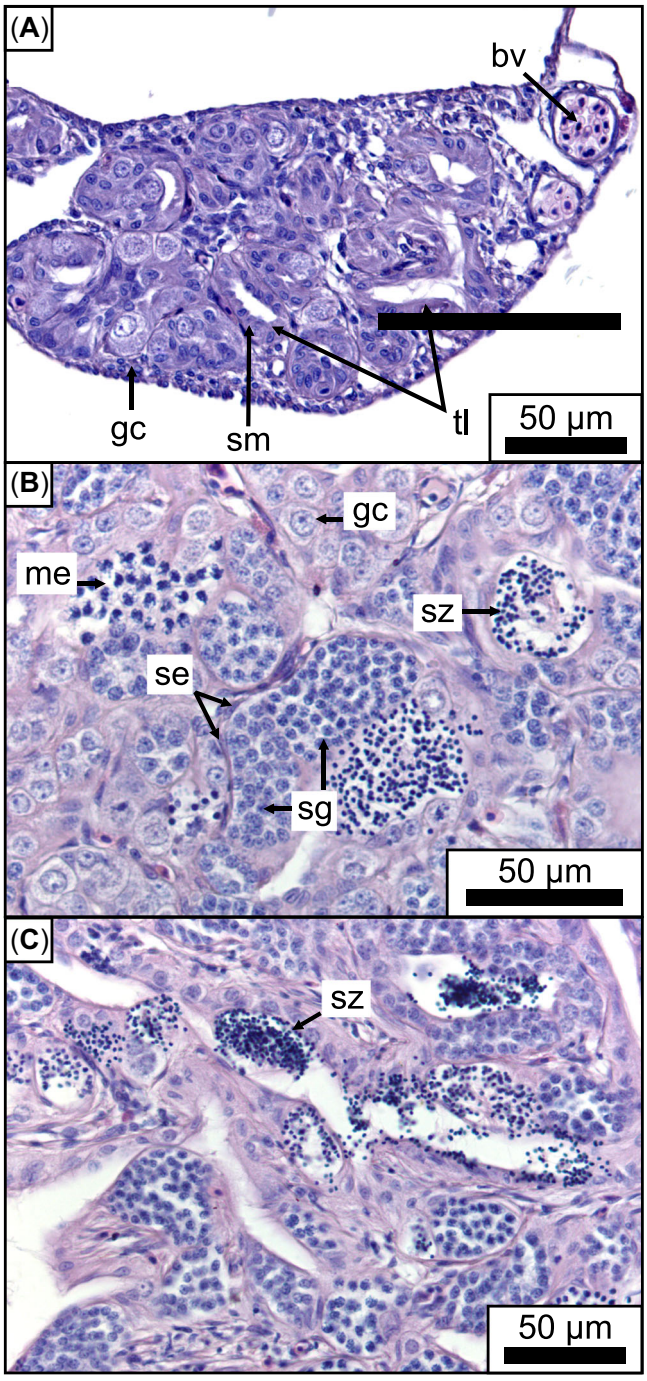

FIGURE 3: Testicular differentiation in swim-up smallmouth bass reared in clean Lake Superior water for 180 days in Experiment 1. (**A**) Test day 92, testis with early spermatic tubules; (**B**) test day 120, spermatocysts enclosing clusters of spermatogonia types A and B, meiotic spermatocytes, and spermatozoa; (**C**) test day 180, testis with mature spermatozoa. bv = blood vessel; gc = germ cell; me = meiotic germ cell cluster; se = Sertoli cells; sg = spermatogonia types A and B; sm = somatic cell; sz = spermatozoa; tl = tubule lumen.

development in the corresponding age-matched cohort of EE2-exposed fish, together with the fact that the proportion of EE2-exposed fish with abnormal ovaries was not significantly different than 0.5 ($p = 0.61$), suggests that the fish with abnormal ovaries were sex-reversed males. In addition, an intersex fish was sampled from the EE2-exposed group at day 180, with a gonad consisting of a central region of ovarian tissue containing several primary oocytes and fully developed ovarian cavities, surrounded by testicular tissue containing all stages of spermatogenesis, including a small number of mature spermatozoa (Figure 6).

Effects of EE2 exposure, Experiment 2. Among fish in the highest EE2 treatment group (3.0 ng/L) in Experiment 2, ovarian phenotypes were observed in 100% of fish collected on test day 90 ($n = 20$; Figure 4A) and 92% of fish collected on test day 120 ($n = 12$; Figure 4B), as well as a single fish with undifferentiated gonads. Abnormal ovarian development was common among 3.0 ng/L EE2-exposed fish at both 90 and 120 days (34%, total $n = 32$). As in Experiment 1, abnormalities included a smaller cross-sectional area compared to controls (480–710 vs. 708–1215 µm, respectively), as well as fewer oocytes and increased pathological findings around the perimeter compared to control ovaries. Ovaries in the remaining EE2-exposed fish in this group were histologically similar to those of control fish.

Among fish exposed to lower EE2 concentrations (0.1, 0.4, and 1.0 ng/L) in Experiment 2, normal ovaries were present in 49% ($n = 57$) on test day 90 (Figure 4A), and in 58% ($n = 31$) on test day 120 (Figure 4B). In these lower EE2 concentration treatment groups, the proportion of fish with ovaries was not significantly different than 0.5 ($p = 0.52$), and no abnormal ovaries were observed. Conversely, all fish that did not have ovaries on day 90 (51%) had undifferentiated gonads that were histologically similar to those in the matched control groups. In the low-EE2 concentration groups on day 120, 42% of the fish displayed testicular differentiation that was histologically similar to testicular tissues observed in the matched control groups. No testicular oocytes were observed in fish from any treatment group in Experiment 2, and the only gonadal anomalies detected were the abnormal ovaries in the high EE2 concentration group (see Figure 5 for representative examples of this phenotype as observed in Experiment 1).

DISCUSSION

The histological evaluations of control fish in Experiment 1 indicate that in early stages, similar undifferentiated gonads are present in putative genetic male and female smallmouth bass. The ovaries and testes develop directly from the undifferentiated stage, with ovarian differentiation occurring earlier than testicular differentiation. Presumptive ovaries were identified by the presence of an ovarian cavity prior to the appearance of oocytes, and testes were identified by the presence of spermatic tubules prior to the appearance of spermatocytes. We were not able to distinguish sex at earlier stages using other markers, such as differences in the organization of germ cells relative to somatic cells or the number of attachment points to the peritoneal wall (Nakamura et al., 1998). The process of ovarian cavity development we observed, in which somatic processes appear to project from the edges of the ovary and fuse together, is similar to that of bluegill sunfish (*Lepomis macrochirus*; Gao et al., 2009) and

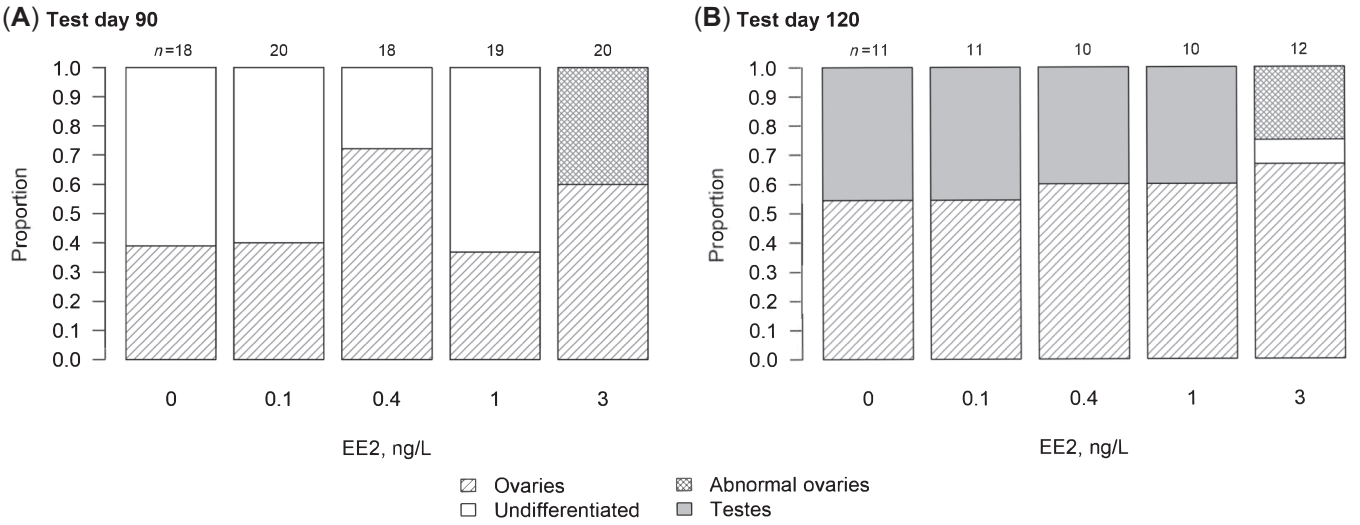

FIGURE 4: Proportions of gonadal phenotypes observed in control and ethinylestradiol (EE2)-exposed groups (0.1 ng/L nominal, 0.4 ng/L nominal, 1.0 ng/L measured, or 3.0 ng/L measured) in Experiment 2 on (**A**) test day 90, and (**B**) test day 120.

pejerrey (*Odontesthes bonariensis*; Strussmann et al., 1996); but different than in other species (e.g., fathead minnow and medaka), where the edges of the gonad fuse directly to the peritoneal wall to form a cavity between the ovary and the peritoneum (Nakamura et al., 1998; van Aerle et al., 2004). After the onset of gonadal differentiation, germ cell development proceeded in a manner similar to that described in other model species (Devlin & Nagahama, 2002; Grier, 1981; Nakamura et al., 1998; Strussmann & Nakamura, 2002).

The co-occurrence of clearly defined male-specific characteristics in males and primary oocytes in females was observed on test day 92 in Experiment 1, and on test day 120 of Experiment 2, suggesting that testicular oocytes or intersex would also have been detectable at those times, if not earlier (e.g., any time at which definitive ovaries were detectable in cohorts). However, testicular oocytes were not observed in any male in either experiment ($n = 49$; total males observed in Experiment 1 on test days 92 through 180 and in Experiment 2 on test day 120). For comparison, in adult smallmouth bass collected from the same lake as the fry used in the present study, testicular oocytes were detected in 2 of 24 (8%) male fish (Kadlec et al., 2017). Although the sampled lake had no known sources of estrogenic compounds and the exposure histories of the adult fish and early fry were not fully characterized, these results suggest a more complex etiology for occurrence of testicular oocytes. In addition, it is not known whether the absence of testicular oocytes in juvenile fish is predictive of a similar absence in adult fish. In laboratory cultures of non-hermaphroditic species (e.g., medaka, fathead minnow, roach, and carp), the spontaneous occurrence of intersex has been reported to range from <1% to 21% (Grim et al., 2007; Jobling et al., 1998; Komen et al., 1989; Niemuth & Klaper, 2015). Moreover, it has been suggested that testicular oocytes are less likely to arise naturally in species (such as smallmouth bass) in which gonadal development progresses directly from an undifferentiated state into either ovaries or testes, compared with species (such as zebrafish) in which the ovary-to-testis transition phase may result in residual oocytes (Beamish & Barker, 2002).

Presumably, all male smallmouth bass exposed to 1.2 or 3.0 ng/L EE2 in Experiments 1 and 2, respectively, were sex reversed. This is consistent with the range of concentrations that has induced sex reversal in other species (Balch et al., 2004; Lange et al., 2001; Zha et al., 2008). In Experiment 2, exposure to 0.1, 0.4, or 1.0 ng/L EE2 had no observed effects on gonadal phenotype or sex ratio. The results of the two experiments are reasonably consistent with each other if we consider one or more of the following: that effect concentrations may exist on a steep response curve, and/or, that sensitivity may vary slightly among juvenile cohorts due to differences in age post-hatch, genetics, or rearing conditions. In addition, chemical analysis methods differed between the two experiments, with higher variability among samples analyzed with ELISA in Experiment 1. These results are also consistent with others reporting a narrow separation between EE2 no-effect concentrations (ranging from 0.3 to 1 ng/L) and concentrations that caused complete sex reversal (e.g., zebrafish, roach; Caldwell et al., 2008).

The finding of an abnormal ovary phenotype, in which ovaries that developed in presumably sex-reversed males and could be distinguished from normally developed ovaries, has not been previously described in any fish species to our knowledge. In both Experiments 1 and 2, the first time point at which abnormal ovaries were observed coincided with the observation of testicular differentiation in control groups, suggesting that the cellular pathways involved in testicular development may not have been completely inhibited by EE2 exposure. Because the abnormal ovary phenotype persisted 90 days after cessation of EE2 exposure in Experiment 1, sex reversal may have been permanent, although we have no empirical evidence related to longer-term outcomes of early life sex reversal. In other fish species, EE2-induced sex reversal or feminization is usually considered permanent (Balch et al., 2004; Devlin & Nagahama, 2002). Intersex gonads were

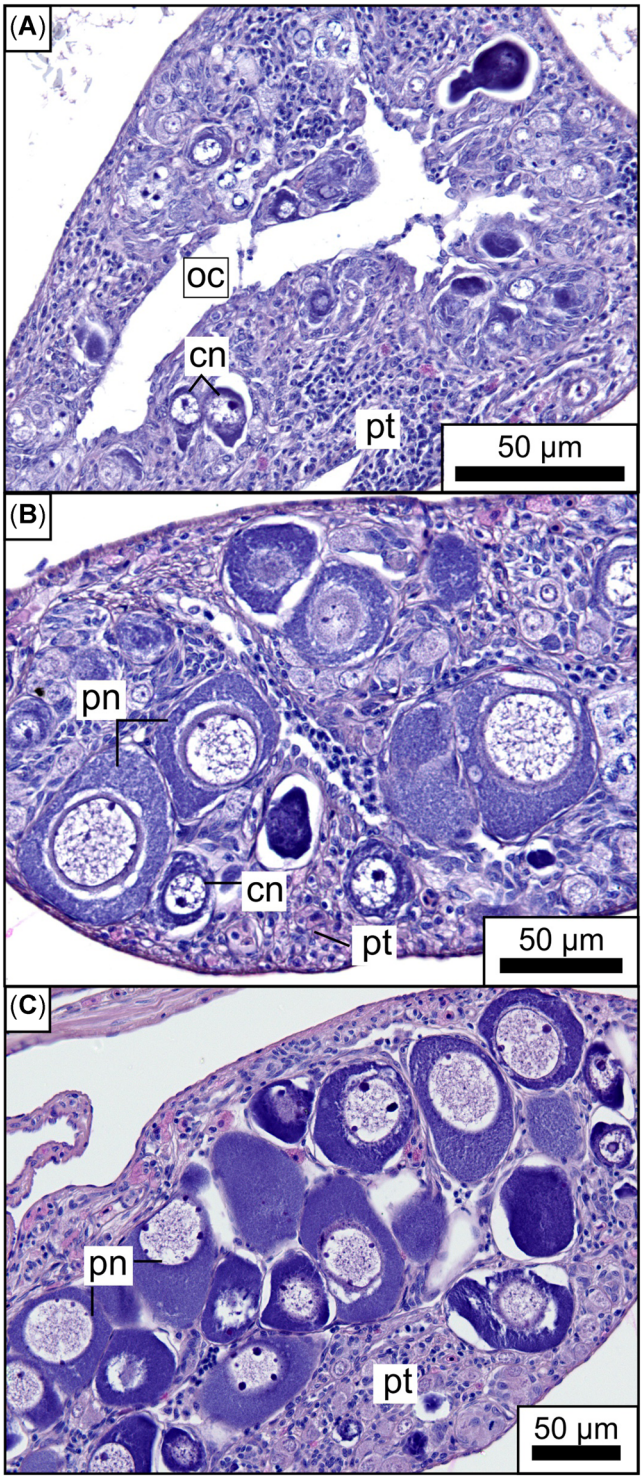

FIGURE 5: Abnormal ovaries in smallmouth bass exposed to 1.2 ng/L ethinylestradiol (EE2; measured), Experiment 1. Abnormal ovaries were observed in 38% of all EE2-exposed fish ($n = 56$) collected on test days 92 (**A**), 120 (**B**), 151 (not shown), 165 (not shown), and 180 (**C**), and were characterized by smaller cross-sectional area, fewer oocytes, and increased observations of pathologic findings (i.e., mononuclear cellular infiltrate, granulomatous inflammation, and fibrotic cells and fibers) compared with normal ovaries in EE2-exposed and control fish. cn = chromatin nucleolar oocytes; oc = ovarian cavity; pn = perinucleolar oocytes; pt = pathologies (including mononuclear cellular infiltrate, granulomatous inflammation, fibrotic cells, and fibers).

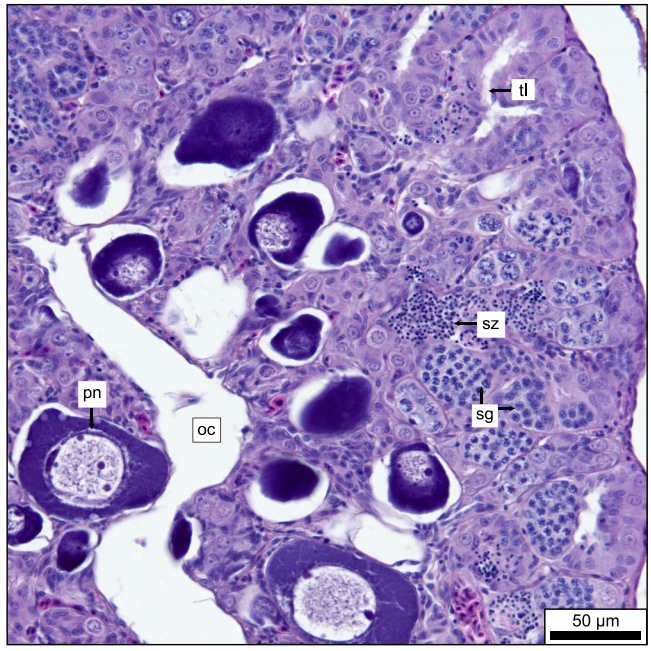

FIGURE 6: Intersex gonad observed in a single smallmouth bass exposed to 1.2 ng/L ethinylestradiol (measured), Experiment 1. Gonad had ovarian tissue in the center (including a small number of oocytes around a central ovarian cavity) and testicular tissue around the perimeter (i.e., spermatic tubules with spermatocysts in all stages of development including small amounts of mature spermatozoa). oc = ovarian cavity; pn = perinucleolar oocyte; sg = spermatogonia type A and type B; tl = spermatic tubule lumen; sz = spermatozoa.

found in only one fish, which was exposed to 1.2 ng/L EE2 and collected at 180 days. However, the segregation of ovarian and testicular tissue in this specimen was unlike the phenotype typically characterized as intersex in field studies of wild adult smallmouth bass, in which a small number of early oocytes are present within otherwise histologically normal testicular tissue (Blazer et al., 2007; Kadlec et al., 2017).

Overall, the present study found no evidence directly linking early life exposure to EE2 with the development of testicular oocytes in smallmouth bass up to the juvenile stage. Although long-term outcomes of EE2 exposures are unknown, we question whether exposure to estrogenic compounds is a unique cause of testicular oocytes in wild populations and whether the presence of testicular oocytes alone is a sufficient indicator of estrogenic exposure in smallmouth bass. The present study adds to the predominant findings in current literature that demonstrate only weak correlations between known human activities and testicular oocytes in this species. We advocate for further understanding of gonadal development in fish reared in the known absence and presence of estrogen as a critical part of interpreting the occurrence of testicular oocytes in wild species.

To our knowledge, ours is the first study to describe early life stage smallmouth bass gonadal differentiation and germ cell development, and to compare gonadal development in the presence of exogenous estrogen. In addition, we have identified developmental stages when abnormal testicular differentiation can be detected. Finally, we have established a range of low EE2

concentrations at which no effects on gonadal development were observed in smallmouth bass. These outcomes increase our understanding of the conditions under which normal and abnormal gonadal developments may occur in smallmouth bass, and provide a foundation for evaluating the reproductive effects of estrogenic exposure in this species.

Supporting Information—The Supporting Information is available on the Wiley Online Library at https://doi.org/10.1002/etc.5320.

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Author Contributions Statement—**Sarah M. Kadlec**: Conceptualization; Data curation; Formal Analysis (lead); Funding acquisition; Investigation (lead); Writing – original draft; Writing – review & editing (lead). **Brett R. Blackwell**: Formal Analysis (analytical chemistry); Investigation (analytical chemistry); Methodology (analytical chemistry); Writing – review & editing. **Chad A. Blanksma**: Formal Analysis (histology); Methodology (histology); Writing – review & editing. **Rodney D. Johnson**: Conceptualization; Supervision; Writing – review & editing. **Jennifer H. Olker**: Funding acquisition (co-lead); Writing – review & editing. **Patrick K. Schoff**: Conceptualization (co-lead); Funding acquisition (co-lead); Supervision (co-lead). **David R. Mount**: Conceptualization (co-lead); Supervision (co-lead).

Data Availability Statement—Data, associated metadata, and calculation tools are available from the corresponding author (kadlec.sarah@epa.gov).

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