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TAMOXIFEN PROTECTS AGAINST 17α-ETHYNYLESTRADIOL–INDUCED LIVER DAMAGE AND THE DEVELOPMENT OF UROGENITAL PAPILLAE IN THE RAINBOW DARTER (*ETHEOSTOMA CAERULEUM*)

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Abstract—Juvenile rainbow darters (*Etheostoma caeruleum*) were exposed to nominal concentrations of 20 to 1,000 ng/L of 17α-ethynylestradiol (EE_2) at 120 d posthatch and in a subsequent experiment to 200 ng/L of EE_2 with 2.0 to 20,000 ng/L of tamoxifen (TMX) at 150 d posthatch to determine the threshold of estrogen-induced morphological and histological changes in a sexually dimorphic benthic fish species ecologically relevant to southern Minnesota (USA). 17α-Ethynylestradiol induced female-associated urogenital papillae in males at 200 ng/L, enlargement and development of fibrosis in male testes, enlargement of ovary and oocyte size in females, and large fatty inclusions in the liver of both sexes. Exposure to 1,000 ng/L of EE_2 caused gross hypertrophy of the liver and kidneys and high mortalities, predominantly in male fish. A low incidence of ovotestes found in all treatment groups was unaffected by EE_2, which may be unusual to this species or a response to unknown water contaminants present during the hatching or early development of the darters. Gonadosomatic index was not altered for either sex by any treatment. A TMX level equal to or less than that of EE_2 decreased fat accumulation in the liver in both sexes, and a TMX level greater than that of EE_2 appeared to prevent urogenital papilla in males. Tamoxifen did not significantly alter fibrosis caused by EE_2 in testes. It appears that the presence of TMX in the environment can mask many signs of estrogen exposure, including secondary sexual characteristics, hypertrophy of ovaries and testes, and fatty infiltration of organs. Ovotestes did not prove to be a good indicator of estrogen exposure at this late stage of juvenile darter development.

Keywords—Rainbow darter Estrogen Tamoxifen Liver Gonads

INTRODUCTION

Numerous studies have been performed in a variety of fish species to assess the effects of a potent environmental estrogen originating from birth control pills, 17α-ethynylestradiol (EE_2), on vitellogenin production [1–4], endocrine and reproductive function [5,6], and cells of reproductive organs and the kidneys and liver [7]. Some studies have examined the antagonistic role of the antiestrogen used in the treatment and prevention of breast cancer, tamoxifen (TMX), in protecting fish against the disrupting action of EE_2 [8–10]. The actions of environmental estrogens in wastewater on aquatic populations appear to confirm the effects seen in controlled laboratory settings [11], including the induction of ovotestes [12]. The amount of TMX in wastewater has generated few studies [13], and its true environmental influence is unclear.

The present study determined the morphological and histological sensitivity of an ecologically relevant native fish species for southern Minnesota (USA), the rainbow darter (*Etheostoma caeruleum*), to estrogenic endocrine disruption. The selection of the rainbow darters as a possible bioindicator species resulted from a number of factors, including its prevalence as a freshwater fish in southern Minnesota rivers and because it is a benthic species that lacks a swim bladder [14]. This increases the likelihood for exposure of darters to hydrophobic estrogenic compounds or TMX bound to sediment [15]. Rainbow darters are sensitive to pollutants, easily maintained in captivity, and sexually dimorphic as adults. Males tend to be colorful and actively guard and defend spawning territories, and cryptic females have a urogenital papilla [16]. Rainbow darters also are found in the same taxonomic family (Percidae) as the walleye, an important Minnesota commercial and sport fish species that has been observed to be affected by environmental estrogens [17]. The objectives of the present study were to determine the threshold concentration of EE_2 for ovotestes and other alterations in the testes of juvenile male rainbow darters, the development of a urogenital papilla in male fish and ovarian hypertrophy in females, and reverse reproductive effects of EE_2 by TMX in both sexes. Liver and kidney histology were examined to provide evidence of overt toxicity of these compounds.

MATERIALS AND METHODS

Chemicals

Tamoxifen citrate (Chemical Abstract Service [CAS] no. 54965-24-1) was provided as a gift from AstraZeneca (London, UK). 17α-Ethynylestradiol (CAS no. 57-63-6) was obtained from Sigma-Aldrich (St. Louis, MO, USA).

Animals

Juvenile rainbow darters of the Lake Phalen (St. Paul, MN, USA) genetic strain were provided by MissFish Aquatics (Onalaska, WI, USA). Wild-type adult specimens were allowed to spawn, and the eggs were raised with treated City of Onalaska tap water after being conditioned in cold temperatures and natural light photoperiod. No fungicides or hormones were used in the culture. Juvenile darters (age, 35 d) were transferred under Minnesota Department of Natural Resources Special Permit 11873 to the laboratory in oxygenated plastic bags that were placed in coolers with minimal ice to reduce stress and were maintained under procedures approved by Minnesota

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Table 1. Tank occupancy (sex ratios) for individual tanks and pooled treatments[a]

Tank	Nominal concentration: 100% ethanol (%vol)	Nominal concentration: EE_2 (ng/L)	Nominal concentration: TMX (ng/L)	Sex: Male	Sex: Female	Sex: Total	Pooled treatment	Sex: Male	Sex: Female	Sex: Total
Estrogen exposure experiment										
C1A	—	—	—	2	2	4	Control	4	4	8
C1B	—	—	—	2	2	4				
SCA	0.002	—	—	2	2	4	Control solvent	3	5	8
SCB	0.002	—	—	1	3	4				
E1A	0.002	20	—	4	0	4	20 ng/L of EE_2	5	3	8
E1B	0.002	20	—	1	3	4				
E2A	0.002	200	—	2	2	4	200 ng/L of EE_2	4	4	8
E2B	0.002	200	—	2	2	4				
E3A	0.002	2,000	—	2	2	4	1,000 ng/L of EE_2	5	3	8
E3B	0.002	2,000	—	3	1	4				
			Total	21	19	40		21	19	40
Estrogen and tamoxifen exposure experiment										
CA	—	—	—	5	4	9	Control	9	8	17
CB	—	—	—	4	4	8				
SCA	0.002	—	—	5	4	9	Control solvent	8	9	17
SCB	0.002	—	—	3	5	8				
EA	0.002	200	—	6	3	9	Estrogen	10	7	17
EB	0.002	200	—	4	4	8				
T1A	0.002	200	2	4	5	9	2 ng/L of TMX	6	11	17
T1B	0.002	200	2	2	6	8				
T2A	0.002	200	20	5	4	9	20 ng/L of TMX	9	8	17
T2B	0.002	200	20	4	4	8				
T3A	0.002	200	200	5	4	9	200 ng/L of TMX	9	8	17
T3B	0.002	200	200	4	4	8				
T4A	0.002	200	2,000	2	7	9	2,000 ng/L of TMX	5	12	17
T4B	0.002	200	2,000	3	5	8				
T5A	0.002	200	20,000	7	2	9	20,000 ng/L of TMX	9	8	17
T5B	0.002	200	20,000	2	6	8				
			Total	65	71	136		65	71	136

[a] These data reflect actual tank occupancies despite effort to segregate sexes. Tank assignments were randomly chosen. EE_2 = 17α-ethynylestradiol; TMX = tamoxifen citrate.

State University's Institutional Animal Care and Use Committee (IACUC 02-04). In the laboratory, the darters were transferred to a 208-L glass aquarium with aeration and biological filtration containing approximately 150 L of City of Mankato (MN, USA) tap water dechlorinated with Kordon Novaqua (Navalek, Haywood, CA, USA). The darters were maintained on a 16:8-h light:dark photoperiod. Live brine shrimp (*Artemia* sp.) were cultured for the purpose of feeding the rainbow darters throughout the holding and experimental phases of the present study. A 70-ml solution of densely populated shrimp was fed to the 300 fish/d during the holding period. Water-quality parameters were measured with a standard colorimetric aquarium water testing kit (Aquarium Pharmaceuticals, Chalfont, PA, USA) and reported (mean ± standard deviation) as follows: Temperature, 23.1 ± 1.0°C (as determined with a mercury thermometer); pH 7.9 ± 0.1; hardness, 211.4 ± 7.1 mg/L; alkalinity, 88.9 ± 3.2 mg/L; undetectable levels of ammonia and nitrate, 0.0 ± 0.0 mg/L. Every two weeks, 10% volume water changes were performed.

Study design

The study designs are reported in Table 1. Note that all treatments were performed in duplicate. Fish were transferred into 75.7-L glass aquaria, fitted with filters and aerators, containing 50 L of dechlorinated City of Mankato tap water at 120 d posthatch for the multiple-concentration EE_2 experiment and at 150 d posthatch for the single-concentration EE_2 and multiple-concentration TMX experiment in the numbers indicated in the table. An attempt was made to put equal numbers of males and females into the tanks by characterizing males after 90 d posthatch according to their dark banding on the first dorsal fin, large pectoral and pelvic fins, dark saddles across the dorsum, green side banding (particularly on the caudal peduncle), golden abdomen, and occasionally, a behavior of swimming higher in the water column when approached by the net as well as by employing color photographs of adult specimens [18]. Clearly, as indicated in Table 1, when the fish sexes were confirmed by dissection following the experiment, this method was not successful this early in their development. Fish were fed 3.0 ml of densely populated brine shrimp solution per day. After 1 d of adaptation, nothing (control), 1.0 ml of 100% ethanol alone (0.002% ethanol final concentration or solvent control), or 1.0 ml of the treatment chemical(s) in ethanol was added into the tank by the box filter to promote thorough mixing. Treatments were started after the lights had gone out to prevent photodegradation, despite data suggesting that EE_2 does not break down at wavelengths greater than 365 nm [19]. Exposures were static following addition of the treatments; only Novaqua-treated tap water was added every 3 d as needed to restore the volumes back to the original 50 L. Daily ambient air temperature was recorded (only initial water temperature was recorded, because water temperature equaled the air temperature), and pH and ammonia concentration were recorded to monitor tank conditions using the

same standard colorimetric aquarium water testing kit mentioned previously. The pH remained fairly constant during the experiments for all treatment groups. Ammonia concentrations did not exceed 1.0 mg/L with the exception of one of the two highest EE_2 tanks, which reached 5.0 mg/L on day 8 of exposure. Three fish died in that tank during the following few days. Dead fish were removed on discovery and placed in labeled vials containing 10% buffered formalin for morphological and histological processing. Nitrite concentrations also were monitored in both experiments by sampling 40 ml of water in U.S. Environmental Protection Agency–certified vials with no headspace and then processed the same day by Hach Program 2630 (Hach Company, Loveland, Colorado, USA) (Nitrite, TNT) at a wavelength of 507 nm. Limit of detection and limit of quantification for nitrite nitrogen were 0.0018 and 0.0060, respectively, employing seven replicates. Nitrite concentrations were less than 1.0 mg/L in the single-concentration EE_2 and multiple-concentration TMX experiment except for one of the two tanks treated only with EE_2 by day 21 of exposure and all tanks containing EE_2 by day 7 of exposure in the multiple-dose EE_2 experiment.

The EE_2 experimental design was achieved by a 21-d exposure of four fish in duplicate control and solvent control tanks and three estrogen treatments created by spiking pairs of tanks with ethanol solutions containing 1, 10, and 50 μg/ml of EE_2, producing nominal concentrations of 20, 200, and 1,000 ng/L, respectively (design based on that described by Zillioux et al. [20]). The exposure protocol was the same for the subsequent experiment with EE_2 and TMX except that nine fish were in the presumed-to-be-male tanks and eight in the duplicate-concentration female tank. Following 1 d of acclimation, five different concentrations of TMX (nominal concentrations, 2.0, 20, 200, 2,000, and 20,000 ng/L) were given to duplicate tanks in a mixture with EE_2 (nominal concentration, 200 ng/L). The EE_2 control tanks received 1.0 ml of 100% ethanol containing 10 μg/ml of EE_2, and solvent control tanks received 1.0 ml of 100% ethanol (0.002% ethanol by volume). The control tanks received nothing. On day 21 of the exposure, the experiment was terminated. At the end of either experiment, specimens were removed from the treatment tanks and anesthetized to death in a dish with 100 mg/L of MS-222 (Finquel; Argent Chemical Laboratories, Redmond, WA, USA). They were gently blotted with Kimwipes EX-L (Kimberly-Clark, Irving, TX, USA) to remove excess moisture and weighed on a calibrated Mettler PC440 (Mettler-Toledo, Columbus, Ohio, USA) analytical balance to the nearest milligram. Their total length and standard length were measured with a dial caliper (Sears Craftsman, Chicago, IL, USA) to the nearest 0.01 mm. Excluding fish that died during the experiments, a strong positive correlation was found between weight (273 ± 53 mg, $n = 152$) and standard length (27.1 ± 1.7 mm, $n = 152$), with no statistical difference between males and females or treatment (standard length [mm] = 18.8 + 0.03·weight [mm], $r^2 = 0.88$). All specimens were then placed in labeled, plastic vials containing enough 10% buffered formalin to adequately cover them.

Histology

After 7 d in fixative, all specimens (control and treatments) were transferred to a 0.1 M phosphate buffer (pH 7.5) for morphological and histological analyses. The specimens were removed from their vials, blotted with Kimwipes, and examined under a dissecting microscope at a total magnification of ×7 to ×30 to evaluate and describe characteristics of the urogenital opening. Additionally, the left pectoral and pelvic fin lengths were manually measured under the microscope using a dial caliper to the nearest 0.01 mm. The urogenital openings were digitally photographed from ventral and lateral positions using SPOT Advance software (Ver 3.5.2 for Windows; Diagnostic Instruments, Sterling Heights, MI, USA) and SPOT digital camera (model 2.3.1) connected to an Olympus BX40 microscope (Leeds Precision Instruments, N. Minneapolis, MN, USA) with a syringe for image-scale calibration for future use. Specimens were promptly returned to the buffer solution. A portion of the specimens were subsequently prepared for paraffin whole-mount embedding (for the estrogen experiment, five fish were used for whole wet mount of individual gonads [all necropsies], and 35 were fixed as whole fish and sectioned for a total of 40 fish; for the TMX experiment, 120 gonads were whole wet mounted [one necropsy], and 16 whole fish, representing one from each treatment, were fixed and sectioned, for a total of 136 fish). Specimens were removed from the buffer and placed in individual vials containing a decalcifying solution (0.88 M acetic acid) for 4 d before being returned to buffer. Three days later, the whole specimens began the process of paraffin (Kendall Paraplast, Harrisburg, PA, USA) embedding using Brown's manual, routine processing protocol (as cited in Hinton [21]), and the resultant blocks were stored in a freezer. Serial sections (thickness, 10 μm), made every 1 to 2 mm, were mounted on glass slides, labeled, and stained with hematoxylin and eosin. Sections were examined microscopically and digitally photographed as before.

The remaining specimens (five fish from the multiple-concentration EE_2 experiment and 120 from the single-concentration EE_2 and multiple-concentration TMX experiment) were removed from the phosphate buffer, and their fins and urogenital characteristics were measured and evaluated under a dissecting microscope. The urogenital openings also were digitally photographed. They were then dissected for the purpose of isolating whole gills, livers, and gonads. The gonads were transferred to separate, labeled, individual glass vials containing phosphate buffer for future staining, gross anatomical evaluation, weighing, and photography. Whole wet mounts of the individual gonads were made by removing them from vials and placing them on slides containing one drop of buffer. A small drop of Wright stain (Biochemical Sciences, Bridgeport, NJ, USA) was added to the slide and allowed to stain the gonad for 30 s. Paraffin-embedded liver sections were stained with hematoxylin and eosin as noted earlier. Repeated rinsing steps using buffer solution removed excess stain before the cover slip was added. The slide was then examined and digitally photographed at a total magnification of ×40 for the creation of a whole-specimen montage image using Adobe Photoshop (Ver 6.0 for Windows; Adobe, San Jose, CA, USA) to capture the overall gonad appearance and for area analysis of tissue. Montage images of liver and gonadal sections were analyzed using the National Institutes of Health Image J software (Java 1.3.1_03; Ver 1.32j; Bethesda, MD, USA). Stained gonad sections were examined microscopically and photographed digitally at a total magnification of either ×40, ×100, or ×400. A calibrated scale was added to each image using a slide micrometer. Specimen slides were examined for the presence of ovotestes, fibrosis, and overall appearance of gonad tissue in males, whereas female slides were examined for the presence of secondary oocytes. General appearance of the kidney and surrounding tissue also was noted. Liver sections were

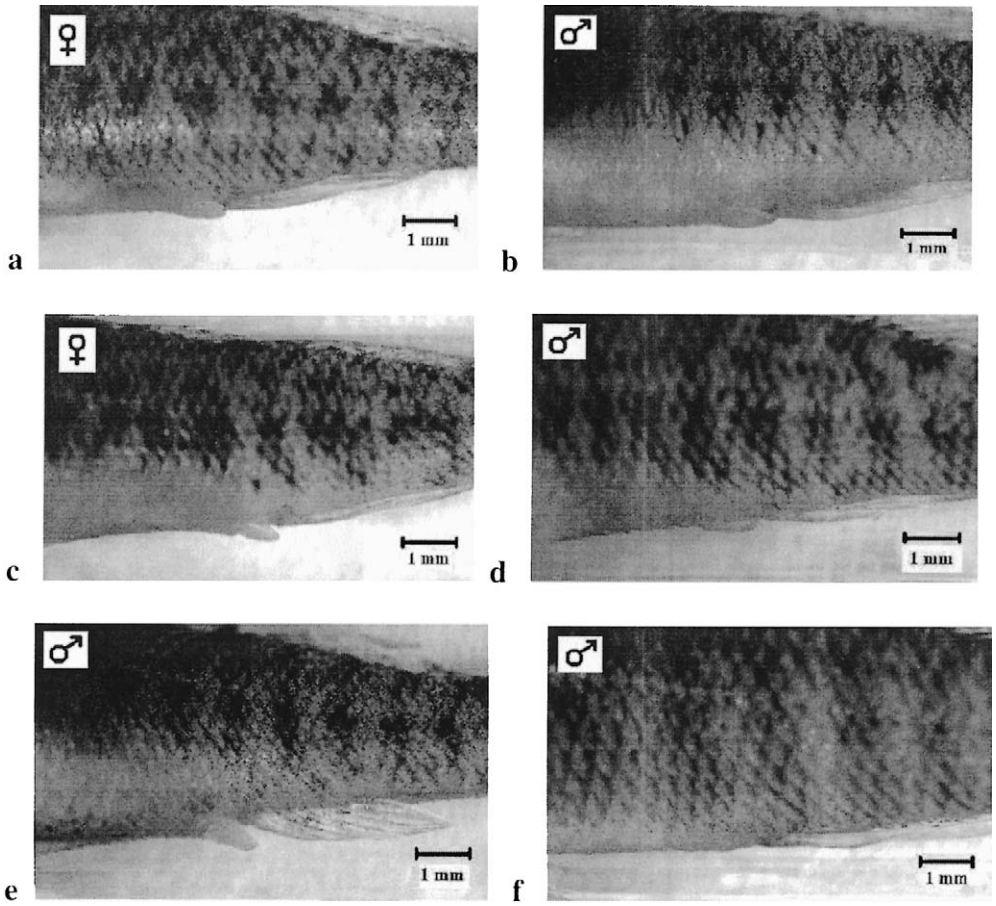


Fig. 1. Photos of rainbow darter (*Etheostoma caeruleum*) urogenital papillae from the estrogen experiment. Modified urogenital characteristics typically found in adult female rainbow darters were observed in juvenile (**a**) females and (**b**) males exposed to 200 ng/L of 17α-ethynylestradiol (EE_2) and in (**c**) females and (**d**) males exposed to 1,000 ng/L of EE_2. Exaggeration of this characteristic was evident in (**e**) one male specimen that died from exposure to 1,000 ng/L of EE_2. This characteristic was not observed in females or (**f**) males in either of the controls or in the 20 ng/L of EE_2 exposure.

examined for signs of fatty areas, inflammatory responses, or infiltration of lymphocytes, fibrotic bands, necrosis, and biliary alterations (determined from descriptions and images provided by Pathologic Images, Cornell University Medical College, New York, NY, USA). Kidneys were examined only for whole-fish sections and were not subjected to the same intensive analyses as the liver.

Preserved gonads eventually were weighed for the purpose of calculating a gonadosomatic index (GSI) for each specimen [22]. The gonads were removed from the buffer solution, carefully blotted using Kimwipes, placed on weigh paper in a calibrated Mettler MX5 analytical balance, and weighed to the nearest 1.0 μg. Gonads were then returned to their vials containing buffer for long-term storage. The GSI was calculated as follows: (preserved gonad wt/blotted wet wt)·100.

Statistical analyses

All statistical analyses were made using Statistical Package for the Social Sciences for Windows, standard version (Release 10.0.5; Chicago, IL, USA) and SYSTAT for Windows (Ver 9; Evanston, IL, USA). Statistical significance was accepted for any parameter for which $p \leq 0.05$ unless noted otherwise. Preplanned comparisons involved the effects of increasing concentrations of EE_2 on standard morphological measures, such as standard length, total length, pectoral fin length, and GSI. Histological comparisons for size of ovaries and testes, the frequency of ovotestes in male gonads, and the general morphology of the gonads (presence of fibrosis or fatty inclusions) were considered. Increases in tank water nitrogen and ammonia were compared not only as a function of water quality but also to determine whether excess protein synthesis in the presence of EE_2 and subsequent catabolism might result in higher excretion of nitrogenous waste products. When TMX was added in the subsequent experiment, the parameters that increased with EE_2 were expected to be reversed by TMX. Analysis of variance (ANOVA) was used for most comparisons, with a least-significant-difference post hoc test performed for individual differences only in the case of $p \leq 0.05$.

RESULTS

Estrogen exposure

The effects of the synthetic estrogen EE_2 on secondary sexual characteristics of the male darter were the most profound morphological change. A χ^2 analysis revealed a statistically significant relationship ($\chi^2 = 21.0$, $df = 4$, $p < 0.001$) between pooled treatments and papillae for males. All males (four at 200 ng/L and five at 1,000 ng/L) developed modified papillae, a secondary sexual characteristic of females, at the two highest concentrations of EE_2 (Fig. 1). In at least one specimen, the papilla was extremely large (Fig. 1e). None of the males in the control, solvent control, or lowest concentra-

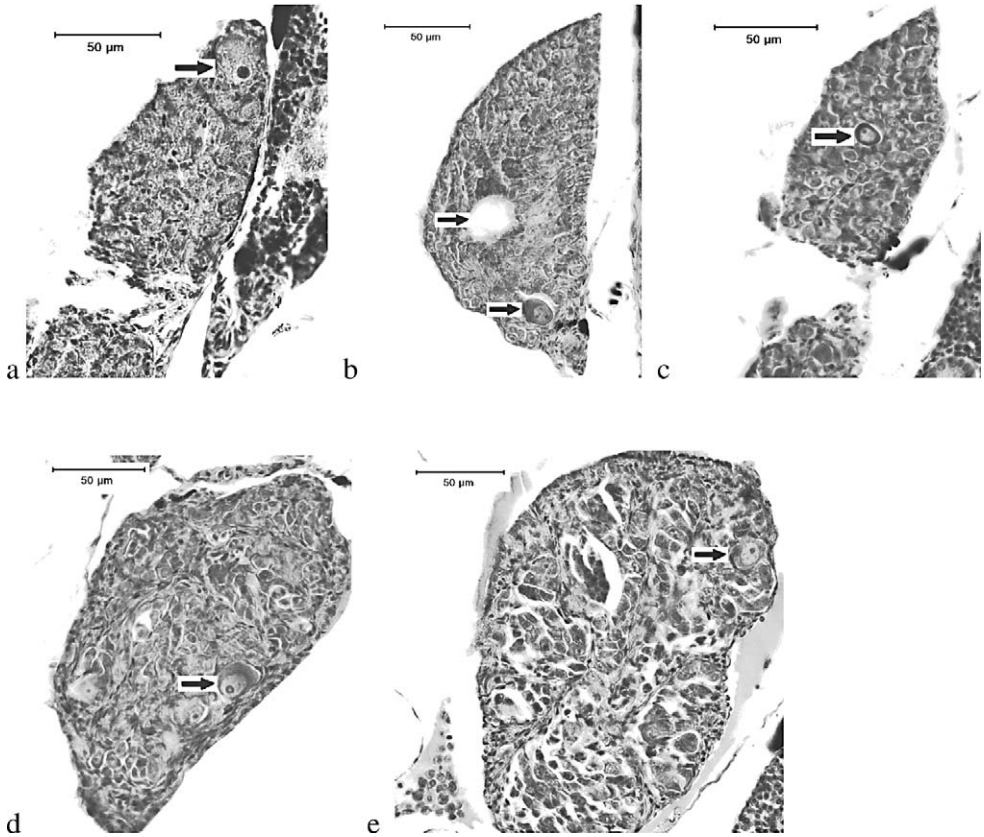


Fig. 2. Appearance of testes and ovotestes condition in juvenile male rainbow darters (*Etheostoma caeruleum*). Ovotestes were observed in all treatment groups to various degrees: (**a**) Control, (**b**) solvent controls (the hole indicates the position of a large ova before sectioning), (**c**) 20 ng/L of 17α-ethynylestradiol (EE_2), (**d**) 200 ng/L of EE_2, and (**e**) 1,000 ng/L of EE_2 (note the presence of fatty deposits surrounding testes and fibrotic appearance). (→) = ova present in testis. Serial sections were 10 µm in thickness.

tion of EE_2 developed papillae; this suggests a concentration-dependent effect. This relationship also was evident in females, but to a slightly lesser degree ($\chi^2 = 15.5$, $df = 4$, $p < 0.01$); however, this may indicate the precocious development of the innate female character rather than abnormal development. Based on these data, the effect threshold lies somewhere between 20 and 200 ng/L of EE_2. Pectoral fin length and pelvic fin length showed no statistical differences between the sexes or pooled treatment. These data are unexpected, because fin size usually is larger in adult males. Five mortalities occurred during the estrogen experiment, all in the highest EE_2 concentration (1,000 ng/L; one female on day 6 of exposure and four males on days 11, 12, 19, and 21 of the exposure). Because autolysis may have affected values, measures of morphometric characteristics for these individuals were not included in related analyses; however, morphological parameters were evaluated.

General histological observations of the serial-sectioned gonads (and whole wet mount gonads from the four mortalities) were made for each individual to assess overall appearance and development (Fig. 2). Control males had small to medium-sized, well-developed testes with no signs of fibrosis. The tissue was dense and well organized in lobules containing some cells in different stages of development (Fig. 2a and b). The testes in solvent control males were similar, with good cellular differentiation, but they appeared to be slightly larger. The EE_2-treated males tended to have a concentration-dependent increase in testes size, lobule disorganization, and overall fibrotic appearance (Fig. 2c–e). At the two highest concentrations of EE_2, fatty material accumulated on the surface of the gonads. Ovotestes condition, as defined by at least one clearly distinguished ova being present in the testis, was found in 11 of 21 males. Each of the pooled treatments had at least one male exhibiting ovotestes (control, two males; solvent control, one male; 20 ng/L of EE_2, three males; 200 ng/L of EE_2, three males; 1,000 ng/L of EE_2, two males). Most specimens had between one and five small, clearly visible oocytes. A χ^2 analysis revealed no significant relationship between pooled treatment and ovotestes condition ($\chi^2 = 1.7$, $df = 4$, $p > 0.05$). No cases of significant ovotestes condition occurred for any treatment. Gonad squashes of whole wet mount preparations might have revealed only slightly higher numbers of oocytes more deeply buried in the lumen, but such preparations were not warranted based on the extremely low presence in any ovotestes male.

Control and solvent control females had medium to large-sized ovaries with primary oocytes of various sizes (Fig. 3). The lowest EE_2 concentration resulted in fairly large ovaries, also with oocytes of various sizes (Fig. 3b). The two highest concentrations showed increased ovary size, slightly increased oocyte size, and the presence of fatty material. This fatty material also coated a large portion of the surface of the ovaries. No overt secondary oocytes were observed in the ovaries of any female specimen for any pooled treatment. The thecal cells between oocytes became more pronounced and increased in abundance at the two highest concentrations (Fig. 3c and d).

Vitellogenic, secondary oocytes were not observed in any female; consequently, other organs, such as the kidney and

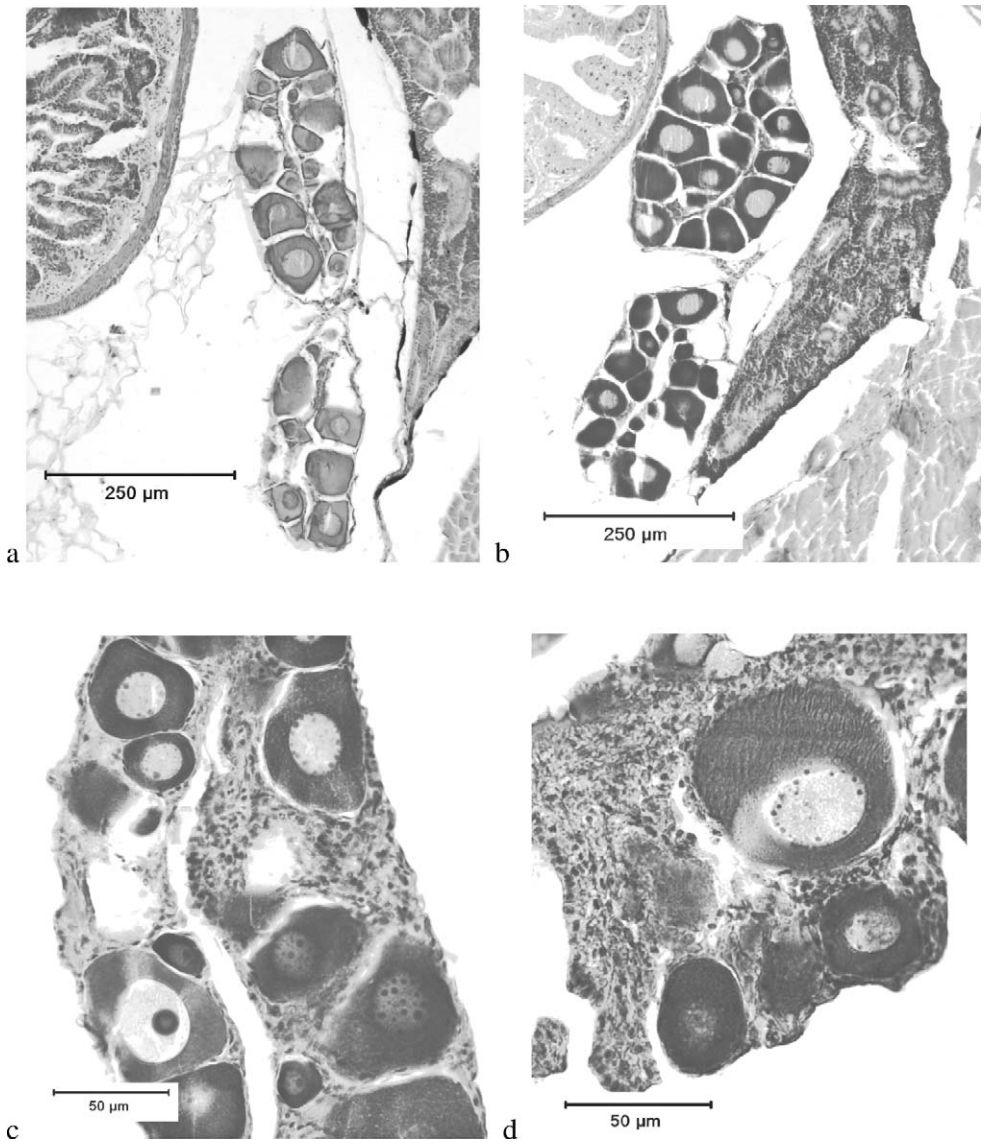


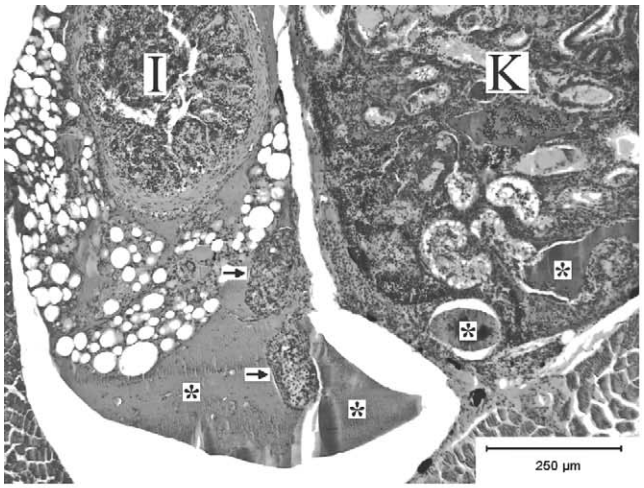
Fig. 3. Ovarian condition in juvenile female rainbow (*Etheostoma caeruleum*): (**a**) Control, (**b**) 20 ng/L of 17α-ethynylestradiol (EE_2), (**c**) 200 ng/L of EE_2 (note the thecal cell proliferation), and (**d**) 1,000 ng/L of EE_2 (abundant thecal cells present). Serial sections were 10 µm in thickness.

liver, were examined for the buildup of lipid/lipoprotein. In some cases, this material completely encased the gonad and filled a large portion of the abdominal cavity (Fig. 4). Histopathology was observed in a concentration-dependent manner for EE_2 in both males and females. Increased vacuolization of fatty material (clear, circular areas in sections) and hypertrophy occurred in the kidney with increasing EE_2 concentration (Fig. 4b). At the highest concentration, the kidney took up a large portion of the abdomen. The intestines sometimes were encased in what appeared to be lipoprotein and, in some cases, had swollen villi. Liver hypertrophy also occurred at higher concentrations; the mortalities all had bloated abdomens because of large, fatty livers (Fig. 4c). The livers of all specimens were examined and exhibited a concentration-dependent relationship between EE_2 and pathologies, vacuolization of fatty material, and overall increased size. Note in Figure 5 that the acinar integrity of the liver is maintained through the 20 ng/L of EE_2 concentration. Fatty deposits are apparent in liver sections of darters exposed to 200 ng/L of EE_2. The livers of darters exposed to 1,000 ng/L of EE_2 show a high disruption of acinar integrity and many large fatty deposits.

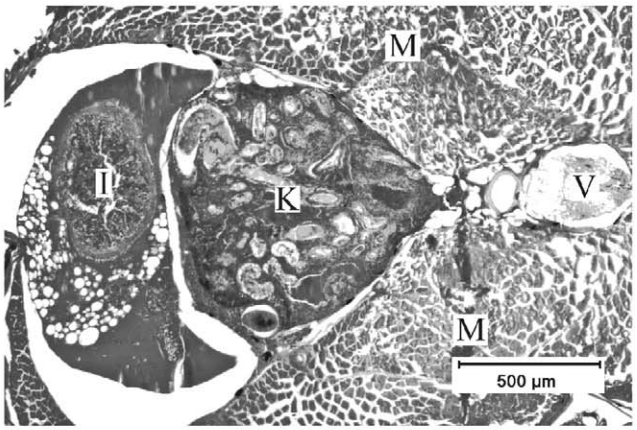
Estrogen and TMX exposure

Based on all the results of the estrogen experiment, 200 ng/L of EE_2 was determined to be the appropriate high-level estrogen concentration in the TMX experiment. This concentration caused gross changes in observed gonad structure and appearance, along with the presence of lipoprotein-like material without resulting in death. This high EE_2 concentration adequately illustrated the disruptive effects of estrogen, which was crucial to evaluating the potential protective effect of TMX. Only one fish died during the EE_2-TMX coexposures. The dead female occurred at the highest concentration of TMX (20,000 ng/L) given concurrently with EE_2 (200 ng/L) on day 11 of the exposure. It is unknown if the specimen succumbed to direct TMX toxicity or to histopathologies related to the TMX and EE_2 mixture. The morphological data for this individual were included in subsequent related analyses, but the morphometric data were omitted because of potential changes caused by autolysis.

None of the treatments appeared to affect general growth. Overall, GSI in males and females was found to be 0.049 ±



a



b

c

Fig. 4. Abdominal cavity and kidney and liver histopathology of juvenile male rainbow darter (*Etheostoma caeruleum*) exposed to 1,000 ng/L of 17α-ethynylestradiol (EE_2). **(a)** Fatty material, likely lipoprotein, can be seen encasing the entire gonads. Note the presence of pockets of similar material in the kidney and partially surrounding the intestine. White vacuoles likely are pockets of fat that were subsequently dissolved during embedding. **(b)** Hypertrophied kidney in male with vacuoles of fatty material and enlarged glomeruli. **(c)** Distended male abdomen, ventral view, illustrating liver size (notice modified urogenital opening). Serial section was 10 µm in thickness. I = intestine; K = kidney; (→) = testis; (*) = fatty material; M = muscle; V = vertebra.

0.048 and 0.391 ± 0.063, respectively. The considerable difference between males and females was expected. Female propagules are significantly larger than male propagules and take up a significant portion of the abdomen in spawning individuals. Because of the gonad size difference between sexes, females usually have higher gonad weight per body mass and, thus, a higher GSI. The highest GSIs in both males and females came from fish that were fairly average in size for their treatment group. The wide standard deviation found in males likely resulted from a few individuals in the highest TMX concentration that had exceedingly high GSIs.

Two-way ANOVAs were performed to determine if significant differences existed between pooled treatments and/or sex based on the parameter in question. No statistical differences were found for blotted wet weight, standard length, and total length. Differences between male and female pelvic fin length were not statistically significant ($p = 0.089$), nor were differences between pooled treatments for pectoral fin length ($p = 0.092$). The GSI was not significantly different for any pooled treatment, but the GSI did show a higher mean value for males at the highest TMX concentration. Four of the seven highest GSIs occurred in the same 20,000 ng/L of TMX exposure.

Urogenital characteristics were evaluated from digital photographs for the presence of a female-related papilla. The χ^2 analysis for the presence of papillae in males based on pooled treatment was not statistically significant; however, papillae were only found in individuals exposed to either EE_2 or to mixtures of EE_2 and TMX in which the TMX concentration was less than or equal to the EE_2 concentration. Females exhibited a statistically significant relationship between presence of papillae and pooled treatment ($\chi^2 = 19.2$, $df = 7$, $p < 0.01$). In similar fashion as in the males, the presence of papillae was restricted to those exposed to EE_2 or to mixtures of EE_2 and TMX in which the TMX concentration was equal to or less than the EE_2 concentration. Taken together, these data indicate that TMX may be play a role in preventing the effects of EE_2 on papilla formation.

The papillae in both sexes were less prominent in shape and size than those in the multiple-concentration EE_2 experiment, in which they were sometimes grossly enlarged. Microscopic histological observations of 56 whole wet mount and nine sectioned male gonads were made to evaluate tissue appearance and to identify the presence/absence of ovotestes. Montages of whole gonads provided the best method for histological evaluation before sectioning. Control and solvent control males had dense testes with good lobule formation and some cellular developmental differentiation. Primary spermatocytes were found in clusters mixed with primary spermatogonia; this would be expected in juveniles that have yet to spawn for the first time. Estrogen-exposed males had only fair lobule formation, with little developmental differentiation of primary spermatogonia. With regulation of testicular development under partial control by estrogen, it was not surprising to see germ cells arrested in their development as predominantly primary spermatogonia. These specimens also seemed to have some fibrosis and general cellular disorganization as compared to controls. The mixtures of EE_2 and TMX did not show a clear concentration-dependent effect for TMX. Fibrosis occurred to some degree in most specimens, and lobule formation was variable. At equal concentrations of EE_2 and TMX, some of the specimens had mushy-looking testes. The highest concentration of TMX showed the most devel-

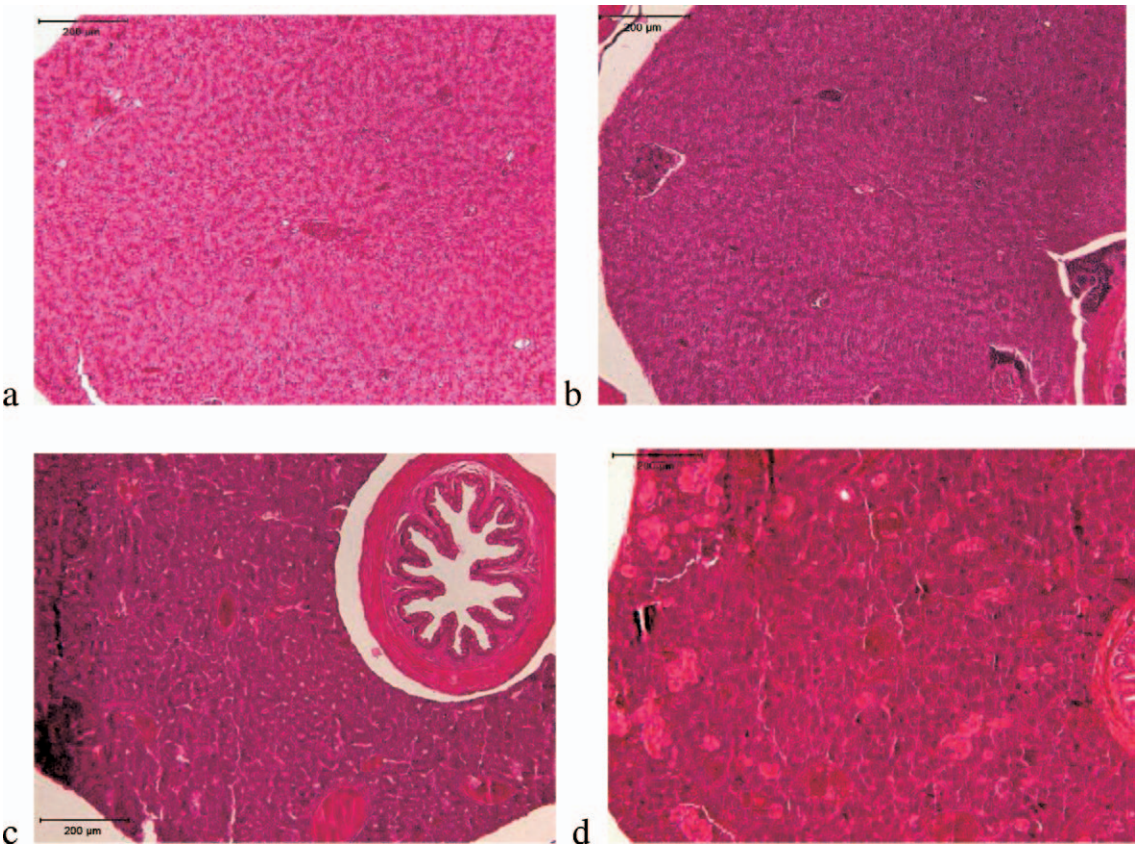


Fig. 5. Appearance of juvenile male rainbow darter liver sections: (**a**) Control, (**b**) 20 ng/L of 17α-ethynylestradiol (EE_2), (**c**) 200 ng/L of EE_2 (note the appearance of fatty deposits), and (**d**) 1,000 ng/L of EE_2 (note the prevalence of fatty deposits and disruption of acinar organization). Serial sections were 10 μm in thickness.

opmental differentiation of all pooled treatments (which also coincided with the highest GSI). These specimens contained large testes with very densely packed primary spermatogonia, primary and secondary spermatocytes, and what appeared to be an abundance of spermatids. Analyses of gonad montage images revealed no relationship between pooled treatment and gonad area, which was not surprising considering that differences in gonad size likely were related to corresponding differences in body size. This is why GSI usually is a better measure of fecundity.

Ovotestes occurred in 31 of the 65 males examined. Ovotestes were found in more than one individual in every control and pooled treatment. No males with ovotestes condition contained even moderate numbers of primary oocytes. No secondary oocytes were observed in any specimen. The χ^2 analysis did not reveal a statistically significant relationship between ovotestes and pooled treatment ($\chi^2 = 2.968$, $df = 7$, $p > 0.05$). One individual exposed to 2,000 ng/L of TMX and 200 ng/L of EE_2 had the most widespread occurrence of ova in any ovotestes male, with an estimated count of more than 20 easily identified primary oocytes. The ova were found scattered throughout the testis, with some localized pockets in which a few were found in close proximity to each other. They were approximately the same size as the smallest primary oocytes found in female ovaries; otherwise, the primary oocytes found in all other male specimens were much smaller than the smallest primary oocytes in females.

Sixty-four whole wet mount and seven serial-sectioned ovaries were examined microscopically for general gonad appearance, size of largest primary oocytes, and the presence of secondary oocytes (or spermatocytes) related to pooled treatments. Most ovaries in each exposure group were well developed and contained primary oocytes of various sizes. The maximum size range was between 544 and 635 μm ($n = 49$). Atretic cells were occasionally found in specimens from all pooled treatments. No overt relationship existed between general appearance and pooled treatments. Two-dimensional ovarian area analysis of montage ovary images also did not reveal any statistically significant differences based on pooled treatment. Secondary, vitellogenic oocytes were present in 18 of the 71 females. At least one female from each pooled treatment contained one or more secondary oocytes. A χ^2 analysis revealed no significant relationship between their presence and the pooled treatments ($\chi^2 = 2.825$, $df = 7$, $p > 0.05$). Digital photographs of secondary oocytes showed different stages in the process of vitellogenin uptake. Circumferences of these oocytes also were measured wherever possible to estimate their average size. The minimum, maximum, and average circumference of secondary oocytes ($n = 60$) was 563, 916, and 720 ± 84 μm, respectively. No overt signs of spermatogonia were present in any female. An unusual observation of multiple germinal vesicles in single primary oocytes was made during microscopic evaluation of ovaries. This intriguing condition occurred in eight specimens (control, three specimens; EE_2, one specimen; 2.0 ng/L of TMX, one specimen; 200 ng/L of TMX, two specimens; 2,000 ng/L of TMX, one specimen), in which less than five individual oocytes from a single ovary exhibited the characteristic. One specimen was found to contain an oocyte with four germinal vesicles. One individual also had an unusual cluster of what appeared to be primordial germ

Table 2. Percentage fatty liver, mean individual fat area, and number of fat areas observed in rainbow darters treated with 17α-ethynylestradiol (EE_2) and tamoxifen citrate (TMX)

Treatment	TMX concentration	% Fatty liver[a]	Mean individual fatty area	
			μm^{2b}	No. fatty areas[c]
None	None	0 A	0 A	0 A
0.002% Ethanol (solvent control)	None	0 A	0 A	0 A
200 ng/L of EE_2	2 ng/L	1.9 A	2,758 B	2.8 B
200 ng/L of EE_2	20 ng/L	4.6 B	2,700 B	9.6 B
200 ng/L of EE_2	200 ng/L	1.6 A	1,097 A	3.2 A
200 ng/L of EE_2	2,000 ng/L	0.5 A	239 A	1.2 A
200 ng/L of EE_2	20,000 ng/L	0 A	0 A	0 A

[a] Means represent 17 samples examined (mean area examined ± standard error of the mean [SEM], 9,794,415 ± 85,094 μm^2; those with different uppercase letters are significantly different at $p < 0.05$ (least-significant-difference [LSD] post hoc test following a significant analysis of variance [ANOVA] of $p < 0.001$; pooled SEM, 0.6%).

[b] Means represent 17 samples examined; those with different uppercase letters are significantly different at $p < 0.05$ (LSD post hoc test following a significant ANOVA of $p = 0.019$; pooled SEM, 508 μm^2).

[c] Means represent 17 samples examined; those with different uppercase letters are significantly different at $p < 0.05$ (LSD post hoc test following a significant ANOVA of $p < 0.001$; pooled SEM, 0.92 fatty areas).

cells sandwiched between primary oocytes. This was the only individual with such tissue, and the cluster was extremely small. Therefore, the treatment was not likely to be a causal agent. Overall, it is apparent that female reproductive end points and functional ability seemed to be largely unaffected by the treatments.

The liver data showed a clear protective effect of TMX on the EE_2-induced accumulation of fat in the liver. As seen in Table 2, 200 ng/L of EE_2 caused fat accumulation in the liver to approximately 4%. Although the lowest concentration of TMX (2.0 ng/L) appeared to reduce the percentage of fat in the liver, no statistically significant decrease in the mean fat area was found until the TMX concentration was equal to the EE_2 concentration (200 ng/L). No signs of fat accumulation were observed at the highest concentration of TMX (20,000 ng/L).

DISCUSSION

The primary purpose of these two laboratory exposure experiments was to assess the sensitivity of rainbow darters as a potential bioindicator species for reproductive endocrine disruption using both EE_2 and TMX. The discovery in all treatment groups of ovotestes, which was not affected by EE_2 or TMX, was unexpected. Several possible explanations for the widespread incidence of ovotestes exist. One explanation is that natural intraspecific variation may include normal, low-level occurrence of ovotestes. The condition may not result in any measurable effect on reproduction, or it may be transient, disappearing after the first spawning season in this short-lived fish. Another explanation is that laboratory temperature was at the high end of the environmental range normally experienced by this species. Ovotestes have been related to elevated temperature during early development in the Japanese medaka [23]. An alternative reason is that inadequate nutrition could have lead to ovotestes, because high mortalities occurred during the acclimation period. Studies of the Japanese medaka also have linked ovotestes condition with periods of prolonged starvation [24].

The possibility of ovotestes occurring before experimental exposure must be considered as well. The City of Onalaska culturing/rearing water and the City of Mankato acclimation/experimental water were not evaluated for estrogenic equivalents. It is entirely possible that these water sources contained low levels of estrogenic substances. Exposure during embryonic or earlier juvenile stages of development may have been responsible for this observation, because subsequent exposure to estrogen did little to induce this phenomenon. It is surprising that the 200 and 1,000 ng/L concentrations in the multiple-concentration EE_2 experiment did not stimulate more pronounced ovotestes. The window of sensitivity for the formation of other primary reproductive characteristics, such as ovarian-like ducts and reduced testicular development, occurred most noticeably in male fathead minnows exposed to 10 ng/L at 20 d or less posthatch [25]. The darters in the EE_2 study were exposed around 120 d posthatch; most primary sexual characteristics already may have been largely determined. Furthermore, the window of sensitivity for the protective effect of TMX had probably closed before the beginning of the exposure (~150 d posthatch). In contrast, the window of sensitivity for the development of urogenital papillae may have still been open, because pooled treatment effects were observed in the EE_2 experiment. Although the specific timing and duration of this window are unknown, it was reasonable to expect the delayed formation of secondary sexual characteristics as postembryonic maturation occurred.

Only high EE_2 levels resulted in urogenital papillae in juvenile males. Likewise, papillae only occurred in females at the high estrogen concentrations. The observation that TMX treatments prevented urogenital papillae in females at concentrations greater than or equal to the EE_2 concentrations should be interpreted with caution, however, because development of papillae would not have been dictated solely by the EE_2 treatment but also through endogenous means. Tamoxifen could have blocked endogenously produced estrogen from binding with the estrogen receptor and prohibiting the natural formation of the papillae. Despite the ability of TMX to prevent urogenital papillae, it did not significantly alter the GSIs (except for a slight stimulatory effect at the highest exposure concentrations in males); EE_2 also did not affect this parameter. This is unusual, because EE_2 reduced the developmental increase in GSI in rainbow trout [26] and reduced GSI in female medaka exposed to EE_2 for two months following hatching [27].

The multiple-concentration EE_2 experiment generated more than 50% mortalities in 21 d at 1,000 ng/L of EE_2 for the juvenile rainbow darter. Similarly, high mortalities were observed at EE_2 concentrations of 800 ng/L or greater for the sheepshead minnow [20]. In the present study, 80% of the

mortalities were male, which is consistent with studies of Japanese medaka [28]. The functional state of the testes is unknown, but the presence of primary spermatogonia and primary spermatocytes indicate that functional males likely existed at all levels of EE_2 exposure. All testes appeared to have functional cells despite the presence/absence of ovotestes and their general morphology; therefore, it superficially appears that males from any treatment group would have the necessary male reproductive structures to carry out spawning. It is unknown whether the presence of a papilla would interfere with this process. The ability of exposed males to successfully establish and defend territories is beyond the scope of the present study.

It can be speculated that the ability of the specimens in the 1,000 ng/L exposures to reproduce successfully would have depended largely on their ability simply to survive. The females had changes in ovarian size, fat surrounding the ovaries and liver, and kidney fatty inclusions as well at the highest concentrations of EE_2. This suggests that vitellogenesis proceeded at a rate that grossly exceeded the female's natural pathways for sequestering the protein in developing secondary, vitellogenic oocytes. The stimulatory effect of the synthetic estrogen may be responsible for the proliferation of the thecal and granulosa cells, because they typically are responsible for steroidogenesis in fish and, possibly, would increase in abundance to help initiate and regulate associated pathways. Because the oocytes are asynchronous in their development, measuring small areas of the gonads would not yield appropriate data for analysis. Additionally, it was not feasible to measure all the oocytes for all the specimens. Therefore, circumferences of the largest oocytes were measured digitally from individuals for each pooled treatment to estimate the relative maximum size.

Liver and kidney toxicity and excess ammonia/nitrate most likely were responsible for the demise of fish exposed to the highest concentration of EE_2, as identified by swollen abdomens containing hypertrophied organs with microscopic histopathologies and high levels of waste nitrogen products in the tanks. Swollen abdomens also were observed in juvenile fathead minnows [29] and before death in Japanese medaka exposed to EE_2 [30]. It appeared that individuals who were able to survive the organ toxicity continued to grow in a fashion similar to that of unexposed individuals. Both experiments showed strong positive correlations for blotted wet weight and standard length despite the treatment concentrations for both sexes. Tamoxifen's protection against EE_2-induced lipid accumulation in fish liver is a novel portion in the present study, although a number of other studies have examined TMX as an antagonist of estrogen's induction of vitellogenin in this organ [31]. This protective action of TMX is opposite to findings of TMX-induced nonalcoholic hepatic steatohepatitis in the rat [32]. The presence of EE_2 in the present study may highlight the protective action of TMX and diminish its toxic influences. The organ pathologies, observed mortalities, and elevated tank nitrite (and ammonia) levels of EE_2-exposed rainbow darters and the reversal by TMX are of interest, but these results are preliminary in nature. Low levels of estrogenic compounds often are associated with million-fold increases in vitellogenesis [26]. Excess protein catabolism may result in high levels of nitrite and ammonia (or of other nitrogenous waste products) in the fish, which may increase the toxicity of high concentrations of nitrogenous compounds in the aquatic environment.

The presence of TMX in the environment appears to mask many of the impacts of environmental estrogens in the rainbow darter. Use of the rainbow darter showed that morphological changes elicited in males by EE_2, such as the presence of urogenital papilla, were preventable by TMX at concentrations approaching those of EE_2. Even toxic responses to EE_2 (i.e., fatty infiltration of organs) were prevented by the presence of TMX. Some impacts of estrogens, including the fibrotic appearance of the male testes, appear to be unaltered by the presence of TMX. The present study also was concerned with the development and prevention of ovotestes in male darters. It appears that the development of ovotestes in male darters may require estrogen exposure at a different stage of development, and ovotestes may be present at low frequencies in this species either innately or because of various stressors present before EE_2 or TMX exposure.

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