

THESIS

EXPOSURE TO 17β -ESTRADIOL ALTERS REPRODUCTION OF THE ADULT
RED SHINER (*CYPRINELLA LUTRENSIS*)

Submitted by

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WE HEREBY RECOMMEND THAT THE THESIS PREPARED UNDER OUR SUPERVISION BY MICHELLE M. MCGREE ENTITLED EXPOSURE TO 17 β -ESTRADIOL ALTERS REPRODUCTION OF THE ADULT RED SHINER (*CYPRINELLA LUTRENSIS*) BE ACCEPTED AS FULFILLING IN PART REQUIREMENTS FOR THE DEGREE OF MASTER OF SCIENCE.

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ABSTRACT OF THESIS

EXPOSURE TO 17 β -ESTRADIOL ALTERS REPRODUCTION OF THE ADULT RED SHINER (*CYPRINELLA LUTRENSIS*)

Endocrine disrupting compounds (EDCs) are prevalent in aquatic ecosystems worldwide and can lead to developmental and reproductive problems in fishes. Concern exists regarding how exposure to EDCs may be contributing to declines in Great Plains fishes in eastern Colorado. We conducted a study using the red shiner as a model organism to examine how estrogenic EDCs might adversely affect Plains fish populations. Male red shiners were exposed to 17 β -estradiol (estradiol), a natural estrogen found in wastewater effluent. Our objectives were to characterize the effects of estradiol exposure on morphometric and behavioral reproductive traits of males, to investigate changes in female mate choice, and to determine whether estradiol exposure reduces reproductive success. We also measured whether reproductive responses could be reversed when exposures were discontinued. Adult males were exposed to nominal concentrations of 120 ngL⁻¹ estradiol, 2.4 ngL⁻¹ estradiol, a solvent control, and a water control for at least one month. Exposures to the highest estradiol concentration resulted in alterations in plasma vitellogenin concentrations, changes in gonadal tissues, and inhibition of mating coloration and tubercles. Furthermore, mating behaviors were altered and reproductive success was reduced; exposed males fertilized fewer eggs and produced no viable progeny. All reproductive endpoints improved when males were

removed from the estradiol treatment and allowed to mate in control water. Estradiol had significant adverse effects on adult male red shiners, indicating that wild populations may have low reproductive success if they are chronically exposed to estrogenic compounds in the field.

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CHAPTER 1: EXPOSURE TO 17 β -ESTRADIOL ALTERS MATING BEHAVIORS AND
REPRODUCTIVE SUCCESS OF THE ADULT RED SHINER (*CYPRINELLA*
LUTRENSIS)

ABSTRACT

Endocrine disrupting compounds (EDCs) in aquatic ecosystems can lead to developmental and reproductive disruption in fishes. Exposure to EDCs from urban and agricultural land-use may be contributing to declines in Great Plains fishes in eastern Colorado. We conducted laboratory studies using the red shiner (*Cyprinella lutrensis*) as a model organism and the estrogen 17 β -estradiol (estradiol) to examine how estrogenic compounds might affect reproductive success of plains fishes. Our objectives were to characterize the effects of estradiol on gonadal histology and secondary sexual characteristics of males, and to determine whether estradiol exposure reduced reproductive success when exposed males mated with unexposed females. We also measured the reversibility of adverse reproductive effects when estradiol exposures were discontinued. Adult males were exposed to nominal concentrations of 120 ngL⁻¹ estradiol, a solvent control, and a water control for at least 84 d. Exposures to estradiol resulted in elevated plasma vitellogenin concentrations, changes in gonadal tissues, and inhibition of mating coloration and tubercles. Furthermore, male mating behaviors were altered and reproductive success was reduced: males fertilized fewer eggs than control males (mean 4% vs. 86% and 88%, for exposed, solvent control, and water control, respectively) and no viable progeny were produced in exposure treatments. Reproductive endpoints improved when males were removed from estradiol treatment and were allowed to mate in control water. Exposure to estradiol had significant adverse effects on red shiners, indicating that wild populations may face developmental and reproductive difficulties if they are chronically exposed to estrogenic compounds in the field.

INTRODUCTION

Contaminants known as endocrine disrupting compounds (EDCs) are an emerging water quality problem worldwide because they interfere with developmental and reproductive processes in fishes (Jobling et al. 1998; Kime 1998; Jobling and Tyler 2003; Bjerregaard et al. 2006; Cheek 2006). The effects of estrogenic EDCs on male fishes are well known and include production of vitellogenin in males, male gonadal abnormalities (including development of oocytes), alterations in hormone concentrations, reduction in sperm quality and quantity, the loss of secondary sexual characteristics, and reduction in courtship behaviors (Bjerselius et al. 2001; Bringolf et al. 2004; Pawlowski et al. 2004; Doyle and Lim 2005; Mills and Chichester 2005). Cellular and physiological changes can cause reductions in reproductive success via lower egg production, reduced egg viability, or poor hatching success (Arcand-Hoy and Benson 1998; Shioda and Wakabayashi 2000; Sohoni et al. 2001; Zillioux et al. 2001; Mills and Chichester 2005; Sumpter 2005; Brown et al. 2007). A recent study demonstrated that experimentally-controlled exposures to estrogenic compounds resulted in a collapse of wild fathead minnow (*Pimephales promelas*) populations (Kidd et al. 2007).

Great Plains fishes in rivers of eastern Colorado are exposed to a suite of contaminants associated with heavy urban and agricultural land-use, some of which are known to cause endocrine disruption. Potential sources of EDCs include wastewater treatment plants, livestock feedlots, and agricultural fields (Jobling et al. 1998; Soto et al. 2004), all of which are prevalent in Colorado river basins containing native plains fishes. For example, pharmaceuticals, hormones and other organic contaminants, many known or suspected to be hormonally active, have been documented below wastewater treatment plants

in the South Platte River (Sprague and Battaglin 2005). Fish collected from the South Platte River have shown signs of intersex gonadal tissues (Woodling et al. 2006). In addition, recent experiments have shown that estrogenicity in the effluent of a wastewater facility near Boulder Creek, a tributary to the South Platte River, resulted in dramatic feminizing effects on male white suckers (*Catostomus commersoni*) and fathead minnows. These studies suggest that estrogenic compounds may be contributing to the declines of plains fish populations, which have been observed in the last two decades in Colorado (Propst 1982; Nesler et al. 1997).

To investigate how estrogenic compounds might adversely affect plains fish populations, we conducted experiments to expose adult, male red shiners (*Cyprinella lutrensis*) to environmentally relevant concentrations of an estrogenic compound (120 ngL^{-1} 17β -estradiol). Red shiners were selected as the model species because they are a member of a common fish family in the Great Plains (Cyprinidae), and also because they have been shown to be stress tolerant and to reproduce readily under laboratory conditions (Gale 1986; Matthews 1986; Matthews et al. 2001). Exposed male red shiners were allowed to mate with unexposed females to determine whether estradiol impacted reproductive success. Specifically, the objectives of our study were to (1) determine whether exposure to 17β -estradiol (estradiol) affected egg deposition, fertilization success, hatching success, male courtship behavior, and male secondary sexual characteristics and (2) determine if removal from estradiol exposure would allow recovery of the same reproductive endpoints.

MATERIALS AND METHODS

Our study consisted of two experiments: a spawning exposure experiment and a reversal experiment. The spawning exposure experiment was comprised of three treatment groups: estradiol (E2, 120 ngL⁻¹ in ethanol), solvent control (SC, 0.00002 % ethanol), and water control (WC). We used the naturally occurring hormone estradiol as a surrogate for estrogenic EDCs for the following reasons: it is a widespread estrogen known to show effects in other fish species (Mills and Chichester 2005); it is the major estrogen of female teleosts that stimulates the production of vitellogenin by the liver of female fish (Arukwe and Goksoyr 2003), it is necessary for reproduction (Nimrod and Benson 1998), and it can be found in and downstream of many wastewater effluents. In the United States, aquatic concentrations of estradiol have been estimated to be less than 200 ngL⁻¹, with a median detectable concentration of 160 ngL⁻¹ (Kolpin et al. 2002). In Boulder Creek, Colorado, concentrations of estradiol have been reported to reach 2.9 ngL⁻¹, with average and maximum estrogenicity equivalents (accounting for additional estrogenic EDCs) of 29 and 50 ngL⁻¹, respectively, in the effluent (Vajda et al. In press).

The experimental setup consisted of a flow-through system of three glass head tanks (20.8-L; one per treatment) that continually supplied water to glass exposure aquaria (37.9 L) during batch exposure and also to glass spawning aquaria (20.8 L) that would eventually house one male and two females during the spawning exposure experiment ($n = 5$ per treatment; Fig. 1.1). Male red shiners were batch exposed to each treatment for 84 d, and were then moved to spawning aquaria where they were allowed to spawn with females during the last three weeks of exposure (Fig 1.2). A suite of reproductive endpoints were measured (described below).

The reversal experiment began after 91 d of male exposure to estradiol (Fig 1.2). This study included a control treatment, where fish from the control aquaria remained in control water (WCWC), and a previously-exposed treatment (E2WC), where fish from the estradiol exposure (120 ngL^{-1}) aquaria were removed and placed into control water. The experimental setup and endpoints measured were identical to the spawning exposure experiment, but instead of continuing exposure, control water was provided to all spawning aquaria beginning on day one of spawning. Reproductive effects and spawning were monitored for four weeks.

Spawning exposure experiment

Organisms

Adult red shiners were collected by seine from the Arkansas River on the Granada State Wildlife Area, CO, USA (N 38.05°, W 102.23°) and transported to the Foothills Fisheries Laboratory at Colorado State University (Fort Collins, CO, USA). Prior to exposure, fish were held for approximately 8.5 months in 340 L polyethylene round tanks with continuous flows of UV-disinfected well and lake water and a light regime corresponding to the natural photoperiod of the Arkansas River. During the holding period, water temperatures were altered to correspond with Arkansas River seasonal changes, ranging between 6.1 °C and 23.3 °C. Fish were fed once daily *ad libitum* with Aquatox commercial flake food (Aquatic Eco-systems, Apopka, FL) (Viant et al. 2006) throughout holding and experimental portions of the study. Fish used in this experiment were maintained according to guidelines established by the Institutional Animal Care and Use Committee of Colorado State University (approval numbers 06-091A-01 and 06-272A-01).

Test chemical and exposure treatments

A concentrated stock solution for the E2 treatment was prepared at the beginning of the study by dissolving the appropriate amount of estradiol (Sigma, St. Louis, MO, USA) in ethanol (EtOH). The concentrated stock solution was used to make secondary water-based stock solutions every other day by adding 400 μL of solution to 4 L of well water in a stainless steel container. This solution was continuously distributed by a variable speed peristaltic pump at a rate of 0.34 L min^{-1} through platinum-cured silicone tubing into a glass head tank (20.8 L) and combined with a continuous flow of water (0.14 L min^{-1}), producing the E2 treatment concentration (120 ngL^{-1}). A solvent control (SC) treatment was made and distributed in the same manner, adding 400 μL 99% EtOH stock solution to 4 L of well water (0.00002 % EtOH). The water control (WC) treatment utilized the same well water. Water from the head tanks of the three treatment groups was continuously distributed to individual exposure aquaria through Teflon capillary tubes, glass funnels, and Teflon-lined tubing, achieving 99.99% replacement in 24 h. Water chemistry remained constant between treatments over the duration of the experiment (nitrate = non-detectable, nitrite = non-detectable, ammonia $< 0.2 \text{ mgL}^{-1}$, pH = 8, alkalinity = 180 mgL^{-1} , and total hardness = 300 mgL^{-1}). Incoming water had a dissolved carbon concentration of 2.84 mgL^{-1} .

The concentrations of estradiol in each treatment were measured immediately before exposure began and every 1.5 weeks during the exposure period. A 60-mL syringe was rinsed with methanol (30 mL) and distilled water (30 mL), and then filled with water from each treatment group head tank (60 mL). The water sample was passed through a tC18 cartridge filter (Waters, Milford, MA) attached to the tip of the syringe. The cartridge was refrigerated until analysis. Water samples were extracted with methanol and analyzed by

enzyme-linked immunosorbent assay (ELISA) (Abraxis, Warminster, PA, USA) according to the manufacturer's protocols.

Exposure conditions of the male red shiner

During the summer holding period, we identified mature males using spawning coloration and held them in a separate holding tank for use in this experiment. After a gradual acclimation to winter conditions (8.5 ± 1.2 °C; 8:16 h L:D) and a holding period of five months, males were weighed and randomly allocated to the E2, SC, and WC treatments. Fish began batch exposure in one 37.9-L glass aquarium per treatment with no spawning substrate. Female fish were maintained in a separate aquarium with control water. Male exposure continued and temperature was increased by 1 °C every other day until reaching 22.5 °C, where it remained constant for the duration of the experiment (22.4 ± 0.42 °C). Photoperiod was increased by 20 minutes daily until reaching 16:8 h (L:D) and thereafter remained constant for the duration of the experiment. This temperature and chemical treatment regime was designed to mimic potential exposure during the late spring and early summer spawning seasons in wild populations.

Spawning trials

After 44 d of batch exposure, when a majority of control males displayed spawning coloration and nuptial tubercles, a random sample of 15 males were weighed and allocated to 20.8 L glass spawning aquaria ($n = 5$ per treatment). Each spawning aquarium contained four spawning stacks placed on four 1.5 x 10 cm glass Petri dishes. Individual spawning stacks were made of four 5.1 x 5.1 cm² sand-colored ceramic tiles (Fig. 1.3). Tiles were

drilled through the center, stacked, and held together by a 0.64 cm diameter 3.8 cm long stainless steel bolt. Tiles were separated by two flat 0.64 cm stainless steel washers, and secured by a 0.64 cm stainless steel nut, creating three horizontal crevices averaging 3 x 47 x 47 mm each (Burkhead and Jelks 2001). After one day of male acclimation, two females were weighed, added to each spawning aquarium, and exposure continued for three weeks after the first sign of spawning (Fig. 1.2). After three weeks of spawning, males were immediately removed from the aquaria (84 d total exposure) and females were removed the following day. After removal from spawning aquaria, fish were anesthetized with tricaine methanesulfonate (50 mgL⁻¹). Fish were measured for total length (TL, mm) and weight (g), and condition factor ($CF = 100 \times [\text{weight (g)} \times [\text{total length}^3 \text{ (cm)}]^{-1}]$) was calculated.

Reproductive endpoints

Reproductive success

Spawning aquaria were monitored at least once daily for egg deposition on the spawning stacks. When eggs were observed, the spawning stack(s) containing eggs were moved to glass beakers containing control water at the experimental temperature. Clean spawning stacks immediately replaced those removed and all aquaria contained at least one spawning stack at all times. Spawning stacks were held in beakers (21.7 ± 1.5 °C) with aeration before processing. Water temperature and presence or absence of fungus on spawning stacks were recorded daily. After 60 - 72 h, spawning stacks were removed from the beakers and transferred into stainless steel containers (2.7 L) for processing. The numbers of eyed and un-eyed eggs were counted and removed from the spawning stack using

a razorblade. The total number of eggs counted was used to determine egg deposition and the number of eyed eggs was used to measure percent fertilization success.

All un-eyed eggs and approximately 10% of the eyed eggs were preserved in vials of 10% neutral buffered formalin (20 mL). The remaining eyed eggs were transferred to glass jars with aerated control water (240 mL; 21.7 ± 1.6 °C). Observations of hatched or dead fish in each jar were recorded at least once daily, and hatched and dead fish were preserved in 10% neutral buffered formalin until all eggs and larvae were removed. Numbers of dead and hatched fish were counted to estimate percent hatching success.

Male behavior

Once visual evidence of spawning was observed, male mating behavior was measured on three occasions, with each measurement at least one week apart. The initial aquarium to be observed was selected randomly, and systematically rotated for each behavioral trial. For each behavioral test, a digital video camera was placed approximately 0.45 m from the spawning aquarium, enclosed in a black curtain. Each aquarium was recorded for six minutes. When videotapes were reviewed, the final five minutes were used to record behavioral data. The frequency of each behavior was measured.

The behavioral categories included initiation and pre-spawning behaviors. Initiation behaviors included follows and bites, while pre-spawning behaviors included dances, courts, and quivers. Behaviors were grouped because initiation behaviors tended to precede pre-spawning behaviors, which are associated with strong sexual drive (Ono and Uematsu 1957) and lead to spawning. Individual behaviors were chosen based on previous observations of red shiner (Gale 1986; M. M. McGree pers. obs.) and other species exhibiting courtship

behaviors (Ono and Uematsu 1957; Bjerselius et al. 2001; Oshima et al. 2003; Moretz and Rogers 2004). “Follows” occurred when the male swam behind the female in close vicinity (Ono and Uematsu 1957; Bjerselius et al. 2001; Oshima et al. 2003). “Bites” occurred when the male bit at the anal region of the female (Bjerselius et al. 2001). “Dances” occurred when the male swam in a circle in front of the stationary female (Ono and Uematsu 1957; Oshima et al. 2003). “Courts” occurred when the male was parallel to the female, facing in the same direction, and tried to push the female into a spawning position (Bjerselius et al. 2001). “Quivers” occurred when the male body undulated or vibrated with fins extended, typically into a crevice (Gale 1986; Moretz and Rogers 2004).

Secondary sexual characteristics

Tubercle number and developmental stage was measured on male red shiners preserved in 10% neutral buffered formalin. A microscope (0.8x magnification) connected to Image-Pro Express (Version 5.0.1.26) software was used to take a photograph of the dorsal surface of the head, from the posterior edge of the supraoccipital crest to the anterior tip of the snout. Number of visible tubercles was counted, and tubercle developmental stage was scored according to the most highly developed tubercles. The tubercle developmental stages were as follows: (0) no visible sign; (1) visible as white disks; (2) projecting above body surface; (3) prominent but not sharp; and (4) prominent and sharp (Fig 1.4; Smith 1978).

To document changes in male coloration on the pectoral and caudal fins, digital photographs were taken of the fish on their left, lateral side against a white background in the presence of a color wheel. All images were manually corrected for black and white using Adobe Photoshop 7.0 and analyzed for nuptial coloration with Image-Pro Express software

(Version 5.0.1.26) to obtain data on mean red, blue, green, and saturation. The trace function was used to select the pectoral fin area (area beginning at the base and encircling the edge of the visible fin), and the caudal fin area (the anterior base of the caudal fin where it meets the caudal peduncle to the posterior edges). Mean color and saturation were determined by selecting the region, producing a color histogram of the image, and recording the reported means that were produced based on abundance and composition of pixels present.

Vitellogenin and histological analyses

After each adult was measured, blood was collected into heparinized capillary tubes via caudal transection (Houston 1990). Samples were centrifuged for three minutes at 11,700 rpm (13,700 g) and the plasma was stored at -20°C until assayed for vitellogenin by homologous enzyme-linked immunosorbent assay using an anti-carp kit (Biosense, Bergen, Norway, batch number 0702) in accordance with the manufacturer's protocol. The kit was validated for use with the red shiner.

Fish were sacrificed by rapid decapitation and the liver and gonads were removed from the fish and weighed (g) to determine gonadosomatic index (GSI) and hepatosomatic index (HSI), respectively. Gonadosomatic index was calculated as $[\text{gonad weight (g)}/\text{body weight (g)} \times 100]$ and HSI was calculated as $[\text{liver weight (g)}/\text{body weight (g)} \times 100]$. All tissues were stored in 10% neutral buffered formalin for histological analysis. Fixed gonads were dehydrated through a graded series of EtOH, cleared in xylene, embedded in paraffin, sectioned (thickness, 6 or 10 μm) using a microtome, mounted on glass slides, rehydrated, and stained with hematoxylin and eosin (Presnell and Schreibman 1997; Woodling et al.

2006). Gonadal sections were examined under a light microscope for stage and maturation of each individual without knowledge of treatment group.

Gonadal staging was done using methods utilized by Vajda et al. (In press) and Pawlowski et al. (2004). Sperm abundance within seminiferous tubules of mature males was categorized as presence or absence, where 0 = sperm absent and 1 = sperm present (Fig. 1.5). Staging of ovarian sections was based on Vajda et al. (In press) and was as follows: 1 = pre-vitellogenic, 2 = early-vitellogenic, 3 = mid-vitellogenic, and 4 = late-vitellogenic. Pre-vitellogenic ovaries were identified by a lack of yolk globules and a centrally located nucleus, early-vitellogenic oocytes were identified by densely stained yolk globules present throughout, mid-vitellogenic oocytes were identified by presence of yolk globules and center yolk fusion, and late-vitellogenic oocytes were identified by yolk fusion extending to the thick, vitelline envelope (Blazer 2002). Ovaries were staged by identifying the most mature oocytes present in the histological section. The sections were then categorized according to relative abundance of pre-vitellogenic oocytes: category A (< 25%), B (25-50%), and C (> 50%).

Statistical analyses

All statistical analyses were performed using SAS version 9.1 (SAS Institute, Cary, NC, USA). Reproductive response variables were log or square-root transformed to meet the assumption of normality if necessary. Data were checked for homogeneity of variance across treatments using Levene's test. If parametric, data were analyzed using one-way analysis of variance (ANOVA). If nonparametric, the Kruskal-Wallis test was used to detect differences among treatments. Weight, total length, and condition factor data were tested using both

ANOVA and Kruskal-Wallis tests. When distributions were tested, Fisher's Exact test was used. Behavioral data were assessed for autocorrelation and when no pattern associated with time was observed, data from all observation events were pooled. When necessary, Tukey's HSD method was used to test multiple comparisons. In all tests, the level of significance used was 0.05.

Reversal experiment

Red shiners in the reversal experiment were collected from the Arkansas River site at the same time fish in the spawning experiment were collected, and were held in the same manner (see above). Only fish that were batch exposed to 120 ngL⁻¹ 17 β -estradiol (E2WC) and to the water control (WCWC) were used in this experiment as treatment groups. After 91 d of batch exposure, a total of 10 males (5 males from each treatment) were chosen randomly, weighed, and allocated into the glass spawning aquaria (see above; Fig. 1.2).

Egg deposition, fertilization success, hatching success, male behavior, secondary sexual characteristics, vitellogenin analysis, and histological parameters were measured in the same manner as the spawning exposure experiment. Water chemistry was measured at the start of the experiment, to ensure that estradiol was not detectable in either treatment.

All statistical analyses were performed using SAS version 9.1 (SAS Institute, Cary, NC, USA) in a similar manner to the spawning exposure experiment. Behavioral data analysis utilized only data from the last observation period to represent the ultimate change over time. Reproductive data collected on a daily basis were pooled into four weekly groupings. Egg deposition was analyzed using the general linear mixed model program Proc Mixed for repeated measures ANOVA. Fixed effects were treatment, time and the treatment

by time interaction. Random effects were aquaria within treatment and the residual. Fertilization success and hatching success were analyzed using the generalized linear mixed model program Proc Glimmix with a binomial distribution for repeated measures ANOVA. Fixed effects were treatment, time and the treatment by time interaction. Random effects were aquarium within treatment and the residual. Post-hoc mean comparisons were made using Tukey's HSD method. In all tests, the level of significance used was 0.05.

RESULTS

Spawning exposure experiment

Fish survival was 100% for all treatments during the chemical exposure period. There were no significant differences among treatments in weight, total length, or condition factor of males and of females at the beginning or conclusion of the study (Table 1.1). Waterborne concentrations of estradiol on all sample dates were 28% to 134% (mean = 58%, SD = 50%, $n = 4$) of nominal values and remained relatively constant during the exposure period. Concentrations of estradiol were not detectable in water samples of SC ($n = 4$) and WC ($n = 4$) treatments.

The mean number of eggs deposited by females in aquaria containing E2 treatment male fish was significantly fewer than aquaria containing SC and WC treatment male fish ($p = 0.0168$, ANOVA test; Fig. 1.6A). Total aquarium egg deposition ranged from 0 to 309 eggs, 599 to 1638 eggs, and 308 to 1304 eggs in the E2, SC, and WC treatments, respectively. Percent fertilization success was significantly lower in the E2 treatment than the WC and SC treatments ($p = 0.0398$, Kruskal-Wallis test; Fig. 1.6B). Of the eggs fertilized, the percent that survived to hatching was significantly lower in the E2 treatment

group than the SC and WC treatments ($p = 0.0328$, Kruskal-Wallis test; Fig. 1.6C). None of the eggs fertilized in the E2 treatment survived to hatching. Pre-spawning courtship behaviors were suppressed in males from the E2 treatment. The frequencies of dances ($p = 0.0078$), courts ($p = 0.0180$), and quivers ($p = 0.0078$) in the E2 treatment group were decreased significantly compared with those of both controls (Kruskal-Wallis test; Fig. 1.7A). Initiation courtship behaviors were similar among treatment groups. The frequencies of following and biting were similar among treatments ($p > 0.1$, Kruskal-Wallis test).

Estradiol had an effect on the secondary sexual characteristics of male fish. The number of tubercles on E2 treatment fish was lower than SC and WC treatment fish ($p = 0.008$, Kruskal-Wallis test; Fig. 1.8). Tubercle developmental stage was significantly lower in the E2 treatment than the SC and WC treatments ($p < 0.001$, Fisher's Exact test; Fig 1.9). Tubercles seen on E2 treatment fish reached a developmental stage of one or below, while SC and WC treatment fish exhibited tubercles at stages three or four. The coloration of E2 treatment males was different than SC and WC fish in the pectoral fin and caudal fin regions (Table 1.2). The pectoral fin of E2 treatment males had higher mean blue ($p = 0.0002$) and lower mean saturation than SC and WC males ($p < 0.0001$), mean red was lower than the WC, but not SC males ($p = 0.0532$), and green was higher than the SC treatment, but not the WC treatment ($p = 0.0311$); SC and WC treatments were not different in any test (ANOVA test, $n = 5$ per treatment). The caudal fin of E2 treatment males had higher mean blue ($p = 0.0003$) and lower mean saturation ($p = 0.0042$) than SC and WC males, higher mean green than the SC treatment, but not WC treatment ($p = 0.0087$), and red did not differ between treatments ($p = 0.5239$); WC and SC did not significantly differ in any test (ANOVA test, $n =$

5 per treatment). Visually, males showed obvious differences between treatments (Fig. 1.10).

Male plasma vitellogenin concentration was significantly higher in the E2 treatment, with no differences between the SC and WC treatments ($p = 0.0090$, Kruskal-Wallis test; Fig. 1.11). Male gonadal stage was significantly reduced in the E2 treatment when compared to SC and WC treatments ($p < 0.0001$, Fisher's Exact test; Fig. 1.12). All males in the E2 treatment exhibited stage zero, and males in the SC and WC treatments exhibited stage one.

Plasma vitellogenin concentration was significantly lower in female fish that were in aquaria with E2 treatment males and that were exposed to estradiol; females in SC and WC treatment aquaria did not significantly differ ($p = 0.0042$, Kruskal-Wallis test). Female gonadal stage ($p = 0.5615$; Fig. 1.13) and amount of pre-vitellogenic oocytes ($p = 0.1567$; Fig. 1.14) did not significantly differ between treatment groups (Fisher's Exact test). Females in aquaria with males that were exposed to estradiol exhibited stages 1-3 and females in the control aquaria exhibited stages 2-4, with a majority of females in stage 3 for all treatment aquarium groups. Each treatment group had female gonads in categories A, B, and C, with the females exposed to estradiol having slightly more category C, pre-vitellogenic oocytes.

The GSI of E2 treatment males (2.042 ± 0.291) was not significantly different than the GSI of SC males (1.916 ± 0.238) and WC males (1.680 ± 0.144) ($p = 0.55$). The HSI of E2 treatment males (1.096 ± 0.133) was significantly higher than the HSI of SC males (0.791 ± 0.090) and WC males (0.714 ± 0.054) ($p = 0.04$). Data are reported as the mean \pm SEM (ANOVA test, $n = 5$ per treatment). The GSI of females exposed to estradiol in aquaria with E2 treatment males (7.051 ± 2.230) was significantly lower than the GSI of females in SC

treatment aquaria (11.732 ± 3.710) ($p = 0.02$) but did not differ from females in WC treatment aquaria (10.096 ± 3.193) ($p = 0.19$). The HSI of females in E2 treatment aquaria (0.741 ± 0.072) was not significantly different than the HSI of females in SC aquaria (1.270 ± 0.204) and females in WC aquaria (0.833 ± 0.124) ($p = 0.06$). Data are reported as the mean \pm SEM (ANOVA test, $n = 10$ per treatment).

Reversal experiment

Fish survival was 100% for all treatments. There were no significant differences among treatments in weight, total length, or condition factor of males and of females at the beginning or conclusion of the study (Table 1.3). Waterborne concentrations of estradiol on all sample dates were below detection limits of assay. Water chemistry was similar to the spawning exposure experiment and remained constant between treatments over the duration of the experiment.

The mean number of eggs deposited by females in aquaria containing E2WC treatment male fish was lower than in aquaria containing WCWC treatment male fish, but not significantly different between treatments ($p = 0.0571$) or weeks ($p = 0.0585$) (Proc Mixed; Fig. 1.15A). Percent fertilization success was 0% in the E2WC treatment for weeks one and two of the study, compared to $98 \pm 1.1\%$ and $99 \pm 0.3\%$ (mean \pm SEM) in the WCWC treatment. The E2WC treatment mean percent fertilization success increased in weeks three and four, and the WCWC treatment continued to be $> 95\%$ for the duration of the study. Treatments were significantly different in weeks three and four ($p = 0.0385$); there was not a significant week effect ($p = 0.2825$), and the interaction indicated a significant difference between E2WC weeks three and four ($p = <0.0001$) and nearly significant difference

between E2WC and WCWC treatments during week three ($p = 0.0568$) (Proc Glimmix; Fig. 1.15B).

Eggs were not available to hatch until week three in the E2WC treatment, while the WCWC treatment had hatching success of $87 \pm 0.1\%$ and $82 \pm 0.2\%$ (mean \pm SEM) during week one and week two of the study, respectively. Of the eggs fertilized, the percent that survived to hatching was not significantly different between treatments ($p = 0.0951$) or weeks ($p = 0.2253$) during weeks three or four. There was a significant difference in hatching success between weeks three and four of the WCWC treatment ($p = 0.0023$) (Proc Glimmix; Fig. 1.15B).

Courtship behaviors appeared to recover in males of the E2WC treatment. Frequencies of dancing ($p = 1.0000$), courting ($p = 1.0000$), and quivering ($p = 0.8815$) in the E2WC treatment group were not significantly different than the WCWC treatment (Kruskal-Wallis test, $n = 5$; Fig. 1.7B). Secondary sexual characteristics of male fish also seemed to reverse. The mean number of tubercles on E2WC fish was not significantly different than E2 treatment fish of the spawning exposure experiment ($p = 0.5681$) or the controls of both experiments (SC, $p = 0.0641$; WC, $p = 0.0945$; WCWC, $p = 0.0945$) (ANOVA test; Fig. 1.8). Tubercles seen on E2WC fish exhibited developmental stages between zero and three, while WCWC treatment fish exhibited tubercles at stages three or four. The E2WC fish had a significantly lower developmental stage than the WCWC treatment ($p = 0.0476$, Fisher's Exact test; Fig. 1.16). The coloration of E2WC fish was different than WCWC fish (Table 1.4). The pectoral fin of E2WC treatment males had significantly higher mean blue, higher mean green, and lower mean saturation than WCWC treatment males, with no significant difference in mean red (Table 1.4). The caudal fin of E2WC treatment males had

significantly higher mean green than WCWC males; mean blue was higher and mean saturation was lower in E2WC treatment males, although statistically insignificant. There was no difference in mean red between treatments (Table 1.4).

Male plasma vitellogenin concentration in the E2WC treatment was significantly higher than the WCWC treatment ($p = 0.0090$, Kruskal-Wallis test; Fig 1.11B). The E2WC treatment was not significantly different than the E2 treatment of the spawning exposure experiment ($p = 0.7292$), and both the E2 and E2WC treatments were statistically different from the controls in both experiments ($p < 0.0001$, all comparisons) (Kruskal-Wallis test; Fig. 1.11). Male gonadal stage was similar between the E2WC and WCWC treatments ($p = 0.4444$, Fisher's Exact test; Fig 1.17), with two E2WC treatment fish in stage zero and three fish in stage one and all WCWC treatment fish in stage one. Female gonadal stage ($p = 1.0000$, Fisher's Exact test) and plasma vitellogenin concentration ($p = 0.3642$ Kruskal-Wallis test) were similar between groups.

The GSI of E2WC treatment males (1.398 ± 0.278) was not significantly different than the GSI of WCWC males (1.890 ± 0.155) ($p = 0.1606$). The HSI of E2WC treatment males (0.850 ± 0.231) was not significantly different than the HSI of WCWC males (0.494 ± 0.057) ($p = 0.1928$). Data are reported as the mean \pm SEM (ANOVA test, $n = 5$ per treatment). The GSI of females in aquaria with E2WC treatment males (9.948 ± 1.139) was not significantly different the GSI of females with WCWC treatment males (9.771 ± 1.014) ($p = 0.9091$). The HSI of females in aquaria with E2WC treatment males (0.949 ± 0.079) was not significantly different than the HSI of females in aquaria with WCWC treatment males (1.007 ± 0.149) ($p = 0.8973$). Data are reported as the mean \pm SEM (ANOVA test, $n = 10$ per treatment).

DISCUSSION

Our study clearly demonstrates that estradiol, at an environmentally relevant level of estrogenicity, can adversely impact reproductive success of adult red shiners.

Experimentally-controlled exposures led to reduction in sperm abundance and tubercles, and also resulted in decreased mating coloration. In addition, exposed males showed a decrease in key spawning behaviors, likely resulting in reduced egg deposition by female partners. Most importantly, estradiol greatly limited reproductive success, where eggs fertilized by exposed males showed poor fertilization success and impaired hatching success. Marked improvements in these reproductive endpoints were observed when males were removed from estradiol exposures and were allowed to spawn in control water. Recovery to levels similar to those observed in control fish occurred after four weeks. Our study suggests chronic exposures to endocrine disruptors, which fish may experience below wastewater treatment facilities, could have severe population repercussions for the red shiner by reducing reproductive success, but that effects of acute exposures on adult males may be reversible.

Males exposed to estradiol exhibited reduced fertilization success. Decreased fertilization success was probably due to sperm deficiencies and decreases in circulating androgens that are necessary in spermatogenesis and spawning (Demski and Hornby 1982; Schulz and Miura 2002). In adult guppies (*Poecilia reticulata*), androgen levels decreased after removal of their pituitary glands, resulting regression of the testes, blocked spermatogonial mitosis, and no progression of cells into spermatocytes (Pandley 1969). Reduced fertility of male fish as a result of exposure to estradiol has been shown in previous studies (Schultz et al. 2003), and is associated with less-developed gonads (Bringolf et al. 2004), delayed spermatogenesis (Billard et al. 1981; Kang et al. 2002), decreased amounts of

expressible milt (Bjerselius et al. 2001; Schoenfuss et al. 2002), decreased gonad size (Toft and Baatrup 2001; Bringolf et al. 2004), and reduced sperm motility (Schoenfuss et al. 2002; Casselman et al. 2006). In red shiner, egg mortality has been linked to low fertilization success from weakened or dead sperm (Gale 1986).

Sperm deficiencies and developmental problems in male red shiners were likely responsible for decreased hatching success observed in our study. Our results are consistent with previous studies that found decreased hatching success in fish eggs after exposure to an estrogenic endocrine disruptor (Shioda and Wakabayashi 2000; Sohoni et al. 2001; Zillioux et al. 2001). In contrast, other studies did not find an effect of EDCs on hatching success (Thorpe et al. 2003; Fenske et al. 2005), indicating that effects may be species-, compound-, or concentration-specific.

We found reduced egg deposition following estradiol exposure, a result that has been reported previously (Shioda and Wakabayashi 2000; Toft and Baatrup 2001; Kang et al. 2002; Thorpe et al. 2003; Brion et al. 2004), and could have resulted from reduction of spawning cues associated with decreases in male courtship behavior. Exposed males in our experiment showed fewer courting, dancing, and quivering behaviors than those in control treatments. This is consistent with other studies, where courtship behaviors of male fishes were reduced after exposure to estradiol at levels comparable to this study (Bayley et al. 1999; Bjerselius et al. 2001; Doyle and Lim 2002; Schoenfuss et al. 2002; Oshima et al. 2003; Doyle and Lim 2005) or after exposure to ethynylestradiol (EE2) (Bell 2001; Kristensen et al. 2005). Male Japanese medaka (*Oryzias latipes*) exposed to estradiol showed suppression of sexual behavior when paired with an estradiol-unexposed receptive female (Oshima et al. 2003). Male eastern mosquitofish (*Gambusia holbrooki*) exposed to

estradiol showed decreased impregnation efficiency, which was attributed to reduced sexual behavior (Doyle and Lim 2005).

Functional spawning behaviors are required for final mating cues and onset of spawning (Shioda and Wakabayashi 2000; Oshima et al. 2003) and are synchronized by reproductive hormones (Kobayashi et al. 2002). In goldfish (*Carassius auratus*), reproductive hormones appear to exert strong influences on olfactory mechanisms (Demski and Hornby 1982). Estradiol may alter male behavior by affecting receptors used to detect pheromones released by females (Bjerselius et al. 2001). Estradiol also reduces androgen production that controls aspects of sexual behavior (Toft and Baatrup 2003; Martinovic et al. 2007). Decreased courtship behaviors in our study likely correspond to a lack of interest to the female, and translate to fewer spawning events and eggs deposited. Reduced spawning activity due to EDC exposure has been shown to coincide with decreased reproductive success in other fishes (Bjerselius et al. 2001; Thorpe et al. 2003; Bringolf et al. 2004; Brion et al. 2004).

Changes in secondary sexual characteristics of male fish may also have influenced decreased egg deposition by female red shiners. Males exposed to estradiol had significantly fewer and less developed tubercles, and reduced spawning coloration compared to controls. We do not know whether tubercles regressed or failed to develop due to estradiol exposure; however, because fish were exposed prior to attaining temperatures needed to induce sexual development, it is likely that they failed to develop. Other studies also show that males exposed to estradiol had fewer tubercles (Bjerselius et al. 2001; Bringolf et al. 2004) and reduced coloration (Toft and Baatrup 2001), which has been shown to correlate with fitness and female choice in cichlid (*Pundamilia nyererei*) (Maan et al. 2006).

Since female fish were exposed to estradiol for a short period of time (39 d) in our spawning exposure experiment, decreased egg deposition could have been due to changes in female reproductive condition. Plasma vitellogenin concentration was significantly different between exposed and control females, which could have influenced reproduction; female vitellogenin and reproductive condition have been positively correlated in fathead minnows (Miller et al. 2007). However, oocyte stage and vitellogenic oocyte abundance in females that were exposed to estradiol during mating were not significantly different from female red shiners that mated in control water. Previous studies have found that female changes in fecundity are affected by exposure concentration or that fecundity can be increased with estrogenic exposure (Kristensen et al. 2005). Java-medaka (*Oryzias javanicus*) exposed to 68 ngL⁻¹ estradiol for their full life-cycle increased egg production while concentrations of 159 ngL⁻¹ and 243 ngL⁻¹ decreased egg production (Imai et al. 2005). However, in our study, females were exposed as adults for a shorter period of time, and at a concentration lower than that found to be detrimental to egg production in Java-medaka. Decreased egg deposition in our experiment was more likely a result of alterations in male spawning cues.

We found that estrogenic effects on male morphometric traits, testicular histology, behavior, and reproductive success were reversible when male red shiners were removed from estradiol exposure and were allowed to mate in control water. Improvement in all reproductive endpoints was observed by three weeks of spawning. Others have shown that reversibility of exposure depends on the concentration, duration, and time-period during which the fish are exposed (Fenske et al. 2005; Schafers et al. 2007), and may also be species-specific (Nash et al. 2004). Reversal experiments often result in partial reproductive recovery (Maack and Segner 2004; Nash et al. 2004; Schafers et al. 2007) that still may result

in reduced fitness. In our reversal study, males showed a slight increase in tubercle number and developmental stage, and mating behaviors were indistinguishable from males that were never exposed, which corresponded with an increase in egg deposition by females.

Reproductive success of the red shiner, a native Great Plains fish in eastern Colorado, was dramatically altered by exposure to an estrogenic compound. Other studies have suggested that reproduction of other Plains fish species may be compromised due to estrogenic compounds in wastewater effluent. For example, white sucker in Boulder Creek, CO (which has a total estrogen equivalence as high as 50 ngL^{-1}), have shown gonadal intersex, altered sex ratios, reduced gonad size, disrupted ovarian and testicular architecture or histological lesions in ovaries and testes, and vitellogenin induction (Vajda et al. In press; A. Vajda, University of Colorado, personal communication). This wide spectrum of aberrations in reproductive parameters was probably due to life-long chronic exposures. When exposed to the same effluent, adult male fathead minnow showed fewer, less prominent nuptial tubercles, reduced sperm abundance, and elevated vitellogenin within 14 d with > 50% effluent (A. Vajda, University of Colorado, personal communication). Intersex white suckers have also been found in the South Platte River downstream of Denver, CO (Woodling et al. 2006). Since many streams of the eastern plains of Colorado are dominated by wastewater discharges and agricultural runoff (Woodling et al. 2006), the potential exists for plains fish communities to be chronically exposed to EDCs.

Our study clearly indicates that exposure to estrogenic compounds could cause dramatic declines in reproductive fitness of red shiners, which could have negative implications for wild populations. Kidd et al. (2007) found that when wild populations of fathead minnows were exposed to an estrogenic EDC in a whole-lake experiment, exposed

adult males showed individual effects similar to our study, which ultimately led to a population collapse. We used red shiners as a model for other cyprinid Great Plains fishes because their life history and biology are well known and they survive and reproduce in laboratory situations. However, reproductive biology of other species may be important in determining how EDCs may influence their survival. Red shiners have relatively complex mating behavior and male spawning behaviors were clearly influenced by EDC exposure. Negative effects caused by exposure to estradiol may be intensified in more reproductively complex species or conversely, may be reduced in species with less complex mating systems. The interaction of life history characteristics with EDC exposure needs to be more fully understood before predictions can be made about the long-term effect of EDCs on population dynamics of Great Plains fishes. In addition, the effects of these chemicals on juvenile fishes need to be determined; the reversibility that we observed in adult fishes may not be possible for fish exposed during ontogenetic development.

TABLES AND FIGURES

Table 1.1. Batch exposure weight (g), total length (TL, mm), and condition factor (CF) of adult male red shiner (*Cyprinella lutrensis*), and initial and final experimental weights, TL, and CF for male and female adult red shiner after exposure to 120 ngL⁻¹ 17β-estradiol (E2), solvent control (SC), and water control (WC) treatments. Each treatment group value represents the mean ± SD (ANOVA tests, α = 0.05).

Sex	Variable	Treatment			P-value
		E2	SC	WC	
Male	Batch exposure weight ^a	2.45 ± 0.49	2.75 ± 0.70	2.95 ± 1.22	0.42
	Batch exposure TL ^a	61 ± 5.1	64 ± 5.3	63 ± 6.9	0.52
	Batch exposure CF ^a	1.08 ± 0.12	1.04 ± 0.05	1.11 ± 0.24	0.58
Male	Initial weight ^b	2.78 ± 0.42	3.93 ± 0.68	3.56 ± 1.33	0.16
	Final weight ^b	2.97 ± 0.33	4.04 ± 0.69	3.93 ± 1.53	0.21
	Final TL ^b	64 ± 1.6	69 ± 3.9	68 ± 8.7	0.35
	Final CF ^b	1.12 ± 0.09	1.20 ± 0.09	1.20 ± 0.13	0.42
Female	Initial weight ^c	3.43 ± 0.37	4.11 ± 1.20	3.90 ± 0.78	0.24
	Final weight ^c	3.46 ± 0.68	3.81 ± 0.77	3.48 ± 0.90	0.55
	Final TL ^c	67 ± 3.7	69 ± 4.3	68 ± 3.7	0.53
	Final CF ^c	1.14 ± 0.09	1.16 ± 0.13	1.10 ± 0.15	0.58

^a n = 11 (E2); n = 10 (SC); n = 6 (WC)

^b n = 5 per treatment

^c n = 10 per treatment

Table 1.2. Pectoral fin and caudal fin coloration of male red shiners (*Cyprinella lutrensis*) in the spawning exposure experiment, after 84 d exposure to 120 ngL⁻¹ 17β-estradiol (E2), solvent control (SC), and water control (WC) treatments (n = 5 per treatment). Data are expressed as mean ± SEM; values with different letters indicate statistical significance (p < 0.05; ANOVA tests).

Area	Variable	E2	SC	WC
Pectoral Fin	Red	176.10 ± 9.02 ^a	190.36 ± 3.52 ^{ab}	200.29 ± 4.84 ^b
	Blue	128.37 ± 19.70 ^a	42.73 ± 4.68 ^b	66.5 ± 5.94 ^b
	Green	156.52 ± 13.44 ^a	114.49 ± 6.12 ^b	135.15 ± 7.98 ^{ab}
	Saturation	52.69 ± 14.55 ^a	170.70 ± 8.02 ^b	135.94 ± 8.02 ^b
Caudal Fin	Red	186.91 ± 6.45 ^a	186.33 ± 3.47 ^a	194.96 ± 6.96 ^a
	Blue	133.59 ± 7.75 ^a	73.90 ± 7.62 ^b	96.99 ± 6.33 ^b
	Green	167.88 ± 7.12 ^a	140.51 ± 4.51 ^b	150.92 ± 2.72 ^{ab}
	Saturation	56.05 ± 3.03 ^a	127.01 ± 12.39 ^b	98.71 ± 12.13 ^b

Table 1.3. Initial and final experimental weights (g), total length (TL, mm), and condition factor (CF) for male and female adult red shiners (*Cyprinella lutrensis*) after previous 91 d exposure to 120 ngL⁻¹ 17β-estradiol followed by four weeks of depuration (E2WC) and water control (WCWC) treatments. Each treatment group value represents the mean ± SD. (ANOVA tests, α = 0.05).

Sex	Variable	Treatment		P-value
		E2WC	WCWC	
Male ^a	Initial weight	4.01 ± 1.05	5.67 ± 1.41	0.07
	Final weight	4.03 ± 1.09	5.35 ± 1.25	0.11
	Final TL	71 ± 5.4	77 ± 5.9	0.18
	Final CF	1.08 ± 0.05	1.17 ± 0.07	0.05
Female ^b	Initial weight	3.56 ± 0.43	3.97 ± 0.74	0.15
	Final weight	3.43 ± 0.62	3.37 ± 0.45	0.81
	Final TL	67 ± 2.5	68 ± 3.4	0.55
	Final CF	1.12 ± 0.12	1.06 ± 0.06	0.23

^a n = 5 per treatment

^b n = 10 per treatment

Table 1.4. Pectoral and caudal fin coloration of male red shiners (*Cyprinella lutrensis*) in the reversal experiment, after 91 d exposure to 120 ngL⁻¹ 17β-estradiol and four weeks of depuration (E2WC) and water control (WCWC) treatments. Each treatment group value represents the mean ± SEM. Asterisks denote significant differences from the control (ANOVA tests, α = 0.05, n = 5 per treatment).

Area	Variable	Treatment		P-value
		E2WC	WCWC	
Pectoral Fin	Red	175.52 ± 5.54	184.22 ± 3.19	0.21
	Blue	91.83 ± 18.81*	33.95 ± 2.65	0.02
	Green	134.83 ± 7.28*	101.32 ± 4.05	< 0.01
	Saturation	92.18 ± 26.88*	184.29 ± 5.11	0.01
Caudal Fin	Red	189.06 ± 1.64	188.37 ± 3.07	0.85
	Blue	110.47 ± 16.02	70.12 ± 8.44	0.06
	Green	159.89 ± 7.17*	135.48 ± 3.28	0.01
	Saturation	82.39 ± 18.03	127.71 ± 10.89	0.06

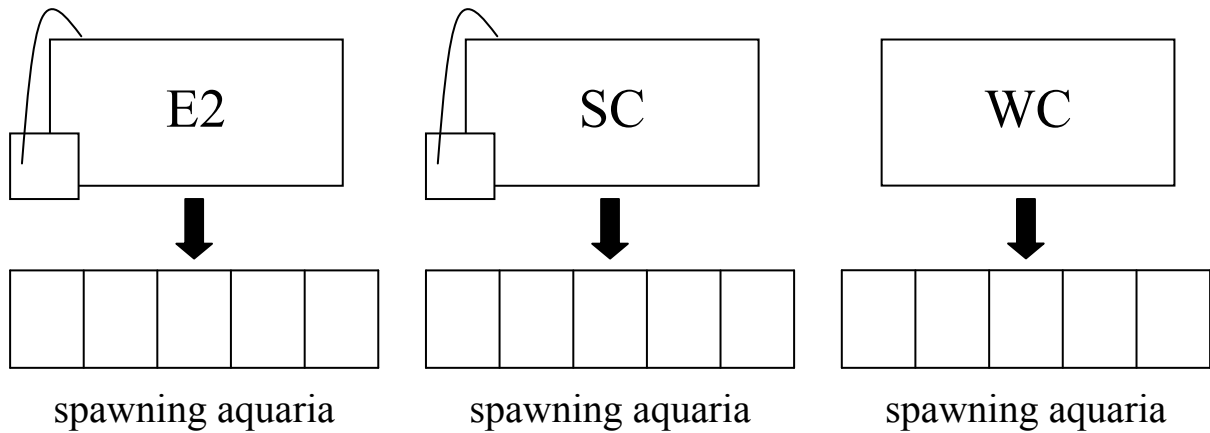


Fig. 1.1. Experimental setup. Water flowed into secondary glass head tanks where it was mixed with stock solutions via peristaltic pumps for the experimental treatments 17- β estradiol (E2, 120 ngL⁻¹) and solvent control (SC), or mixed without a stock solution to produce the water control (WC) treatment. Water from each treatment was delivered to five spawning aquaria, each holding one male and two female fish for the spawning experiment.

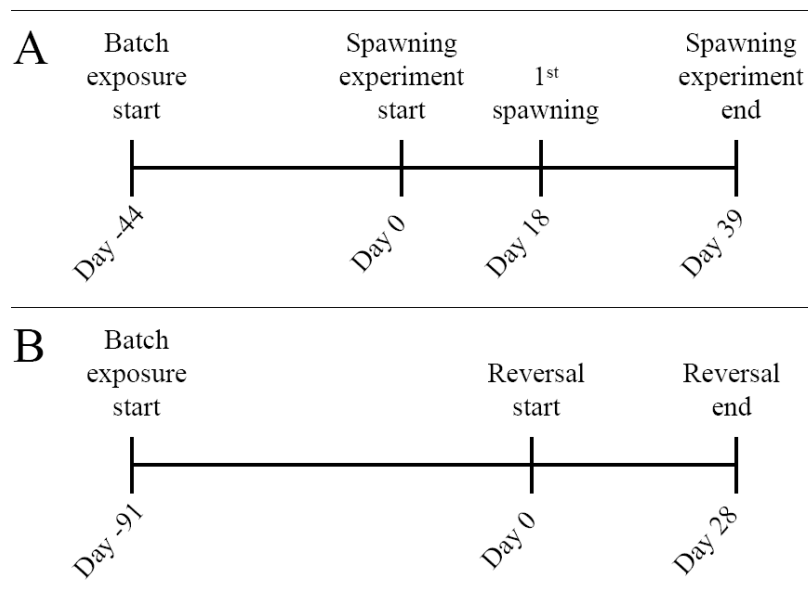


Fig. 1.2. Schematic diagram of experimental protocols used in studies. The axes are relative and not to scale. In the spawning exposure experiment (A) exposure began (120ngL⁻¹ 17 β -estradiol, E2; solvent control, SC, water control, WC) on the date of batch exposure and continued to the spawning experiment end. Reproductive endpoints were measured for 21 d after spawning was first seen (1st spawning). In the reversal experiment (B) exposure began (120 ngL⁻¹ 17 β -estradiol, E2WC) on the date of batch exposure and was discontinued immediately prior to the start of the reversal experiment (day 0). Reproductive endpoints were measured for 28 d after spawning was first seen, which coincided with the start of the reversal experiment (day 0).

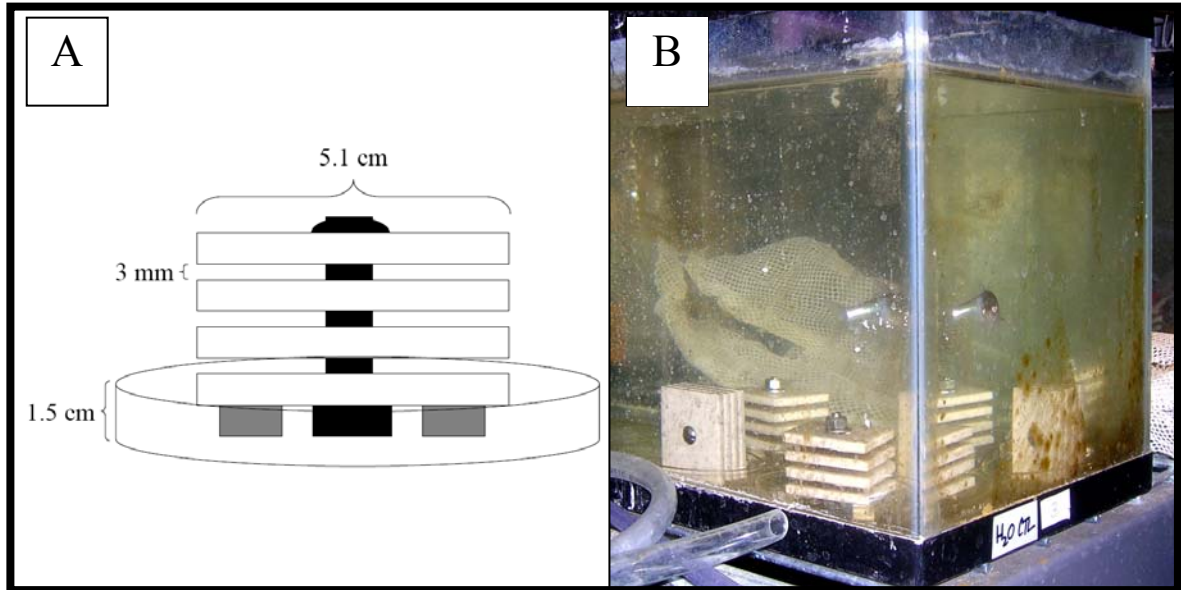


Fig. 1.3. Spawning stacks were composed of four 5.1 x 5.1 cm sand-colored ceramic tiles drilled through the center, stacked, and held together by a stainless steel bolt. Tiles were separated by two flat stainless steel washers, and secured by a stainless steel nut, creating three horizontal crevices averaging 3 x 47 x 47 mm each. Each spawning stack was placed on a 1.5 x 10 cm glass Petri dish and each aquarium contained four spawning stacks. Spawning stacks are illustrated using a schematic drawing (A) and a digital photograph (B) within a spawning aquarium.

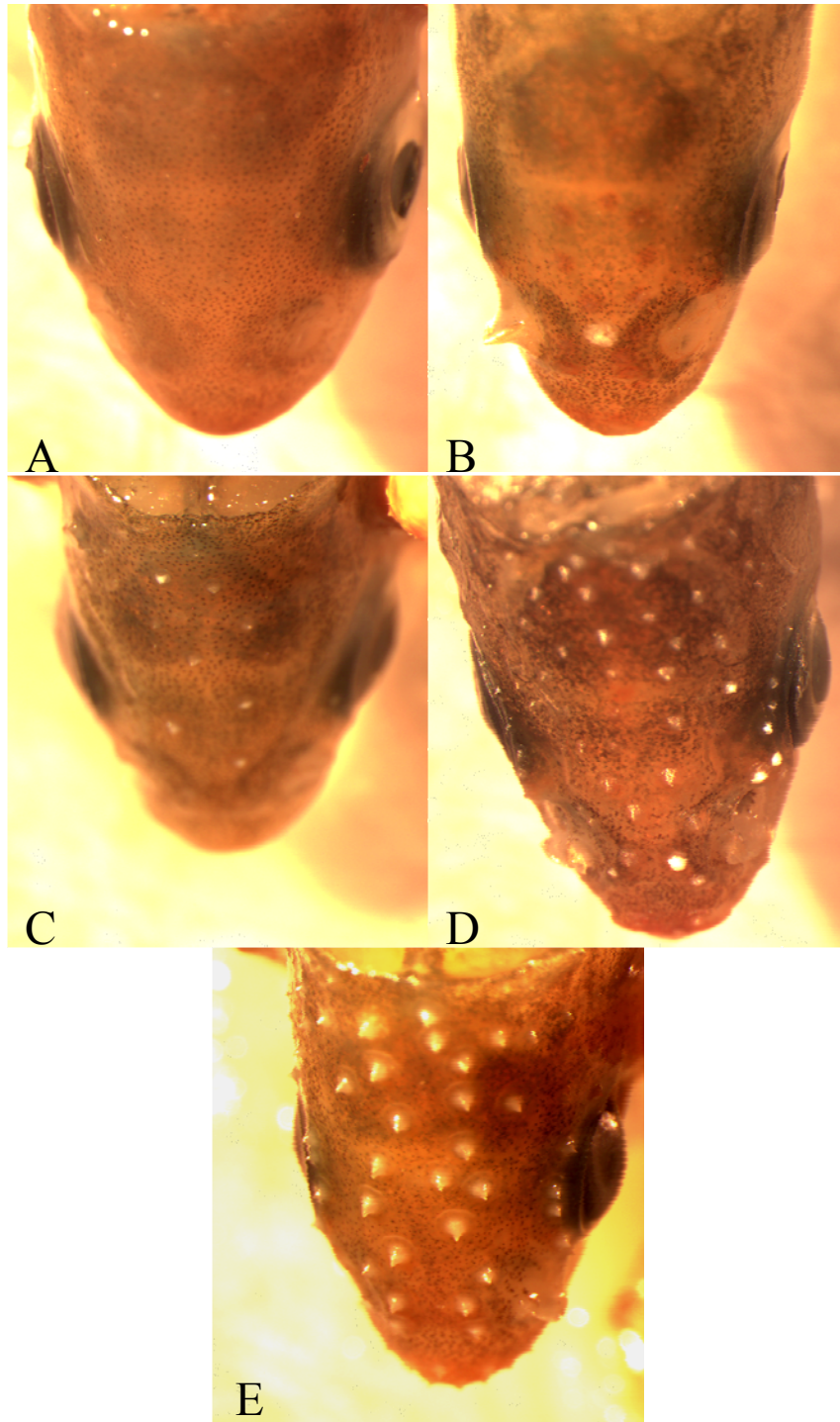


Fig. 1.4. Tubercle developmental stage of male red shiners (*Cyprinella lutrensis*) on the dorsal surface of the head from the posterior edge of the supraoccipital crest to the anterior tip of the snout. Staging was based on Smith (1978) and was assigned according to the most highly developed tubercles using the criteria (0) no visible sign (A); (1) visible as white disks (B); (2) project above body surface (C); (3) prominent but not sharp (D); and (4) prominent and sharp (E).

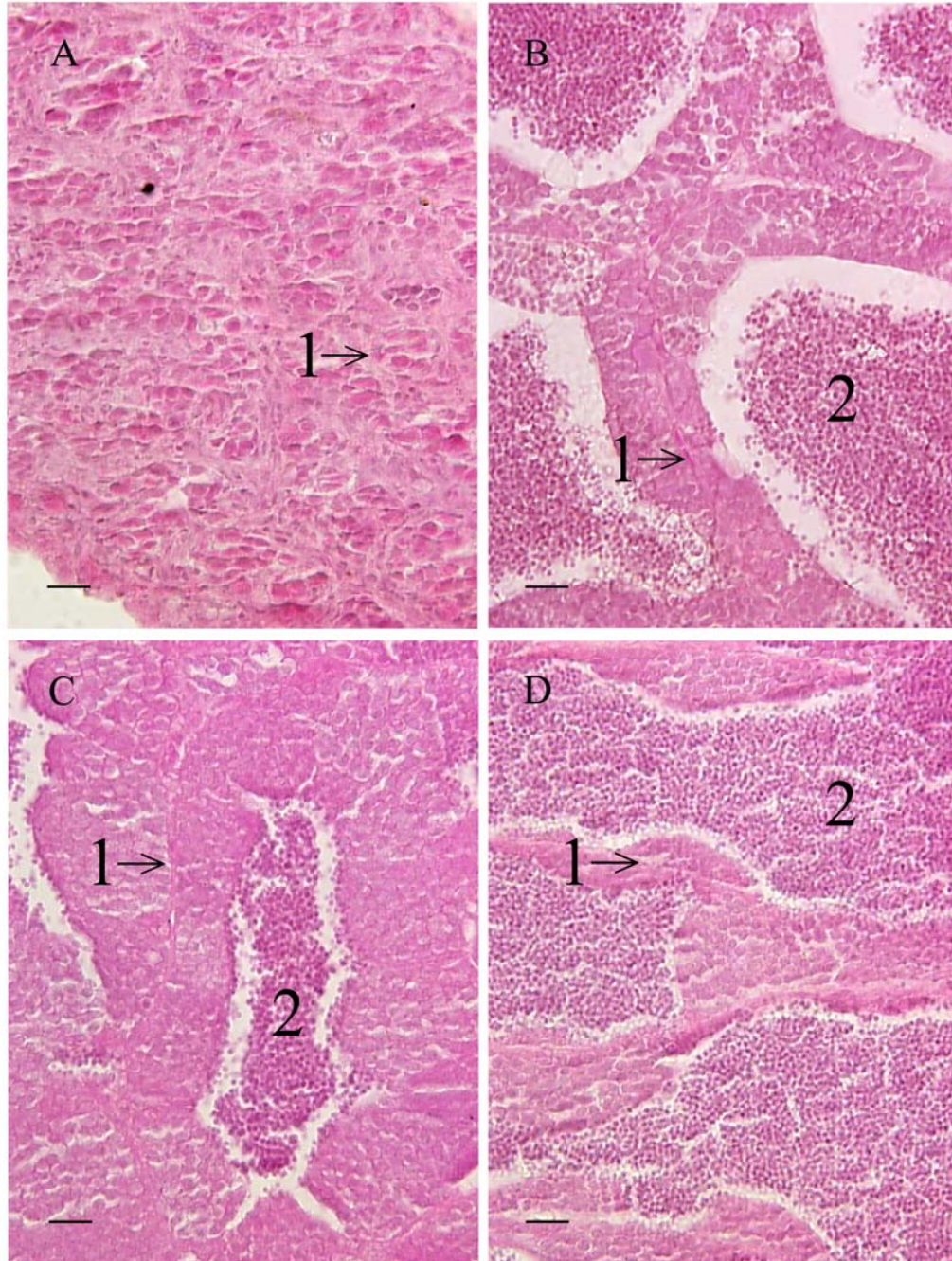


Fig. 1.5. Photomicrographs of representative testicular sections. Panels A and B: Male red shiners (*Cyprinella lutrensis*) exposed for 84 d to 120 ngL^{-1} 17β -estradiol in ethanol (solvent) (A) or solvent control (B). Whereas seminiferous tubules in solvent control fish were replete with sperm, seminiferous tubules were regressed and spermatogenesis was not evident in estradiol-exposed males. Panels C and D: Males exposed to 120 ngL^{-1} 17β -estradiol for 91 d followed by 28 d depuration in water (C) or water control (D). The numbers refer to the outer edge of the seminiferous tubule seminal epithelium (1) and luminal sperm (2). Note the recovery of spermatogenesis in estradiol-exposed fish following depuration. Hematoxylin and eosin staining. Scale bar: $15 \mu\text{m}$.

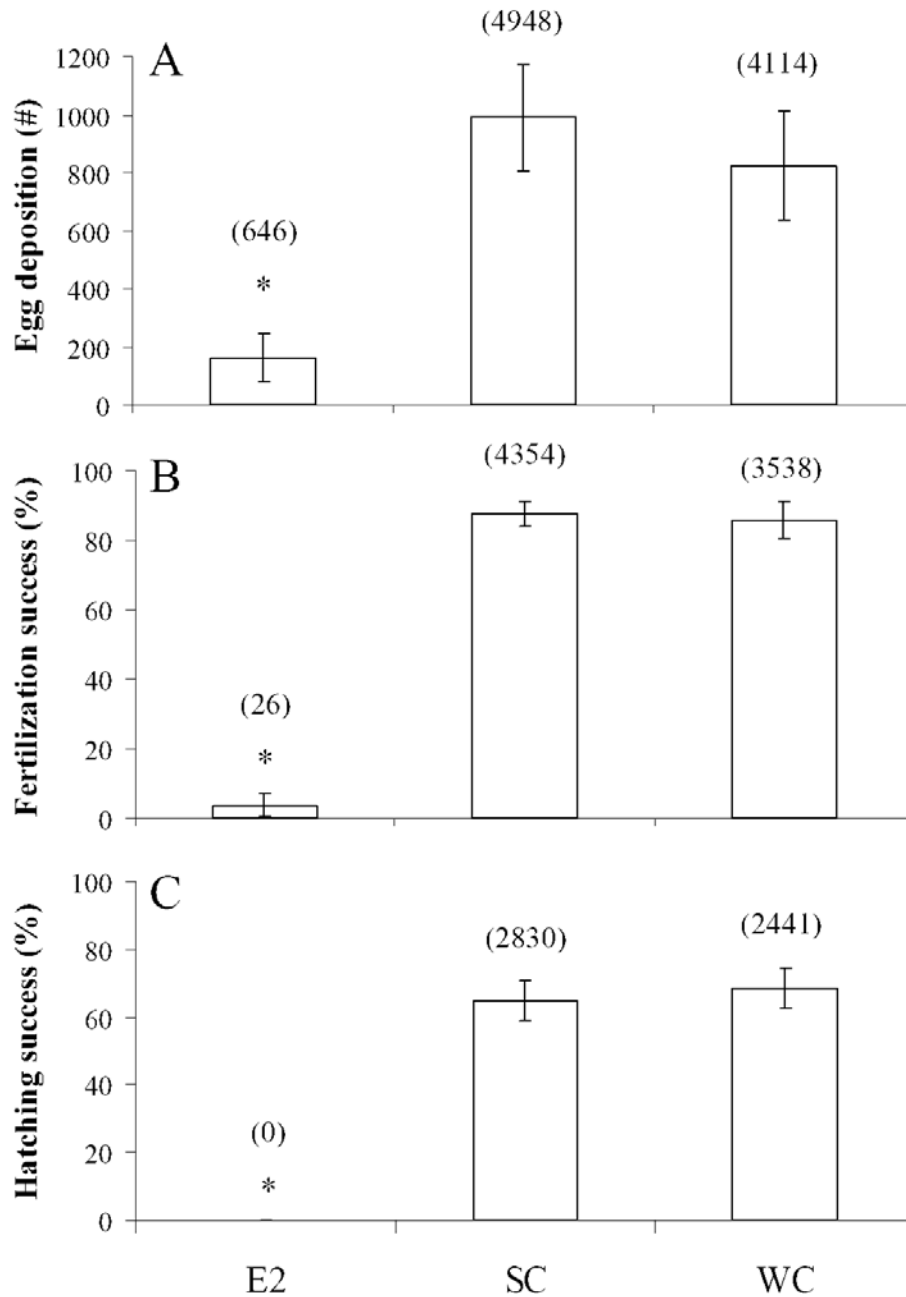


Fig. 1.6. Bar graphs showing effects on the reproductive success of the red shiner (*Cyprinella lutrensis*) after exposure to 17β-estradiol (E2), solvent control (SC), and water control (WC) treatments: egg deposition (A); percent fertilization success (B); and hatching success (C). Means ± SEM are shown. Asterisks indicate statistically significant differences from the water control * $p < 0.01$; $n = 5$ for SC and WC treatments and $n = 4$ (egg deposition) and 3 (fertilization and hatching success) for the E2 treatment. Total eggs per treatment deposited (A), fertilized (B), and hatched (C) are shown in parentheses above each bar.

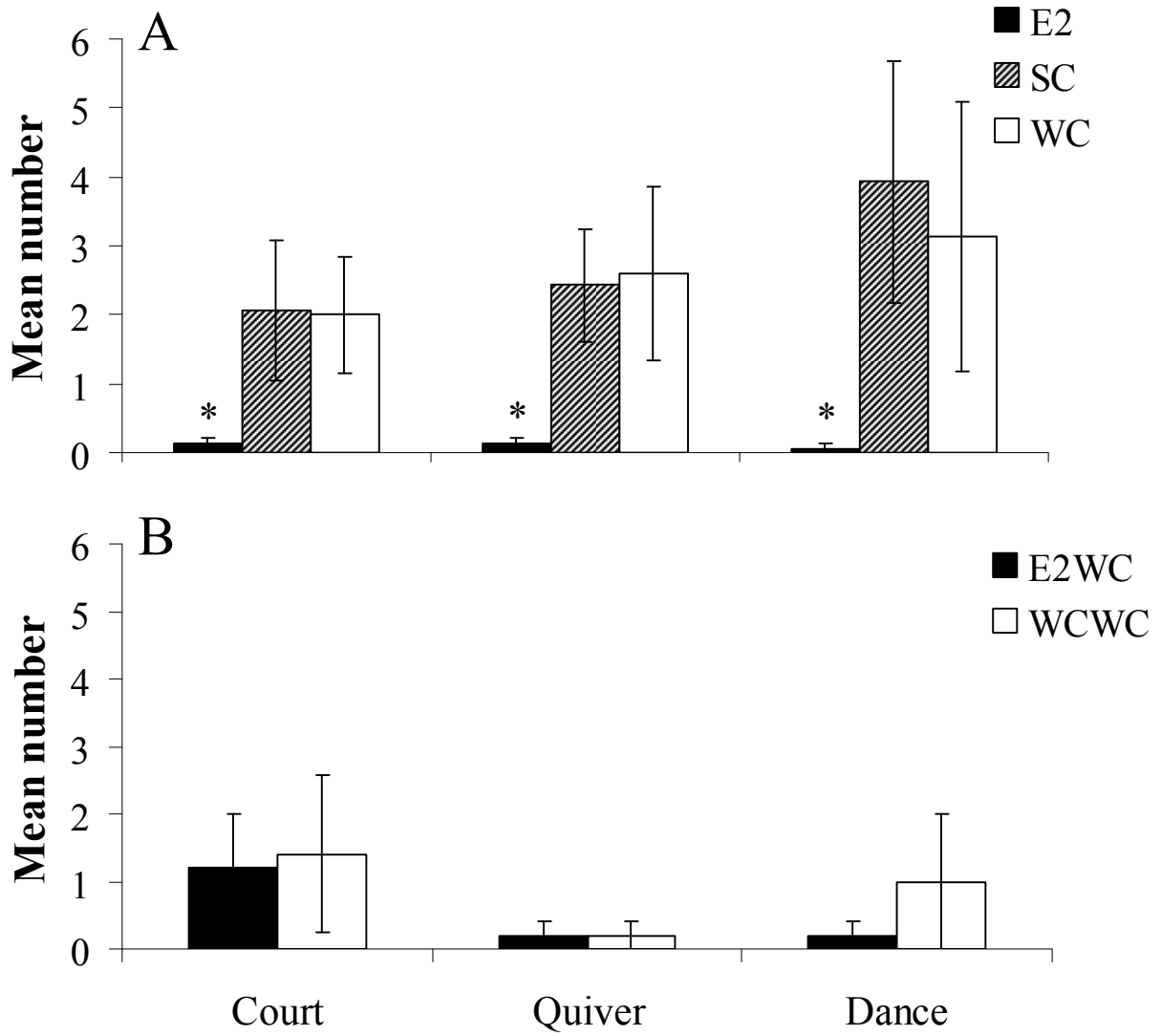


Fig. 1.7. Pre-spawning courtship behaviors of male red shiners (*Cyprinella lutrensis*) in the spawning exposure experiment (A), after males were exposed to 17 β -estradiol (E2), solvent control (SC), and water control (WC) treatments ($n = 15$ per treatment) and in the reversal experiment (B), after males were previously exposed to 17 β -estradiol (E2WC) and water control (WCWC) treatments ($n = 5$ per treatment). Means \pm SEM are shown. Asterisks indicate statistically significant differences from the water control $*p < 0.05$.

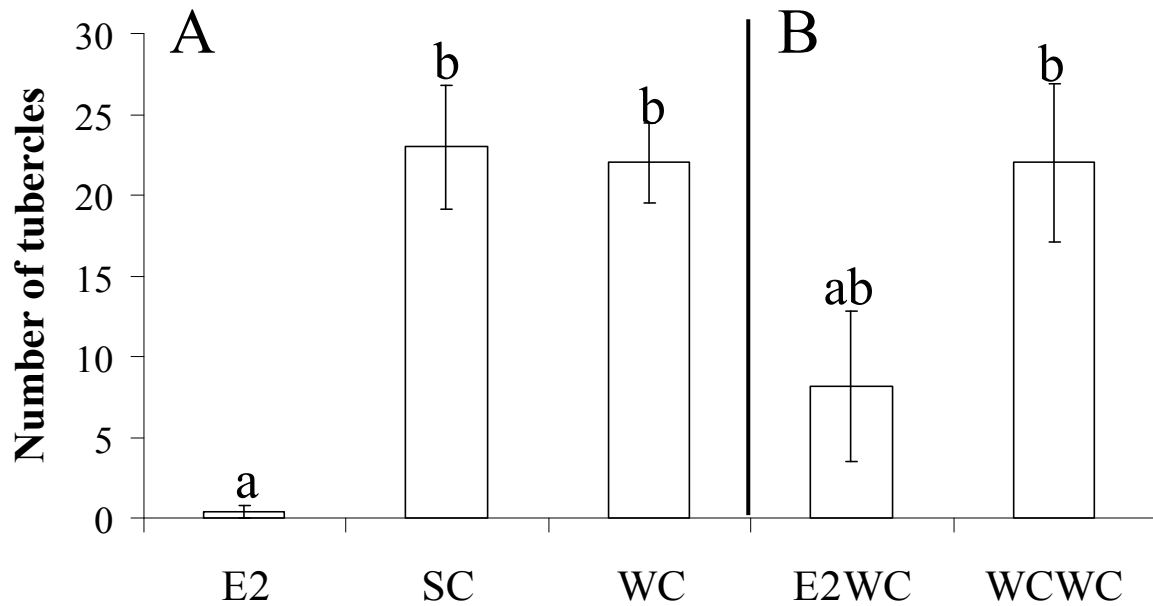


Fig. 1.8. Tubercle numbers of male red shiners (*Cyprinella lutrensis*) in the spawning exposure experiment (A), after males were exposed to 120 ngL⁻¹ 17 β -estradiol (E2), solvent control (SC), and water control (WC) treatments ($n = 5$ per treatment) and in the reversal experiment (B), after a 91 d male exposure to 120 ngL⁻¹ 17 β -estradiol and four weeks of depuration (E2WC) and water control (WCWC) treatments ($n = 5$ per treatment). Means \pm SEM are shown; values with different lowercase letters indicate statistical significance ($p < 0.05$).

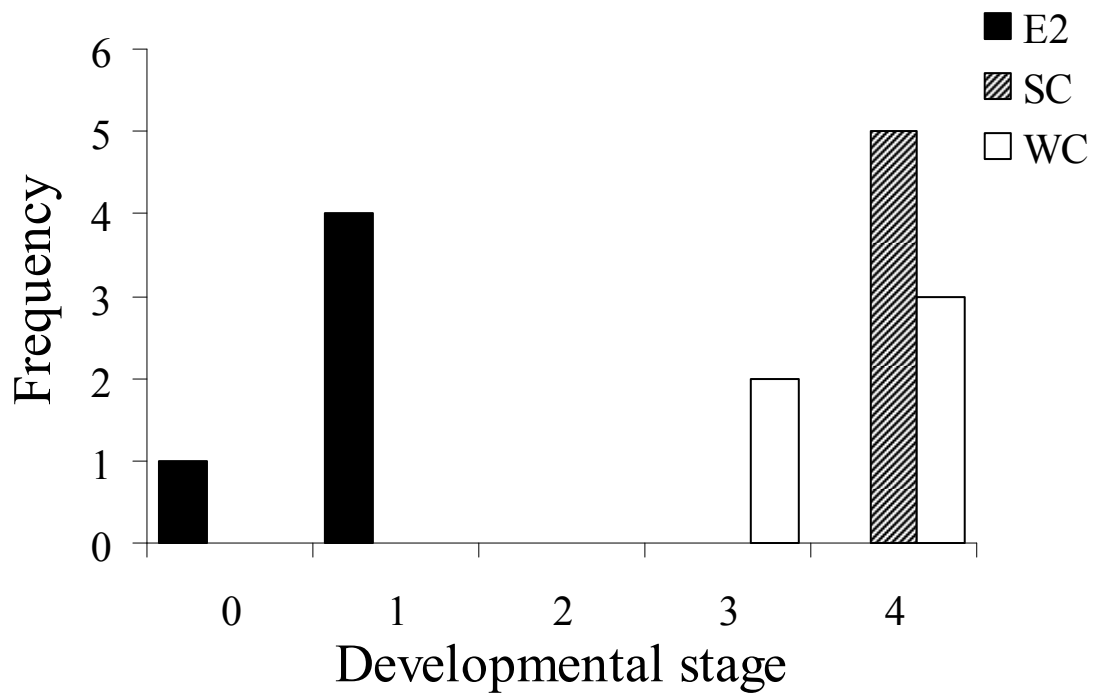


Fig. 1.9. Tubercle developmental stage of male red shiners (*Cyprinella lutrensis*) after 84 d male exposure to 120 ngL^{-1} 17β -estradiol (E2), solvent control (SC), and water control (WC) treatments. Stage was assigned according to the most highly developed tubercles: (0) no visible sign; (1) visible as white disks; (2) project above body surface; (3) prominent but not sharp; and (4) prominent and sharp. Frequency of each stage is shown, by treatment. Fisher's Exact test, $p < 0.0001$, $n = 5$ per treatment.

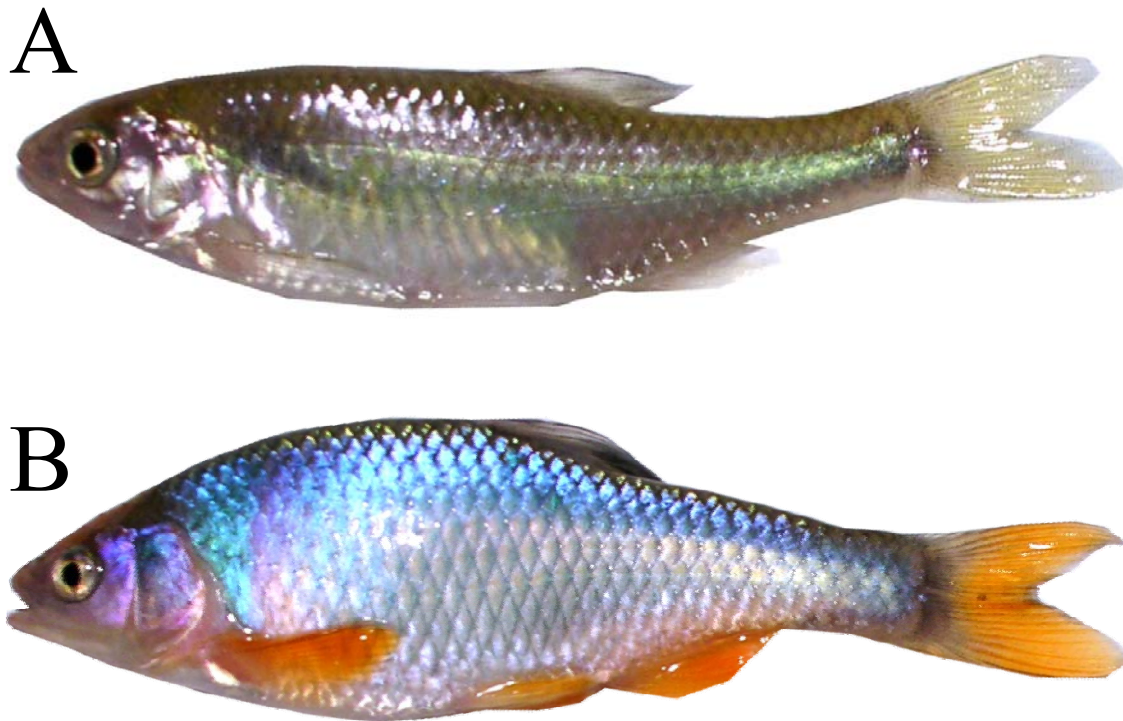


Fig. 1.10. Coloration of adult male red shiner (*Cyprinella lutrensis*) after 84 d exposure to 120 ngL^{-1} 17β -estradiol in ethanol (solvent) (A) or water control (B). Solvent control males were not visually different from water control males.

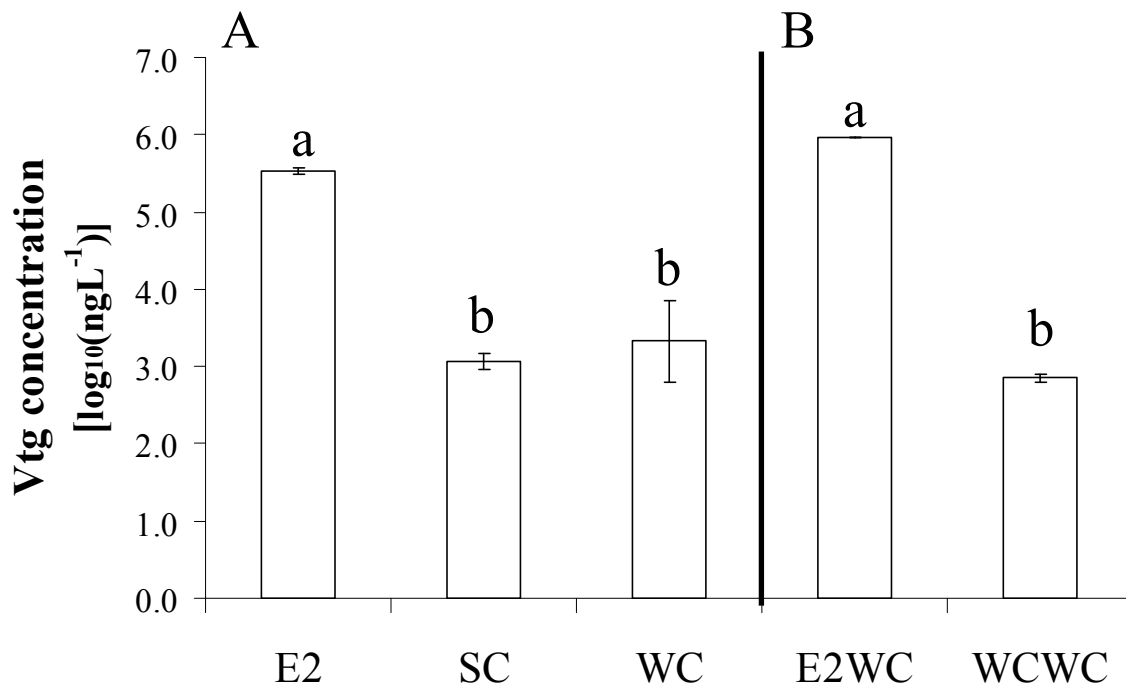


Fig. 1.11. Vitellogenin (Vtg) plasma concentrations in adult male red shiner (*Cyprinella lutrensis*) at the conclusion of the spawning exposure (A) and reversal (B) experiments. Fish were exposed to 120 ngL⁻¹ 17 β -estradiol (E2), solvent control (SC), or water control (WC) treatments for 84 d (A) or exposed to 120 ngL⁻¹ 17 β -estradiol followed by a four-week depuration (E2WC) or water control (WCWC) treatment for 91 d (B) before measurements. Means \pm SEM are shown; values with different lowercase letters indicate statistical significance (ANOVA test, $p < 0.05$).

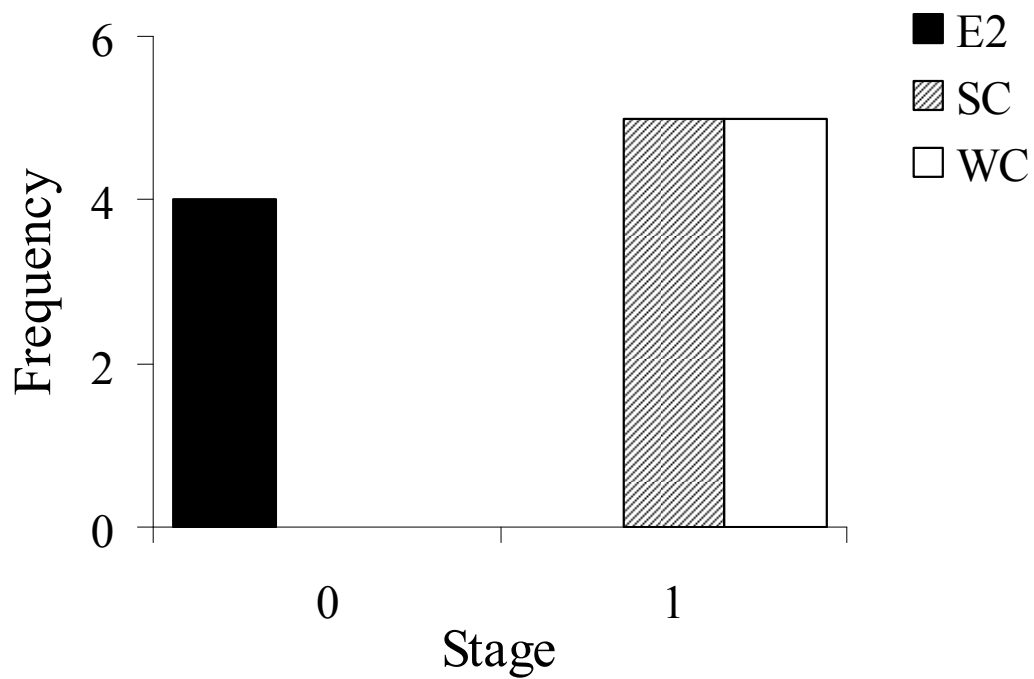


Fig. 1.12. Gonadal developmental stage of male red shiners (*Cyprinella lutrensis*) after 84 d male exposure to 120 ngL^{-1} 17β -estradiol (E2), solvent control (SC), and water control (WC) treatments. Stage was assigned according to presence or absence of sperm, where 0 = sperm absent and 1 = sperm present. Fisher's Exact test, $p = 0.0009$, $n_{E2} = 4$, $n_{SC, WC} = 5$.

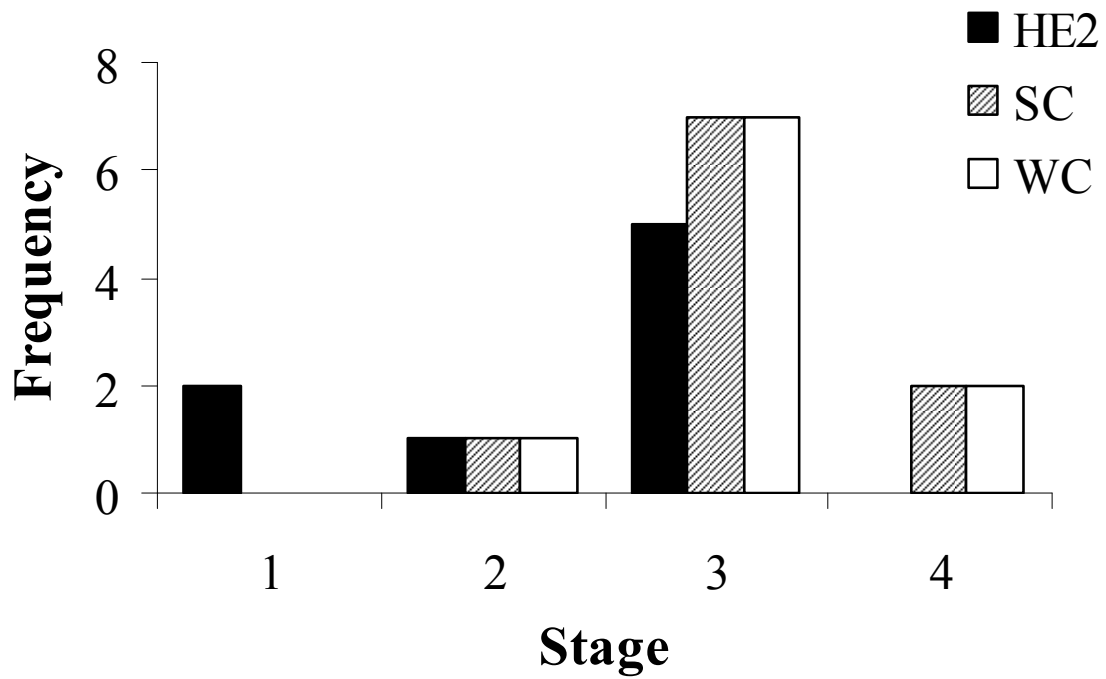


Fig. 1.13. Gonadal developmental stage of female red shiners (*Cyprinella lutrensis*) after 39 d exposure to 120 ngL^{-1} 17β -estradiol (E2), solvent control (SC), and water control (WC) treatments while in spawning aquaria with males. Stage was assigned according to oocyte development and was as follows: 1 = pre-vitellogenic, 2 = early-vitellogenic, 3 = mid-vitellogenic, and 4 = late-vitellogenic. Fisher's Exact test, $p = 0.5615$, $n_{E2} = 8$, $n_{SC, WC} = 10$.

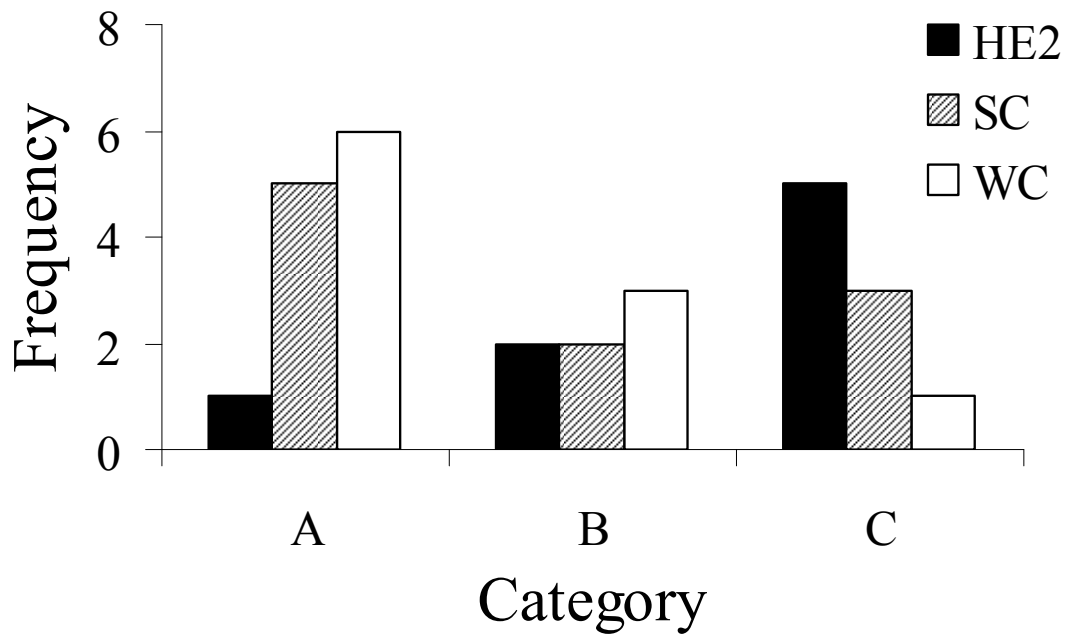


Fig. 1.14. Pre-vitellogenic oocyte abundance of female red shiners (*Cyprinella lutrensis*) after 39 d exposure to 120 ngL^{-1} 17β -estradiol (E2), solvent control (SC), and water control (WC) treatments while in spawning aquaria with males. Category was assigned according to relative abundance of pre-vitellogenic oocytes: A (< 25%), B (25-50%), and C (> 50%). Fisher's Exact test, $p = 0.1567$, $n_{\text{E2}} = 8$, $n_{\text{SC, WC}} = 10$.

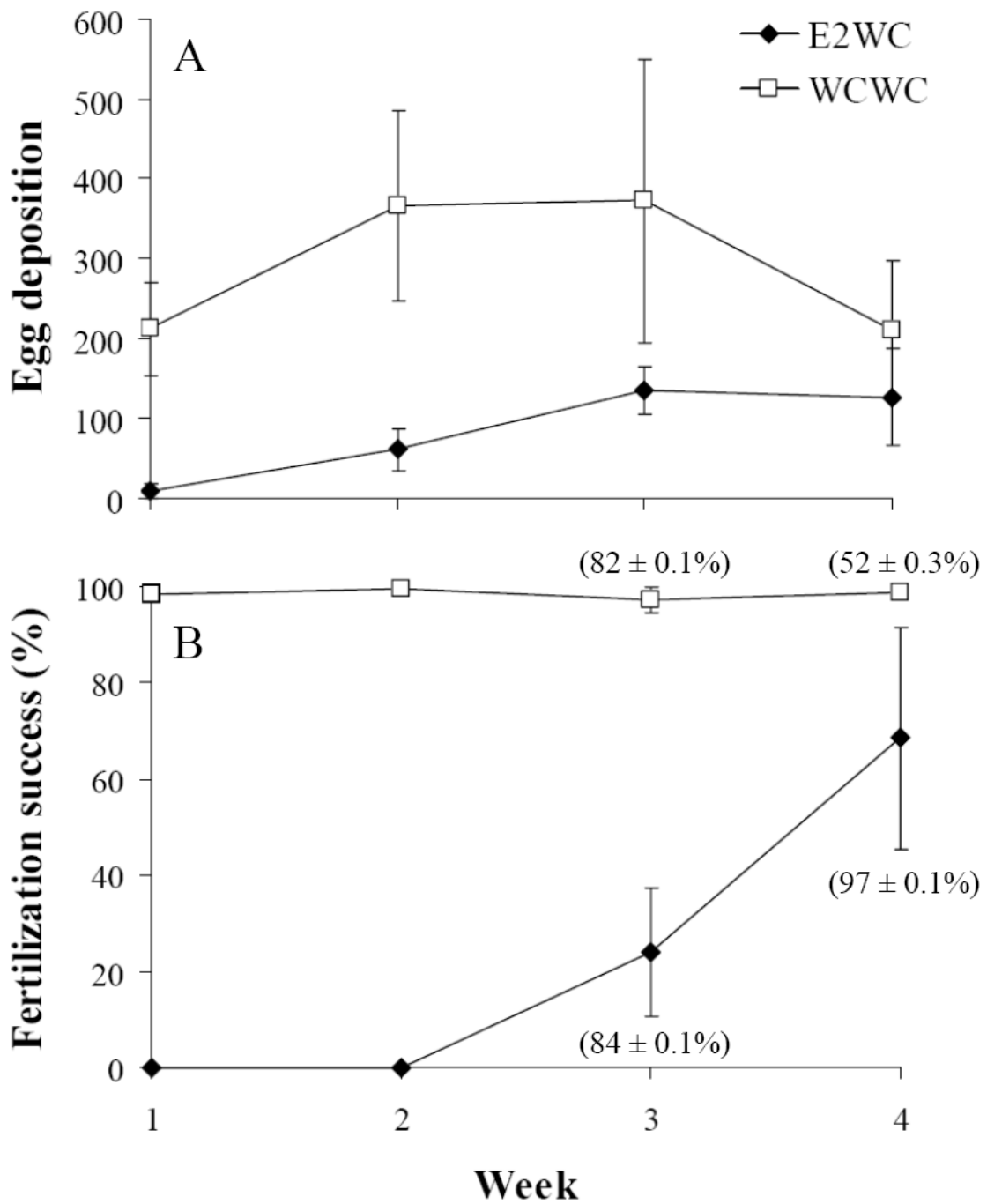


Fig. 1.15. The reproductive success of the red shiner (*Cyprinella lutrensis*) after exposed males were removed from the 120 ngL^{-1} 17β -estradiol exposure (E2WC) and were allowed to mate in control water. Previous control males were maintained in control water (WCWC) treatments in the reversal experiment. (A) Egg deposition (mean eggs \pm SEM) and (B) percent fertilization success (mean percent \pm SEM) and hatching success (mean percent \pm SEM; shown in parentheses above and below the WCWC and E2WC treatments, respectively, for weeks three and four).

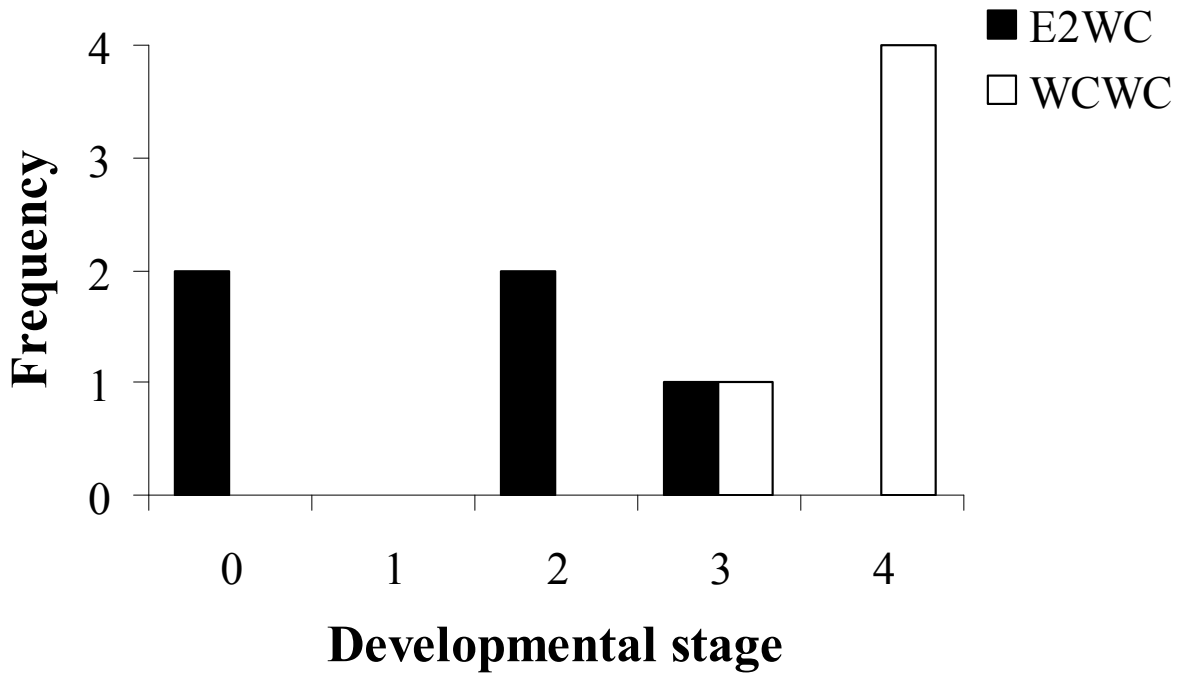


Fig. 1.16. Tubercle developmental stage of male red shiners (*Cyprinella lutrensis*) after four week depuration from 120 ngL⁻¹ 17β-estradiol exposure (E2WC) and water control (WCWC) treatments. Stage was assigned according to the most highly developed tubercles: (0) no visible sign; (1) visible as white disks; (2) project above body surface; (3) prominent but not sharp; and (4) prominent and sharp. Frequency of each stage is shown, by treatment. Fisher's Exact test, $p = 0.0476$, $n = 5$ per treatment.

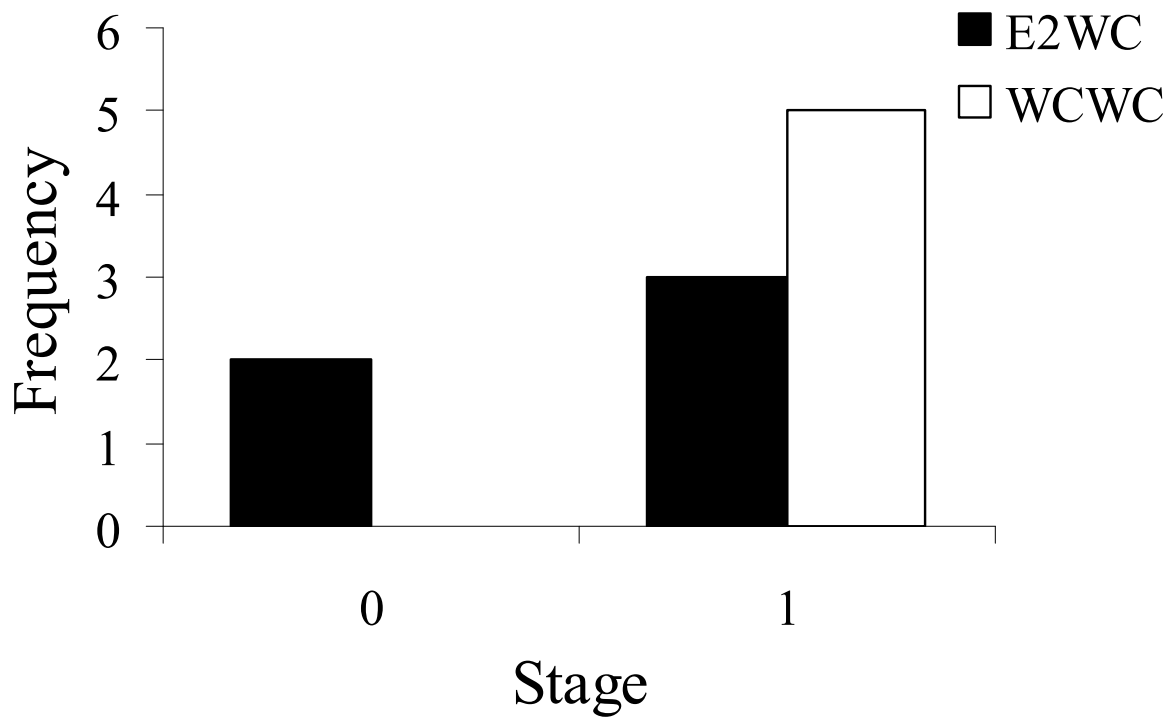


Fig. 1.17. Gonadal developmental stage of male red shiners (*Cyprinella lutrensis*) after four week depuration from 120 ngL⁻¹ 17β-estradiol exposure (E2WC) and water control (WCWC) treatments. Stage was assigned according to presence or absence of sperm, where 0 = sperm absent and 1 = sperm present. Fisher's Exact test, $p = 0.4444$ $n = 5$ per treatment.

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CHAPTER 2: EFFECTS OF 17β -ESTRADIOL ON MATING BEHAVIOR AND FEMALE
MATE CHOICE OF THE RED SHINER (*CYPRINELLA LUTRENSIS*)

ABSTRACT

Endocrine disrupting compounds (EDCs) are found worldwide in aquatic ecosystems and can lead to developmental and reproductive disruption in fishes. The estrogenic EDC 17 β -estradiol (estradiol) is a natural hormone found in wastewater effluent-treated waters, and thus may impact Great Plains fishes in the urban Front Range of Colorado. The goals of my study were to determine how estradiol affected physical, morphometric, and behavioral mating characteristics of male red shiners (*Cyprinella lutrensis*), and to assess whether such changes elicited changes in female mate choice. For a period of four weeks, mature males were exposed to estradiol concentrations of 2.4 ngL⁻¹ or 120 ngL⁻¹. Subsequently, mate choice tests were conducted, male mating behavior was assessed, and morphometric and histopathological characteristics of males were compared with untreated control fish. We found that plasma concentrations of vitellogenin and hepatosomatic index values were increased in males exposed to the highest estradiol concentration. Spermatogenesis was reduced and gonadosomatic index was lower in males exposed to the highest concentration of estradiol, which may indicate lower reproductive potential. Behavioral trials indicated that the highest concentration of estradiol reduced male mating behaviors and was associated with fewer, less developed nuptial tubercles on male fish. However, females did not selectively choose control males over exposed males. Our study suggests that exposure to estradiol influences reproductive behavior, secondary sexual traits, and developmental processes of male red shiners, potentially having consequences for populations in the field.

INTRODUCTION

Chemicals known as endocrine disrupting compounds (EDCs) interfere with developmental and reproductive processes in fishes (Jobling et al. 1998; Kime 1998; Jobling and Tyler 2003; Bjerregaard et al. 2006; Cheek 2006). The effects of estrogenic EDCs on male fishes are well known and include production of vitellogenin, gonadal abnormalities (including development of female oocytes), alterations in hormone concentrations, reduction in sperm quality and quantity, the loss of secondary sexual characteristics, and reduction in courtship behaviors (Bjerselius et al. 2001; Bringolf et al. 2004; Pawlowski et al. 2004; Doyle and Lim 2005; Mills and Chichester 2005). Ultimately, these cellular and physiological changes in fish result in reduced reproductive success (Arcand-Hoy and Benson 1998; Shioda and Wakabayashi 2000; Sohoni et al. 2001; Zillioux et al. 2001; Mills and Chichester 2005; Sumpter 2005; Brown et al. 2007).

Concern has been raised over the possible detrimental effects of EDCs on endangered and threatened fish populations on the Great Plains of Colorado. These communities are increasingly being exposed to urban wastewater, cattle feed lots, and agricultural fields, which are known sources of EDCs (Jobling et al. 1998; Soto et al. 2004). A recent survey of the lower South Platte River in Colorado found detectable levels of pharmaceuticals, hormones, and other organic wastewater contaminants that are known or suspected to be hormonally active (Sprague and Battaglin 2005). The environmental concentration of the estrogenic hormone 17β -estradiol (estradiol) has been estimated to be less than 200 ngL^{-1} , with a median detectable concentration of 160 ngL^{-1} in the United States (Kolpin et al. 2002). In Boulder Creek, Colorado, concentrations of estradiol have been reported to reach 2.9

ngL⁻¹, with average and maximum estrogenicity equivalents of 29 and 50 ngL⁻¹, respectively (Vajda et al. In press).

Sexual behaviors are closely tied to spawning potential and can be reduced with exposure to EDCs, which affects population structure and dynamics (Oshima et al. 2003). Morphometric characteristics such as tubercle development also influence mating efficiency and sexual selection (Jalabert et al. 2000), and can serve as cues for a high-quality mate (Kortet et al. 2004). In this study, we conducted experiments to assess the effects of estradiol on the red shiner (*Cyprinella lutrensis*), exposing them to nominal concentrations of 2.4 and 120 ngL⁻¹. Red shiners were selected as the study species because they are native to Colorado and have advantages for laboratory-based studies utilizing reproduction as an endpoint; they readily reproduce in the laboratory, are a highly tolerant species, and are a member of a common family (Cyprinidae) of Great Plains fishes (Gale 1986; Matthews 1986; Matthews et al. 2001). The objectives of the present study were to determine whether exposure to estradiol affected male development and courtship behavior in the red shiner, and if changes translated to alterations in mate choice by females.

MATERIALS AND METHODS

Our study consisted of a 28-32 d adult male exposure, and was comprised of four treatment groups: 120 ngL⁻¹ estradiol (HE2), 2.4 ngL⁻¹ estradiol (LE2), solvent control (SC, 0.00002 % ethanol), and water control (WC). Concentrations are expressed as target, or nominal, concentrations. The experimental setup used a flow-through system of four glass head tanks (20.8-L; one per treatment) that continually supplied water to glass exposure aquaria holding five males (20.8 L; $n = 4$ aquaria per treatment; Fig. 2.1). Twenty male red

shiners were exposed to each treatment for a total of 28-32 d and were examined for individual changes, behavioral effects, and how the effects translated to female mate choice.

Organisms

Adult red shiners were seined from the Arkansas River on the Granada State Wildlife Area, CO, USA (N 38.05°, W 102.23°) and transported to the Foothills Fisheries Laboratory at Colorado State University (Fort Collins, CO, USA). Fish were held for approximately 8.2 months before exposure, in 2.8 L glass aquaria (females and supplementary males in 110 L glass aquaria) with continuous flows of UV-disinfected well and lake water. The photoperiod corresponded to the natural Arkansas River photoperiod, reaching 16:8 L:D prior to the experiment. During the holding period, water temperatures corresponded with Arkansas River seasonal changes, gradually increasing from 7.8 °C to 22.0 °C over a six month period. Fish were fed once daily *ad libitum* with Aquatox commercial flake food (Aquatic Eco-systems, Apopka, FL) (Viant et al. 2006) throughout holding and experimental portions of the study. Fish used in this experiment were maintained according to guidelines established by the Institutional Animal Care and Use Committee of Colorado State University (approval number 05-264A-01).

Test chemical and exposure treatments

Concentrated stock solutions for the HE2 and LE2 treatments were prepared at the beginning of the study by dissolving the appropriate amount of estradiol (Sigma, St. Louis, MO, USA) in ethanol (EtOH). The concentrated stock solutions were used to make secondary water-based stock solutions every other day by adding 400 µL of solution to 4 L of

well water in a 4 L covered glass flask. These solutions were continuously distributed (rate 0.34 L min^{-1}) by variable speed peristaltic pumps through platinum-cured silicone tubing into glass head tanks (20.8 L) and combined with continuous flows of water (0.14 L min^{-1}), producing the HE2 and LE2 treatment concentrations (120 and 2.4 ngL^{-1} , respectively). A solvent control (SC) treatment was made and distributed in the same manner, adding $400 \mu\text{L}$ ml 99% EtOH stock solution to 4 L of well water (0.00002% EtOH). The water control (WC) treatment utilized the same well water. Water from treatment head tanks was continuously distributed to individual exposure aquaria through Teflon capillary tubes, glass funnels, and Teflon-lined tubing, achieving 99.99% replacement in 24 h. Water chemistry remained constant between treatments over the duration of the experiment (nitrate = non-detectable, nitrite = non-detectable, ammonia $< 0.3 \text{ mgL}^{-1}$, pH = 8, alkalinity = 150 mgL^{-1} , and total hardness = 240 mgL^{-1}). Incoming water had a dissolved carbon concentration of 2.84 mgL^{-1} .

The concentration of estradiol in each treatment was measured immediately before exposure began and every week during the exposure period. Water chemistry was analyzed by enzyme-linked immunosorbant assay (ELISA) and also by radioimmunoassay (RIA). When analyzed by ELISA, a 60 mL syringe was rinsed with methanol (30 mL) and distilled water (30 mL), and then filled with water from each treatment group head tank (60 mL). The water sample was passed through a tC18 cartridge filter (Waters, Milford, MA) attached to the tip of the syringe. Cartridges were refrigerated until analysis, when they were extracted with methanol and analyzed by ELISA (Abraxis, Warminster, PA, USA) according to the manufacturer's protocols. When water chemistry was analyzed by RIA (England and Niswender 1974; Niswender et al. 1976), it was completed by the Animal Reproduction and

Biotechnology Laboratory at Colorado State University. Samples of exposure tank water were pipetted into glass vials (10 mL), and extractions were done using 6 mL per sample, reconstituted in 600 µl of PBS-Gel and with diethyl ether as the solvent.

Exposure conditions of the male red shiner

At the completion of the holding period, we identified mature males using spawning coloration. On day one of the experiment, 20 mature males were chosen randomly, measured (total length, mm), weighed (g) and allocated into one glass spawning aquarium (20.8-L) per treatment. Thereafter, every week for four weeks, 20 mature males were chosen at random and allocated to one aquarium per treatment (five fish per aquarium, four treatments, four aquaria per treatment) to facilitate staggered evaluation. Female fish were maintained in separate aquaria with control water. Male exposure continued for 28 d before testing began. During exposure, photoperiod was held at 16:8 (L:D) and temperature was 22.1 ± 0.6 °C.

Mate choice tests

Mate choice arenas were constructed within glass aquaria (75.7 L). The inside of each aquarium was separated into three compartments by partitions of clear glass edged with stainless steel screen (Fig. 2.2) to allow water exchange. The center compartment contained the female and 50% of the arena space. Within the center compartment, the outer 20% of the space on both sides was called a preference zone, divided by black lines on the outside of the aquarium. These zones were used to indicate a preference for the male on the other side of the partition. The inner 60% of the center compartment was considered a “no-preference” zone and indicated that the female had no male preference (Ryan and Wagner 1987; Howard

et al. 1998). Mate choice arenas were static systems with uniform lighting, had no food dispensed, and were aerated only during the acclimatization period. A brick was placed in each outside compartment to provide spawning substrate for the males. Water was changed prior to every mate choice trial. Each aquarium was visually isolated by black curtain and observations were made via digital video camera.

Each treatment combination (Table 2.1) was tested four times. For each test, males of comparable size were randomly chosen from exposure aquaria and placed into one of the two outside compartments. An unexposed female was gently placed into a removable opaque cylinder in the center of the aquarium (Ryan and Wagner 1987). Fish were allowed to acclimate without visual contact for 20 minutes (Reichard et al. 2005). The opaque cylinder was removed at the end of the non-visual acclimation period, and fish were allowed to acclimate visually for an additional five minutes. During the 20-minute trial, the time the female spent in each male's preference zone was measured. Each trial was run in duplicate with males rotated between arena sides to test for side preferences (Ryan and Wagner 1987; Howard et al. 1998; Wong and Jennions 2003).

Male behavior

Mate choice test video recordings were reviewed to quantify male behavior during the intermediate ten minutes of each trial. The frequency of each behavior was measured. Behaviors assessed included quivers, charges, up/downs, and back/forths. These behaviors were chosen based on observations of red shiner (Gale 1986) and other species exhibiting courtship behaviors (Ono and Uematsu 1957; Bjerselius et al. 2001; Oshima et al. 2003; Moretz and Rogers 2004), but were modified due to lack of physical contact between males

and females in our study. “Quivers” occurred when the male body undulated or vibrated with fins extended, typically into a crevice (Gale 1986; Moretz and Rogers 2004). “Charges” occurred when the male rapidly accelerated toward the female until reaching the glass partition. “Up/downs” occurred when the male moved in a vertical direction up and down the aquarium, covering at least one-half of the vertical distance of the aquarium, often following the female. “Back/forths” occurred when the male moved in a horizontal direction from the glass partition to the spawning substrate and returned to the partition. Only quivers were analyzed statistically; because there was no direct contact between male and female, they were the only behavior unquestionably tied to courtship.

Secondary sexual characteristics

Tubercle number and developmental stage were measured on male red shiners preserved in 10% neutral buffered formalin. A microscope (0.8x magnification) equipped with Image-Pro Express (Version 5.0.1.26) software was used to capture a digital image of the dorsal surface of the head, from the posterior edge of the supraoccipital crest to the anterior tip of the snout. Number of visible tubercles was counted, and tubercle developmental stage was scored according to the most highly developed tubercles. Tubercle developmental stages were as follows: (0) no visible sign; (1) visible as white disks; (2) project above body surface; (3) prominent but not sharp; and (4) prominent and sharp (Smith 1978; Chapter 1, Fig. 1.4).

Vitellogenin, morphometric, and histological analyses

After each adult was measured and weighed, blood was collected in heparinized capillary tubes via caudal transection (Houston 1990). Samples were centrifuged for three minutes at 11,700 rpm (13,700 g) and the plasma was stored at -20°C until assayed for vitellogenin by homologous enzyme-linked immunosorbent assay using an anti-carp kit (Biosense, Bergen, Norway; batch number 0701) in accordance with the manufacturer's protocol. The kit was validated for use with the red shiner.

Fish were sacrificed by rapid decapitation and the liver and gonads were removed and weighed (g) to determine gonadosomatic index (GSI) and hepatosomatic index (HSI), respectively. All tissues were stored in 10% neutral buffered formalin for histological analysis. Fixed gonads were dehydrated through a graded series of ethanol, cleared in xylene, embedded in paraffin, sectioned (thickness, 10 µm) using a microtome, mounted on glass slides, rehydrated, and stained with hematoxylin and eosin (Presnell and Schreiber 1997; Woodling et al. 2006). The gonadal sections were examined under a light microscope for stage and maturation of each individual without knowledge of treatment group. Gonadal staging was done based on Vajda et al. (In press) and Pawlowski et al. (2004). Sperm abundance within seminiferous tubules of mature males was assessed semi-quantitatively, where 0 = sperm insignificant (< 25%), 1 = low (25-49%), 2 = moderate (50-75%), and 3 = high (> 75%).

Statistical analyses

All statistical analyses were performed using SAS version 9.1 (SAS Institute, Cary, NC, USA). Variables were log or square-root transformed to comply with the assumption of

normality if necessary. Experimental data were checked for homogeneity of variance across treatments using Levene's test. If parametric, data were analyzed using one-way analysis of variance (ANOVA). If nonparametric, the Kruskal-Wallis test was used to detect differences among treatments. When distributions were tested, Fisher's Exact test was used. When necessary, post-hoc Tukey's HSD method was used to test multiple comparisons. Mate choice tests analyzed time spent with a female using the general linear model program Proc Mixed for an ANOVA. Fixed effects were by treatment, week, and treatment of male tested against. Random effects were aquarium within treatment and the residual error. Only weeks two, three, and four were used in mate choice and behavioral analysis due to changes in methodology after week one. In all tests, the level of significance used was 0.05.

RESULTS

Fish survival was > 90% for all treatments during the exposure period. There were no significant differences among treatments in weight, total length, or condition factor of males at the beginning or conclusion of the study (Table 2.2). Waterborne concentrations of estradiol in the HE2 treatment for the first three weeks of the study (RIA method) were 83% to 246% (mean = 136%, SD = 76%, $n = 4$) of nominal values. Concentrations of estradiol in the LE2 treatment for the first three weeks of the study (RIA method) were 30 to 295% (mean = 150%, SD = 113%, $n = 4$) of nominal values. Concentrations of estradiol were not detectable in water samples of SC ($n = 4$) and WC ($n = 4$) treatments (detection limit 1.51 ngL⁻¹). Waterborne concentrations of estradiol in the HE2 treatment during the final six weeks were higher than expected at 206 to 444% (mean = 322%, SD = 90%, $n = 6$) of

nominal values. In the LE2, SC, and WC treatment groups, concentrations of estradiol were 16.1 ± 9.5 , 4.5 ± 2.1 , and 2.2 ± 4.9 ngL⁻¹, respectively, with a detection limit of 2.5 ngL⁻¹.

Estradiol had an effect on the behavior and secondary sexual characteristics of male fish. Quiver behaviors were decreased in males of the HE2 treatment ($p = 0.0513$, Kruskal-Wallis test; Fig. 2.3). See Fig. 2.4 for means associated with other behaviors. The number of tubercles on HE2 treatment fish was significantly lower than LE2 and the WC treatment fish; SC, LE2, and WC treatment fish were similar ($p = 0.0085$, Kruskal-Wallis test; Fig. 2.5). Tubercles seen on HE2 treatment fish exhibited a developmental stage at or below two, while LE2 and SC treatment fish exhibited tubercles at ranging from stage zero to four, and WC treatment fish exhibited tubercles ranging from stage one to four (Fig 2.6). The distribution of tubercle stages between treatments was significantly different ($p = 0.0012$, Fisher's Exact test).

Male plasma vitellogenin concentration was significantly higher in the HE2 treatment than the LE2, SC, and WC treatments ($p = 0.0002$, Kruskal-Wallis test; Fig. 2.7). Male gonadal stage ranged between one and three for all treatments, but the majority of gonads were at stage one in the HE2 treatment, compared with a majority in stage two in the LE2, SC, and WC treatments (Fig. 2.8). The distribution of gonad stage was significantly different among treatments ($p = 0.0345$, Fisher's Exact test). The GSI of HE2 treatment males (1.363 ± 0.095) was significantly lower than the GSI of LE2 males (1.925 ± 0.122), SC males (1.669 ± 0.114), and WC males (1.777 ± 0.101) ($p = 0.0002$, ANOVA test). The HSI of HE2 treatment males (1.788 ± 0.143) was significantly higher than the HSI of LE2 males (1.170 ± 0.102) and WC males (1.222 ± 0.058), but not SC males (1.669 ± 0.069) ($p = 0.0054$, ANOVA test). Data are reported as the mean \pm SEM.

Exposure to estradiol did not significantly affect female choice, as the female time spent with LE2 and HE2 treatment males did not differ from controls; however, there was an unexplained significant difference between the SC and WC treatments ($p = 0.0169$, Proc Mixed; Fig. 2.9).

DISCUSSION

Our study shows that exposure of adult male red shiners to an environmentally relevant level of an estrogenic compound causes pronounced changes in reproductive characteristics and reductions in courtship behavior. Male exposure to our HE2 treatment led to adverse physiological effects such as elevated vitellogenin, decreased sperm abundance and gonad size, inhibition of development of mating tubercles, and decreased spawning behavior. Our findings are similar to other studies where estradiol exposure resulted in less-developed gonads (Bringolf et al. 2004), delayed spermatogenesis (Billard et al. 1981; Kang et al. 2002), decreased amounts of expressible milt (Bjerselius et al. 2001; Schoenfuss et al. 2002), and decreased gonad size (Toft and Baatrup 2001; Bringolf et al. 2004). Exposed male red shiners also exhibited reduced gonadosomatic index and gonad developmental stage, which was probably due to decreases in androgens that are necessary for spermatogenesis (Demski and Hornby 1982; Schulz and Miura 2002). In adult guppies (*Poecilia reticulata*), experimental reduction in androgen resulted in regression of the testes, blocked spermatogonial mitosis, and no progression of cells into spermatocytes (Pandley 1969).

Secondary sexual traits, such as coloration and tubercles, and mating behavior of male fish are important cues to female fish during the mating season. Male red shiners

exposed to the highest concentration of estradiol had significantly fewer and less developed tubercles when compared to the other treatments. Other studies show that estradiol inhibits tubercles in male fish (Bjerselius et al. 2001; Bringolf et al. 2004). Male red shiners also showed fewer quivering behaviors in our high estradiol concentration. This is consistent with other studies, where courtship behaviors of males were reduced after exposure to estradiol (Bayley et al. 1999; Doyle and Lim 2002; Schoenfuss et al. 2002; Doyle and Lim 2005) or ethynylestradiol (EE2) (Bell 2001; Kristensen et al. 2005). For example, male Japanese medaka (*Oryzias latipes*) exposed to estradiol showed suppression of sexual behavior when paired with an estradiol-unexposed receptive female (Oshima et al. 2003). Estradiol may alter male behavior by affecting receptors used to detect pheromones released by females (Bjerselius et al. 2001) and reducing androgen production that controls aspects of sexual behavior (Toft and Baatrup 2003; Martinovic et al. 2007).

Spawning behaviors are critical for final mating cues and onset of spawning (Shioda and Wakabayashi 2000; Oshima et al. 2003) and are synchronized by reproductive hormones (Kobayashi et al. 2002). Therefore, decreased mating behaviors in males exposed to estrogenic compounds should be associated with a lack of female interest, because mate choice is driven by physical and behavioral characteristics (Ishikawa and Mori 2000; Maan et al. 2004; Maan et al. 2006). However, we did not find that females preferred control males over estradiol-exposed males in our mate choice experiments. This result was counter-intuitive, given the dramatic reproductive effects observed in males from the highest estradiol concentration. Lack of female mate choice difference may have been due to the fact that the experimental setup did not allow for physical contact between male and female fish. Such contact may be necessary for pheromone detection and reproductive stimulation. In addition,

some females appeared to be more gravid than others (deeper body shape), which may have contributed to the high level of variability in female behaviors measured in the experiment.

The adverse effects on secondary sexual traits and mating behaviors observed in male red shiners suggest that estrogenic EDCs have the potential to impact Great Plains fishes in eastern Colorado. Other studies have suggested that development and reproduction of Plains fishes may be impacted by estrogenic wastewater treatment plant (WWTP) effluent. For example, Boulder Creek near Boulder, CO has a total estrogen equivalence as high as 29 ngL⁻¹, and white suckers (*Catostomus commersonii*) exposed to this water have shown gonadal intersex, altered sex ratios, reduced gonad size, disrupted ovarian and testicular histopathology, and vitellogenin induction (Vajda et al. In press). Adult male fathead minnows (*Pimephales promelas*) showed fewer, less prominent nuptial tubercles, reduced sperm abundance, and elevated vitellogenin within 14 d with > 50% effluent below a Boulder WWTP (A. Vajda, University of Colorado, personal communication). Endocrine disruption has also been observed in male white suckers in the South Platte River downstream of a WWTP in Denver, CO (Woodling et al. 2006). In general, streams in the Eastern plains of Colorado are dominated by WWTP discharges, which may result in chronic exposure of plains fishes to estrogenic compounds (Woodling et al. 2006). Our study suggests that exposure to an EDC may alter reproductive fitness which could have implications for wild populations of Great Plains fishes.

TABLES AND FIGURES

Table 2.1. Mate choice test trial treatment combinations, where the adult male red shiner (*Cyprinella lutrensis*) experimental treatments include 120 ngL⁻¹ 17β-estradiol (HE2), 2.4 ngL⁻¹ 17β-estradiol (LE2), solvent control (SC, 0.00002 % ethanol), and water control (WC) treatments. Trial combinations tested are denoted by an “X” and duplicate trial combinations are denoted by “-.”

Treatment	HE2	LE2	SC	WC
HE2	X	-	-	-
LE2	X	X	-	-
SC	X	X	X	-
WC	X	X	X	X

Table 2.2. Exposure weight (g), total length (TL, mm), and condition factor (CF) of adult male red shiner (*Cyprinella lutrensis*) prior to the experiment (initial) and at the conclusion of the study (final) after exposure to 120 ngL⁻¹ 17β-estradiol (HE2), 2.4 ngL⁻¹ 17β-estradiol (LE2), solvent control (SC, 0.00002 % ethanol), and water control (WC) treatments. Each treatment value represents the mean ± standard deviation. Variables were tested using ANOVA. All tests used $\alpha = 0.05$.

Time	Variable	Treatment				P-value
		HE2	LE2	SC	WC	
Initial ^a	Weight (g)	4.34 ± 1.30	4.07 ± 1.20	4.07 ± 1.12	3.93 ± 1.00	0.89
	TL (mm)	71 ± 6.3	70 ± 6.4	70 ± 6.6	69 ± 6.3	0.96
	CF	1.18 ± 0.08	1.15 ± 0.12	1.17 ± 0.17	1.15 ± 0.11	0.79
Final ^b	Weight (g)	3.94 ± 1.16	4.22 ± 1.38	3.94 ± 1.11	3.95 ± 0.89	0.90
	TL (mm)	71 ± 6.0	71 ± 6.6	71 ± 6.2	71 ± 5.9	0.98
	CF	1.08 ± 0.11	1.12 ± 0.11	1.07 ± 0.13	1.10 ± 0.10	0.51

^a n = 20 per treatment

^b n = 18 (HE2), 19 (LE2), 20 (WC and SC)

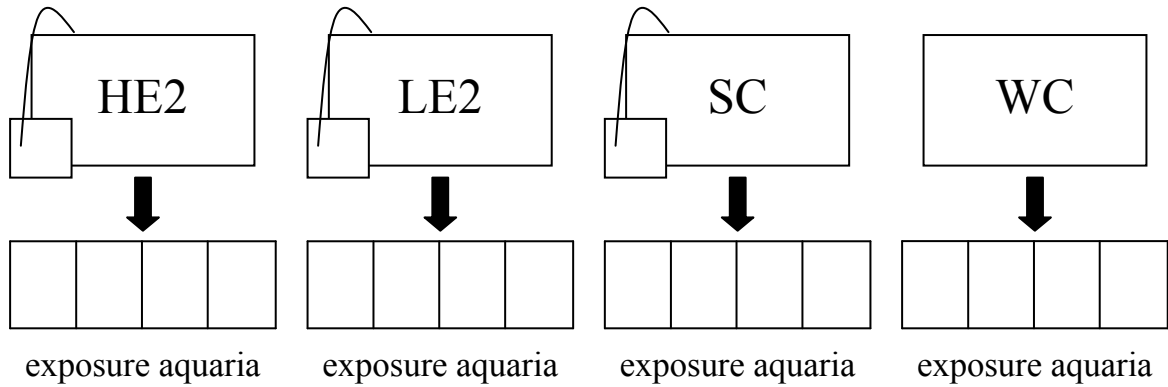


Fig. 2.1. Experimental setup. Water flowed into secondary glass head tanks where it was mixed with stock solutions via peristaltic pumps for the experimental treatments 120 ngL^{-1} 17β -estradiol (HE2), 2.4 ngL^{-1} 17β -estradiol (LE2), and solvent control (SC, 0.00002 % ethanol), or mixed without a stock solution to produce the water control (WC) treatment. Water from each treatment was delivered to four spawning aquaria, each holding five male fish for the experiment.

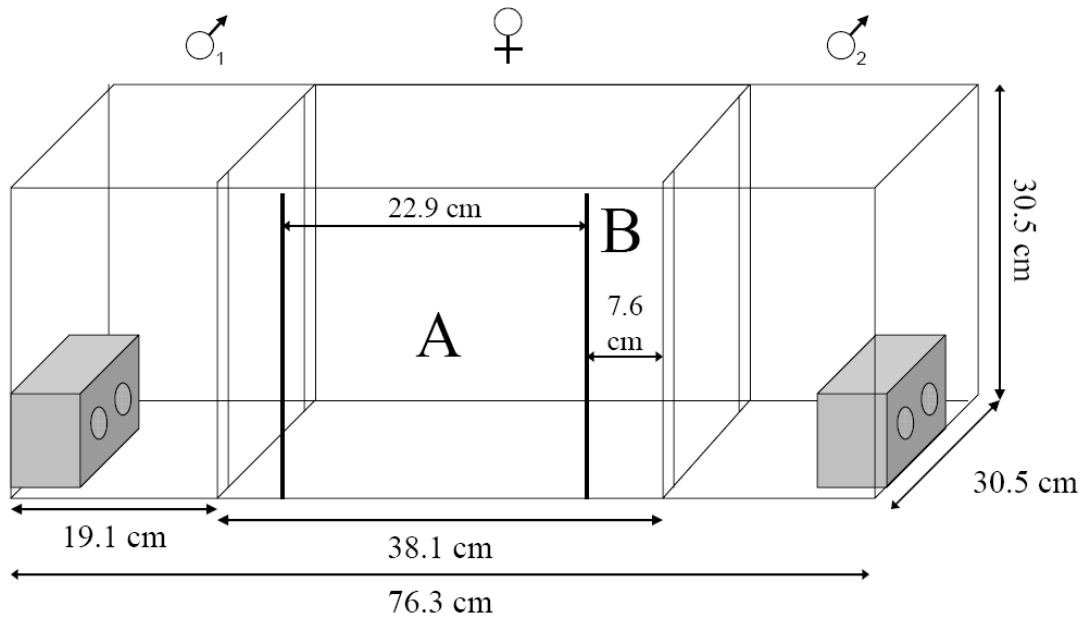


Fig. 2.2. The aquarium setup for mate choice trials comprised of a center area and two side compartments. The female was released into the center and the time spent in each male's preference zone was recorded. (A) Female no-preference zone, the inner 3/5 of the center compartment. (B) Preference zone for male 2, 1/5 of the center compartment space.

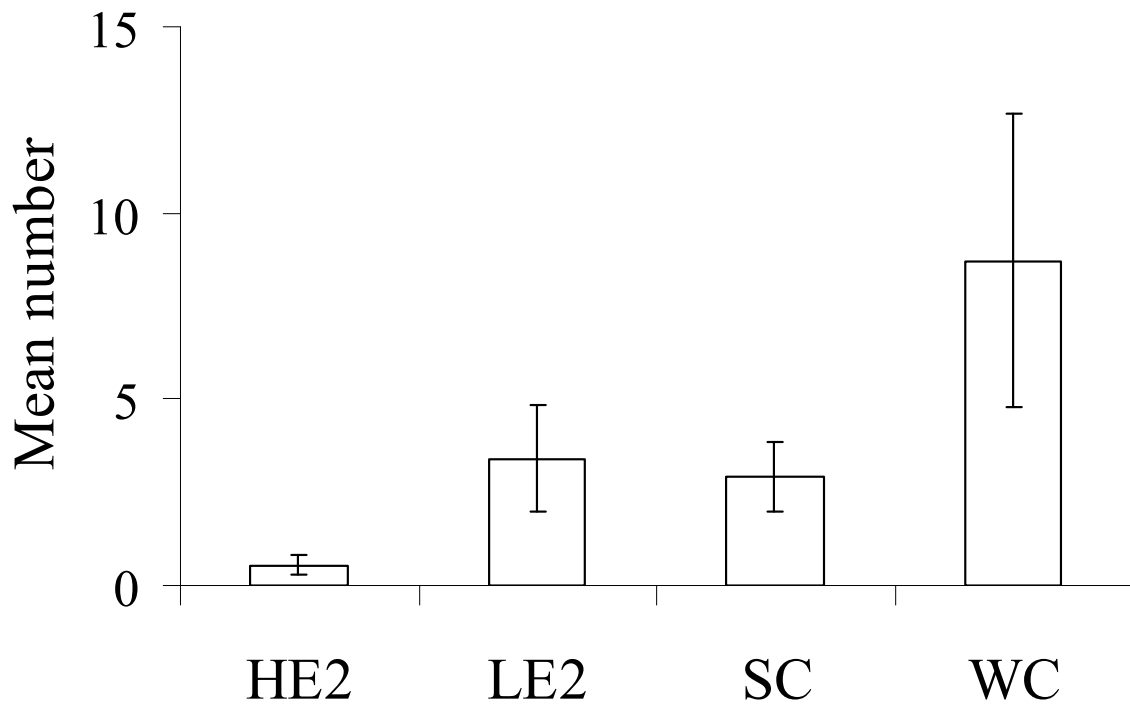


Fig. 2.3. Quiver courtship behaviors of male red shiners (*Cyprinella lutrensis*) after 28-32 d exposure to 120 ngL^{-1} 17β -estradiol (HE2), 2.4 ngL^{-1} 17β -estradiol (LE2), solvent control (SC, 0.00002 % ethanol), or water control (WC). Means \pm SEM are shown. Kruskal-Wallis test, $p = 0.0513$, $n_{\text{LE2, WC}} = 10$, $n_{\text{HE2, SC}} = 11$.

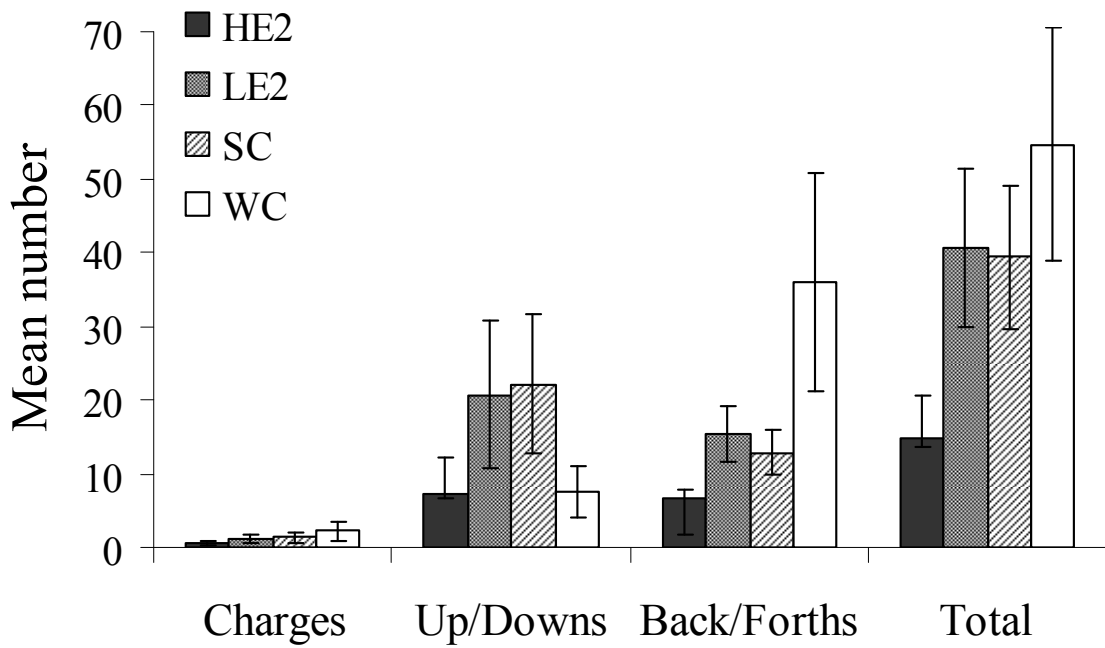


Fig. 2.4. Courtship behaviors of male red shiners (*Cyprinella lutrensis*) after 28-32 d male exposure to 120 ngL^{-1} 17β -estradiol (HE2), 2.4 ngL^{-1} 17β -estradiol (LE2), solvent control (SC), or water control (WC). Charges, up/downs, back/forths, and total behaviors seen are shown by means \pm SEM ($n_{\text{LE2, WC}} = 10$, $n_{\text{HE2, SC}} = 11$).

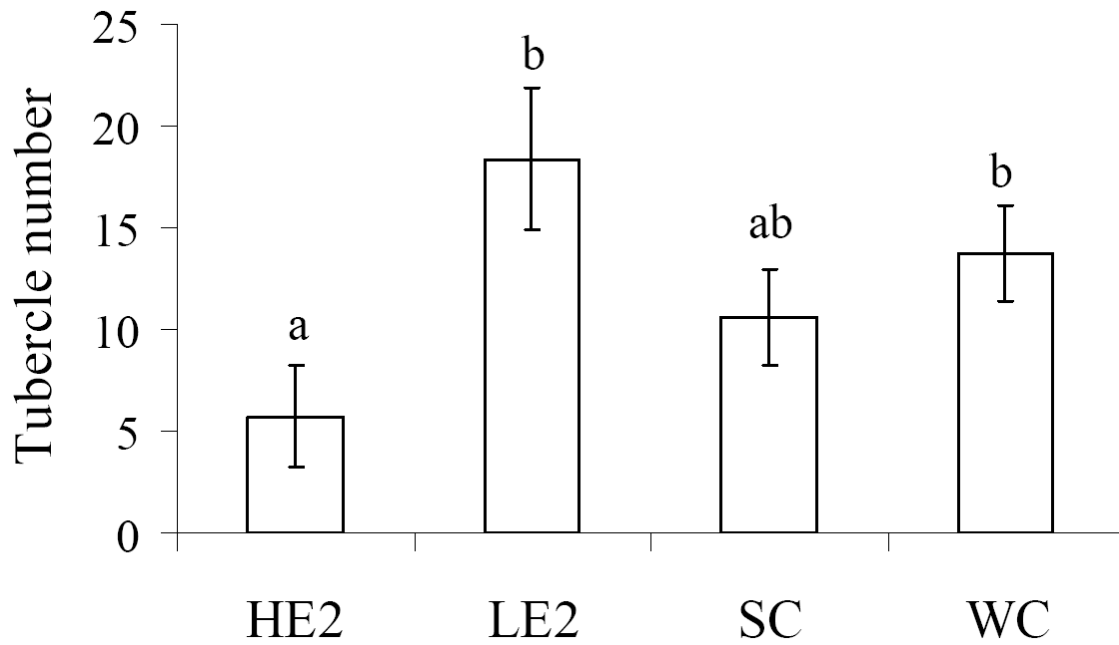


Fig. 2.5. Tubercle numbers of male red shiners (*Cyprinella lutrensis*), after 28-32 d male exposure to 120 ngL^{-1} 17β -estradiol (HE2), 2.4 ngL^{-1} 17β -estradiol (LE2), solvent control (SC, 0.00002 % ethanol), or water control (WC). Means \pm SEM are shown; values with different lowercase letters indicate statistical significance ($p < 0.05$). Kruskal-Wallis test, $n_{\text{HE2}} = 18$, $n_{\text{LE2}} = 19$, $n_{\text{SC, WC}} = 20$.

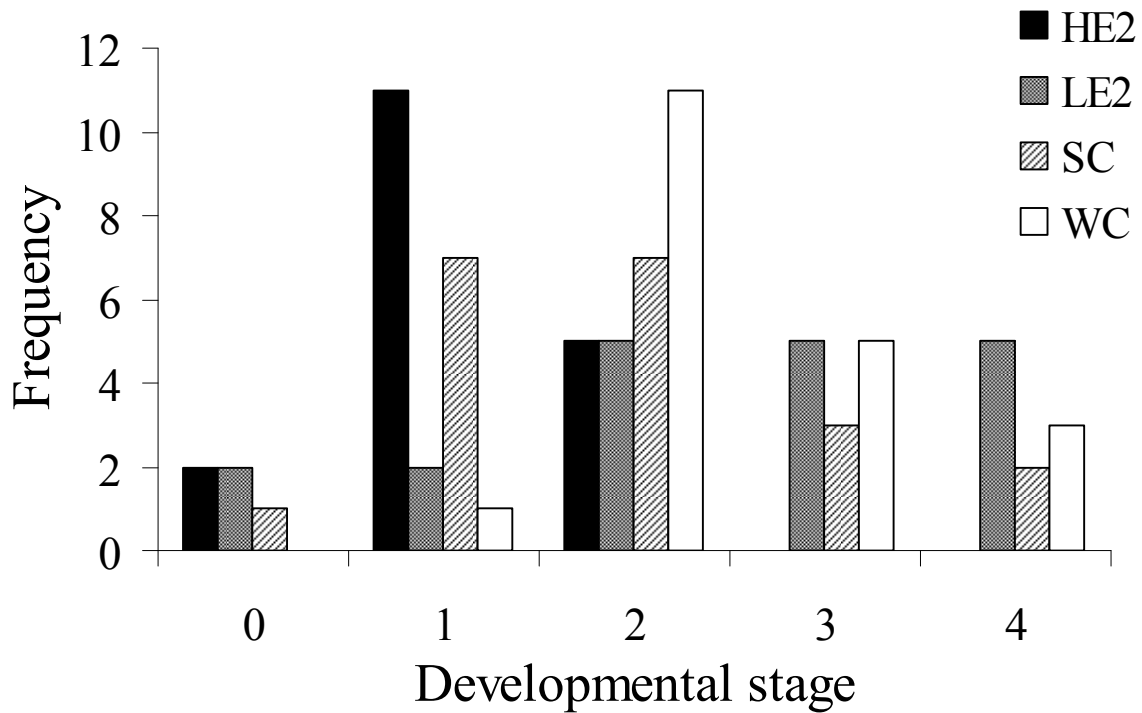


Fig. 2.6. Tubercle developmental stage of male red shiners (*Cyprinella lutrensis*) after 28-32 d male exposure to 120 ngL^{-1} 17β -estradiol (HE2), 2.4 ngL^{-1} 17β -estradiol (LE2), solvent control (SC), or water control (WC). Stage was assigned according to the most highly developed tubercles: (0) no visible sign; (1) visible as white disks; (2) project above body surface; (3) prominent but not sharp; and (4) prominent and sharp. Frequency of each stage is shown, by treatment. Fisher's Exact test, $p = 0.0012$, $n_{\text{HE2}} = 18$, $n_{\text{LE2}} = 19$, $n_{\text{SC, WC}} = 20$.

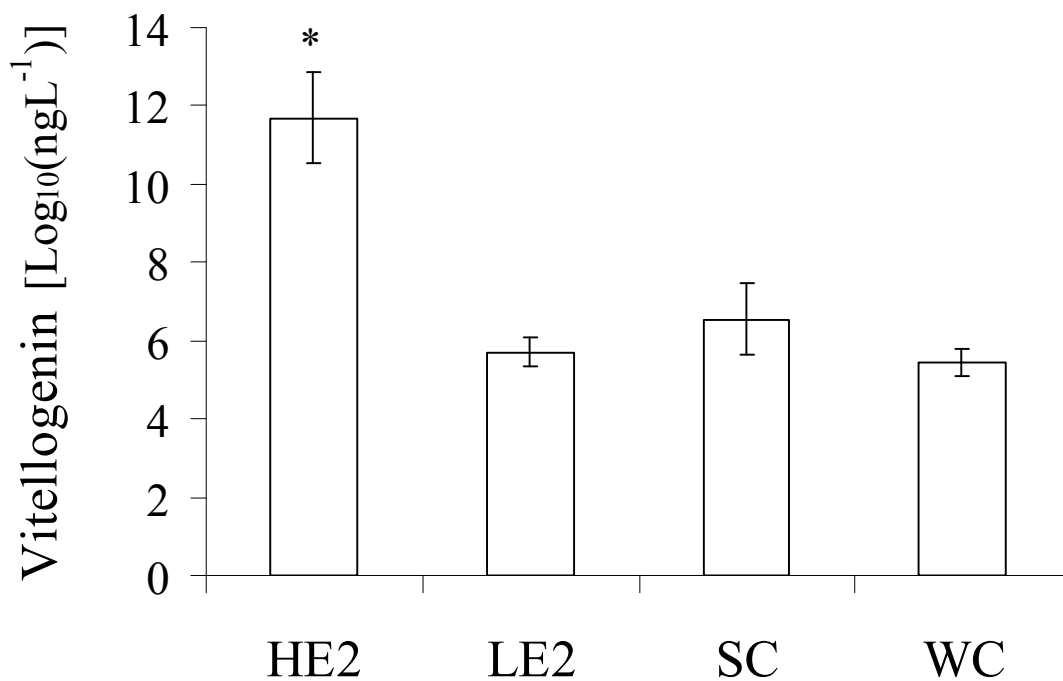


Fig. 2.7. Vitellogenin plasma concentrations in adult male red shiner (*Cyprinella lutrensis*) after 28-32 d male exposure to 120 ngL⁻¹ 17β-estradiol (HE2), 2.4 ngL⁻¹ 17β-estradiol (LE2), solvent control (SC, 0.00002 % ethanol), or water control (WC). Means ± SEM are shown; asterisks indicate statistical differences from the WC treatment ($p < 0.05$). Kruskal-Wallis test, $n_{HE2} = 15$, $n_{LE2} = 20$, $n_{SC} = 17$, $n_{WC} = 19$.

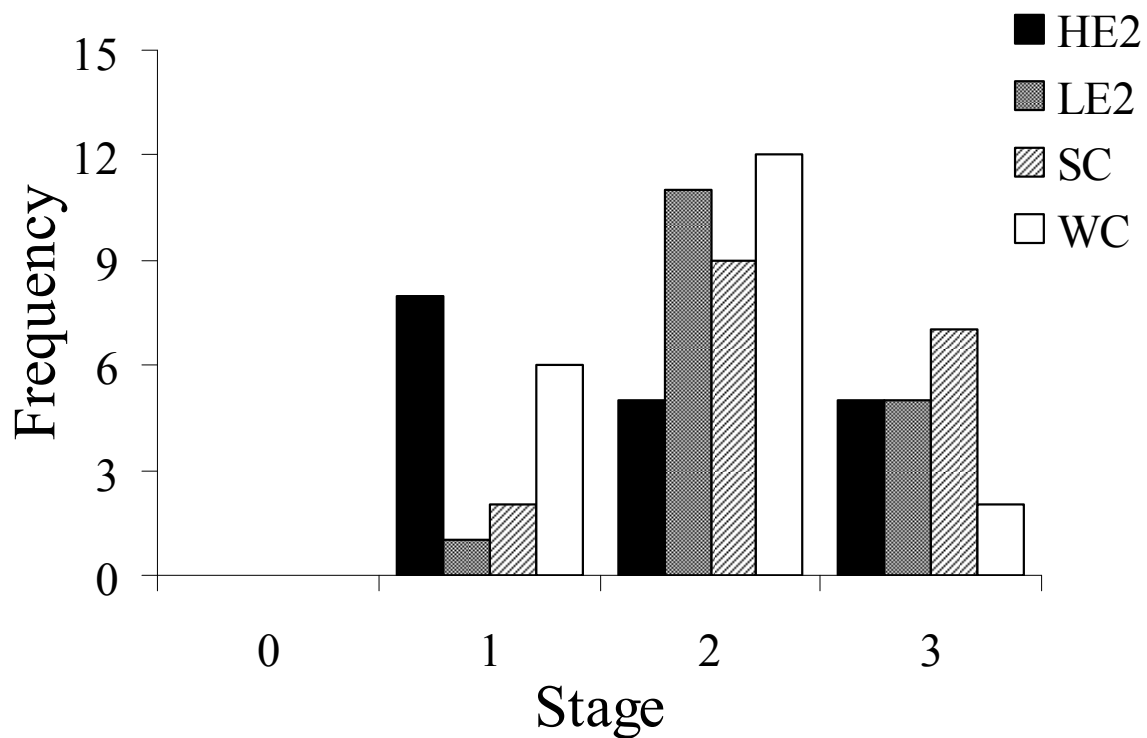


Fig. 2.8. Gonadal developmental stage of male red shiners (*Cyprinella lutrensis*) after 28-32 d male exposure to 120 ngL^{-1} 17β -estradiol (HE2), 2.4 ngL^{-1} 17β -estradiol (LE2), solvent control (SC), or water control (WC). Stage was assigned semi-quantitatively, where 0 = sperm insignificant (< 25%), 1 = low (25-49%), 2 = moderate (50-75%), and 3 = high (> 75%). Frequency of each stage is shown, by treatment. Fisher's Exact test, $p = 0.0345$, n_{HE2} , $n_{\text{LE2}} = 17$, $n_{\text{WC}} = 20$.

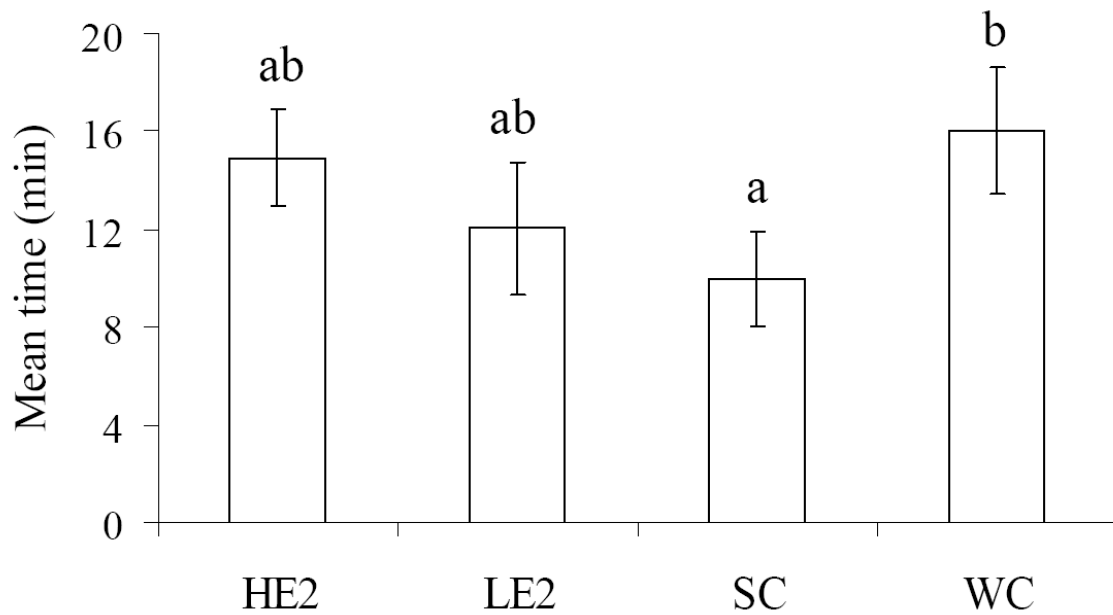


Fig. 2.9. Female time spent with males of each treatment (120 ngL^{-1} 17β -estradiol (HE2), 2.4 ngL^{-1} 17β -estradiol (LE2), solvent control (SC), or water control (WC)) after 28-32 d male exposure. Means \pm SEM are shown; values with different lowercase letters indicate statistical significance ($p < 0.05$). Proc Mixed, $n_{\text{HE2}} = 11$, $n_{\text{LE2}} = 13$, $n_{\text{SC, WC}} = 14$.

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