Reproductive failure of the red shiner (*Cyprinella lutrensis*) after exposure to an exogenous estrogen

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Abstract: Endocrine disrupting chemicals (EDCs) have been detected in surface waters worldwide and can lead to developmental and reproductive disruption in exposed fishes. In the US Great Plains, EDCs are impacting streams and rivers and may be causing adverse reproductive effects. To examine how estrogenic EDCs might affect reproductive success of plains fishes, we experimentally exposed male red shiners (*Cyprinella lutrensis*) to exogenous 17β-estradiol. We characterized the effects of estradiol on male gonadal histology and secondary sexual characteristics, determined whether exposure reduced reproductive success, and examined the effects of depuration. Adults were exposed to a mean concentration of 70 ng·L^{-1} estradiol, a solvent control, or a water control for at least 83 days. Male exposure to estradiol resulted in elevated plasma vitellogenin concentrations, changes in spermatogenesis, reduced mating coloration and tubercles, altered mating behaviors, and reduced reproductive success with no viable progeny produced. Reproductive endpoints improved upon depuration (28 days). 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 estradiol.

Résumé : Partout dans le monde, on a pu détecter des produits chimiques perturbateurs endocriniens (EDCs) dans les eaux de surface qui peuvent causer des dérèglements dans le développement et la reproduction des poissons exposés. Dans les Grandes Plaines des É.-U., les EDCs affectent les ruisseaux et les rivières et peuvent produire des effets négatifs sur la reproduction. Afin d'étudier de quelle manière les EDCs œstrogéniques peuvent affecter le succès reproductif des poissons des plaines, nous avons exposé expérimentalement des ides américains à nageoires rouges (*Cyprinella lutrensis*) mâles à de l'estradiol-17β exogène. Nous avons décrit les effets de l'estradiol sur l'histologie des gonades mâles et les caractéristiques sexuelles secondaires, déterminé si l'exposition réduisait le succès reproductif et observé les effets de la dépuration. Des adultes ont été exposés à une concentration moyenne de 70 ng·L^{-1} d'estradiol, à un témoin avec solvant ou à un témoin d'eau pendant au moins 83 jours. L'exposition des mâles à l'estradiol cause une augmentation des concentrations de vitellogénine dans le plasma, des modifications de la spermatogenèse, une réduction de la coloration nuptiale et des tubercules, un changement dans le comportement d'accouplement et un succès reproductif réduit sans production de rejetons viables. Les effets sur la reproduction s'aménuisent durant la dépuration (28 jours). L'exposition à l'estradiol a des effets négatifs significatifs sur les ides américains à nageoires rouges, ce qui indique que les populations sauvages peuvent avoir à faire face à des problèmes de développement et de reproduction s'ils sont exposés à l'estradiol de manière chronique.

[Traduit par la Rédaction]

Introduction

Endocrine disrupting chemicals (EDCs) have been documented worldwide in freshwater, estuarine, and marine environments (Tyler et al. 1998; Mills and Chichester 2005). The physiological effects of EDCs on the reproductive organs and mating behaviors of individual fish are well known and are associated with reduced reproductive capacity (Kime 1995; van der Oost et al. 2003). However, experimental studies connecting EDCs, reproduction, and population declines are rare (Bruno et al. 2003; Jenkins et al. 2009). A notable exception is a whole-lake experiment that showed the addition of an estrogenic EDC at environmentally relevant concentrations led to reproductive failure and a population-level collapse of fathead minnow (*Pimephales promelas*) (Kidd et al. 2007). Laboratory studies linking fecundity with EDC exposure indicate that lower egg production, reduced egg viability, and poor hatching success could correspond to population-level effects (Shioda and Wakabayashi 2000; Zillioux et al. 2001; Nash et al. 2004). Under-

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standing the potential for EDCs to affect fish populations is a critical requirement for conservation and management strategies.

Potential sources of EDCs include wastewater treatment plants (WWTPs), livestock feedlots, and agricultural fields (Jobling et al. 1998; Soto et al. 2003. For example, pharmaceuticals, hormones, and other hormonally active organic contaminants have been documented below WWTPs worldwide (Desbrow et al. 1998; Jobling and Tyler 2003). Many rivers and streams below urban areas are dominated by WWTP effluent (Woodling et al. 2006), and it is a challenge to manage fish populations in these systems. For example, Great Plains rivers in the western United States are influenced by heavy urban and agricultural use (Dodds et al. 2004), contain EDCs, and are home to threatened populations of native fishes. In the South Platte River drainage, EDCs have been detected (Barnes et al. 2002; Kolpin et al. 2002) and fish with intersex gonadal tissues have been observed downstream from WWTP outfalls (Woodling et al. 2006; Vajda et al. 2008). Colorado plains fish populations have been declining for the last two decades (Propst 1982; Nesler et al. 1997), and EDCs may be contributing to these declines.

Further study of the relationship among EDCs, reproductive success, and population processes of native plains fishes is needed to understand if these compounds are contributing to population declines. Recent studies have demonstrated that male fathead minnow and white suckers (*Catostomus commersonii*), species native to Great Plains rivers, become dramatically feminized after exposure to EDCs in the South Platte basin (Vajda 2006; Vajda et al. 2008). Our goal was to test the response of another sexually dimorphic plains fish, the red shiner (*Cyprinella lutrensis*), to an EDC and determine if similar feminization would occur. Additionally, we wanted to investigate the effects of exposure on fecundity. Red shiners are small-bodied, short-lived fish that mature rapidly and naturally spawn in aquaria (Vives 1993). These traits make them good candidates for laboratory studies. Additionally, *Cyprinella* is one of the most abundant and widespread genera of cyprinids in North America, consisting of approximately 30 species (Schönhuth and Mayden 2010). Therefore, inferences regarding exposure to EDCs might be more broadly relevant to many species in the Great Plains and Southwest and provide important insight into how native plains fish species should be managed in relation to endocrine disruption.

We were also interested in the effects of EDCs on courtship behaviors and secondary sexual characteristics in relation to reproductive success. Red shiners are sexually dimorphic, and males display nuptial tubercles and increased coloration during spawning. Alteration in male appearance and behavior may ultimately affect reproduction. Therefore, we chose to focus our research on male responses to exposure and subsequent effects on reproductive success.

Our study was designed to answer three questions involving exposure of the red shiner to the common estrogenic compound 17β-estradiol (estradiol). First, we wanted to know if exposure affected individual male secondary sexual traits and reproductive physiology. Second, we wanted to know if egg deposition, fertilization success, hatching success, and male courtship behavior and components of reproductive capability were affected by exposure to estradiol. Finally, we wanted to determine if removal from chronic exposure to estradiol would allow male red shiners to recover from any diminished reproduction caused by exposure. Fish population responses following depuration were interpreted in light of possible management strategies in the field to reduce the potential for adverse effects of EDCs on the red shiner and other native plains fishes.

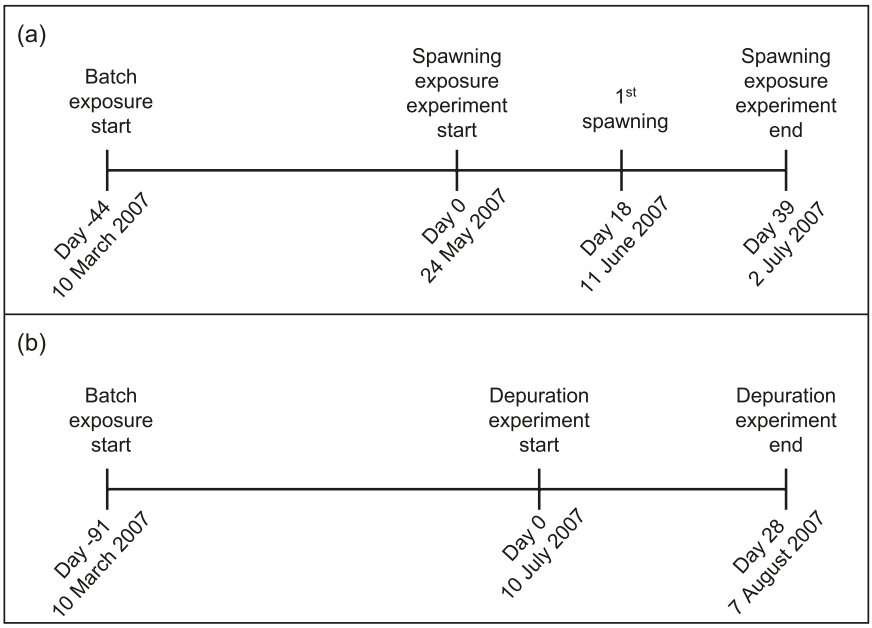
Materials and methods

Our study consisted of two experiments: a spawning exposure experiment and a depuration experiment. The spawning exposure experiment was composed of three treatments: 17β-estradiol (E2, 70 $ng \cdot L^{-1}$ 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 the major estrogen of female teleosts (Arukwe and Goksøyr 2003), it is necessary for reproduction in male and female teleosts (Nimrod and Benson 1998), it can be found in and downstream of many WWTP effluents, and exposure to exogenous estradiol can induce adverse reproductive effects in diverse fish species (Mills and Chichester 2005). In the United States, aquatic concentrations of estradiol have been estimated to be less than 200 $ng \cdot L^{-1}$, with a median detectable concentration of 160 $ng \cdot L^{-1}$ (Kolpin et al. 2002). In Boulder Creek, Colorado, concentrations of estradiol have been reported to reach 2.9 $ng \cdot L^{-1}$, with average and maximum estrogenicity equivalents (accounting for additional estrogenic EDCs) of 29 and 50 $ng \cdot L^{-1}$, respectively, in the effluent (Vajda et al. 2008). Our goal was to mimic the total estrogenicity seen in Boulder Creek using estradiol. Previous research indicated that actual concentrations could be 10%–30% less than nominal concentrations (Nimrod and Benson 1998). Therefore, we chose a nominal concentration of 120 $ng \cdot L^{-1}$ that would result in a measured concentration similar to the total estrogenicity seen in Boulder Creek.

The experimental setup consisted of a flow-through system of three glass head tanks (20.8 L; one per treatment) that continuously 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). Male red shiners were batch exposed to E2, SC, or WC for 44 days. Then they were moved to spawning aquaria where they continued exposure and were allowed to spawn with females for 3 weeks (83 days total exposure; Fig. 1*a*). A suite of reproductive endpoints was measured (described below). Batch exposure was logistically necessary to ensure that all male fish within and between each treatment were receiving similar social and environmental cues (light regime, temperature, visual contact with females) to initiate spawning readiness. In addition, red shiners are a social species that occur in large schools, and we felt holding males in isolation for 44 days would cause excessive stress.

The depuration experiment began after 91 days of male exposure to estradiol. This study included a control treatment, where fish from the control aquaria remained in control water (WCWC), and a previously exposed treatment

Fig. 1. Schematic diagram of experimental protocols. Axes are relative and not to scale. In the spawning exposure experiment (*a*), exposure (70 ng·L^{-1} 17β-estradiol, E2; solvent control, SC; water control, WC) began on the date of batch exposure and continued to the spawning experiment end. Reproductive endpoints were measured 21 days after spawning was first seen (first spawning). In the depuration experiment (*b*), exposure (70 ng·L^{-1} 17β-estradiol, E2WC) began on the date of batch exposure and was discontinued immediately prior to the start of the depuration experiment (day 0). Reproductive endpoints were measured 28 days after spawning was first seen, which coincided with day 0.



(E2WC), where fish from the estradiol exposure (70 ng·L^{-1} actual concentration, see above) 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 0 (Fig. 1*b*). Reproductive effects and spawning were monitored for 4 weeks.

Spawning exposure experiment

Organisms

Adult red shiners were collected by seine from the Arkansas River on the Granada State Wildlife Area, Colorado, USA (38.05°N, 102.23°W), and transported to the Foothills Fisheries Laboratory at Colorado State University (Fort Collins, Colorado) on 29 November 2005 and 9 February 2006. 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 every other day to correspond with Arkansas River seasonal changes, ranging between 6.1 and 23.3 °C. Fish were fed once daily ad libitum with Aquatox commercial flake food (Aquatic Eco-systems, Apopka, Florida) (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, Missouri) in ethanol. 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}) to produce the E2 treatment concentration. The E2 treatment nominal concentration was 120 ng·L^{-1}; however, the actual waterborne concentration was expected to be 10%–30% of nominal (Nimrod and Benson 1998). A solvent control (SC) treatment was made and distributed in the same manner, adding 400 μL 99% ethanol stock solution to 4 L of well water (0.00002% ethanol). The water control (WC) treatment utilized the same well water. Water from the head tanks of the three treatments was continuously distributed to individual exposure aquaria through Teflon capillary tubes, glass funnels, and Teflon-lined tubing, achieving 99.99% replacement for each aquarium every 24 h. Water chemistry remained constant between treatments over the duration of the experiment (nitrate = nondetectable, nitrite = nondetectable, ammonia < 0.2 mg·L^{-1}, pH = 8, alkalinity = 180 mg·L^{-1}, and total hardness = 300 mg·L^{-1}). Incoming water had a dissolved carbon concentration of 2.84 mg·L^{-1}.

The concentrations of estradiol in each treatment were measured immediately before exposure began and every 1.5 weeks during the exposure period (n = 4) and combined to calculate a mean concentration during the experiment (ac-

tual concentration). 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, Massachusetts) 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, Pennsylvania) according to the manufacturer's protocols (detection limit 2 $ng \cdot L^{-1}$, determined by manufacturer).

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 °C ± 1.2 °C; 8 h light : 16 h dark) and a holding period of 5 months, males were weighed and randomly allocated to three 37.9 L glass aquaria with no spawning substrate. One group of males was exposed to estradiol (E2, described above) and the other two groups received either the solvent (SC) or water control (WC) treatment. Batch exposure lasted 44 days. 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 °C ± 0.42 °C). Photoperiod was increased by 20 min daily until reaching 16 h light : 8 h dark 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

Fifteen 20.8 L glass spawning aquaria were randomly assigned to one of the three treatments. After 44 days of batch exposure, when a majority of control males displayed spawning coloration and nuptial tubercles, five males were taken from each batch exposure aquarium, weighed, and placed in separate aquaria (n = 5 per treatment, 15 total). Each spawning aquarium contained four spawning stacks placed on four 1.5 cm × 10 cm glass Petri dishes. Individual spawning stacks were made of four 5.1 cm × 5.1 cm sand-colored ceramic tiles. 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 mm × 47 mm × 47 mm each (Burkhead and Jelks 2001). After 1 day of male acclimation, females were weighed and added to each spawning aquarium holding one male (two females per male). Exposure continued for 3 weeks after the first sign of spawning. After 3 weeks of spawning, males were immediately removed from aquaria (83 days total exposure), and females were removed the following day. After removal from spawning aquaria, fish were anesthetized with tricaine methanesulfonate (50 $mg \cdot L^{-1}$). 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 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 °C ± 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 uneyed 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 uneyed 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 °C ± 1.6 °C). Observations of hatched or dead fish in each jar were recorded at least once daily. Dead fish were opaque, compared with the translucent appearance of live fish, and were unresponsive to disturbance. 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 observed on three occasions, with each observation at least 1 week apart. On each occasion the initial aquarium to be observed was selected randomly and alternated systematically. Male behavior was recorded in all 15 aquaria on each occasion. For each behavioral observation, a digital video camera was placed approximately 0.45 m from the spawning aquarium, enclosed in a black curtain. Each aquarium was recorded for 6 min. When videotapes were reviewed, the final 5 min were used to record behavioral data because other studies suggest waiting at least 1 min after disturbance (Winkelman 1996; Doyle and Lim 2005), and our preliminary observations suggested that red shiners recovered from visual disturbance within 1 min. The frequency of each behavior was measured.

Prespawning behavioral categories included dances, courts, and quivers. "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 were measured on all male red shiners in the mating experiment, and specimens were preserved in 10% neutral buffered formalin. A microscope (0.8× magnification) connected to Image-Pro

Express (version 5.0.1.26, Media Cybernetics, Bethesda, Maryland) 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 (Smith 1978).

To document changes in male coloration on the pectoral and caudal fins, digital photographs were taken before preservation 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 3 min 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 by parallelism.

Fish were sacrificed by rapid decapitation and the gonadsand liver were removed from the fish and weighed (g) to determine gonadosomatic index (GSI) and hepatosomatic index (HSI), respectively. GSI was calculated as [gonad weight (g) × body weight (g)$^{-1}$ × 100], and HSI was calculated as [liver weight (g) × body weight (g)$^{-1}$ × 100]. 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, 6 or 10 μm) using a microtome, mounted on glass slides, rehydrated, and stained with hematoxylin and eosin (Presnell and Schreibman 1997; Blazer 2002). Gonadal sections were examined under a light microscope for stage and maturation of each individual without knowledge of treatment.

Gonadal staging was done using methods utilized by Pawlowski et al. (2004) and Vajda et al. (2008). Sperm abundance within seminiferous tubules of mature males was classified as presence or absence, where 0 = sperm absent and 1 = sperm present. Staging of ovarian sections was based on Blazer (2002) and was as follows: 1 = previtellogenic, 2 = early vitellogenic, 3 = mid-vitellogenic, and 4 = late vitellogenic. Previtellogenic 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 previtellogenic oocytes: A (<25%), B (25%–50%), and C (>50%).

Statistical analyses

All statistical analyses were performed using SAS version 9.1 (SAS Institute Inc. Cary, North Carolina, USA). Several response variables were log-transformed (coloration, vitellogenin) to meet the assumption of normality. Data were checked for homogeneity of variance across treatments using Levene's test. Parametric data were analyzed using one-way analysis of variance (ANOVA) and are denoted by an F statistic. Both an ANOVA test and a nonparametric alternative, the Kruskal–Wallis test, were used when parametric assumptions were not met. Although we report the χ^2 statistic to be conservative, both ANOVA and Kruskal–Wallis tests always yielded similar main effects probabilities. Therefore, Tukey's honestly significant difference (HSD) method was used to make multiple comparisons for all response variables where the main effects model was significant. For ease of data interpretation in the results, we report statistical significance of the main effects models (F or χ^2 and associated probabilities). In all multiple comparisons, the level of significance used was 0.05. 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. Means are reported ± standard error (SEM) and n = 5 unless otherwise noted.

Depuration experiment

Red shiners in the depuration 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 and exposed to the same water as fish in the spawning exposure experiment. Only fish that were batch exposed to E2WC and to WCWC were used in this experiment as treatments. After 91 days of batch exposure, a total of 10 males (five males from each treatment) were chosen randomly, weighed, and allocated into glass spawning aquaria.

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 Inc., Cary, North Carolina) in a similar manner to the spawning exposure experiment. Response variables were log-transformed (coloration, percent fertilization success, vitellogenin) to meet the assumption of normality if necessary. Behavioral data analysis utilized only data from the last observation period to represent the ultimate change

over time. Egg deposition, fertilization success, and hatching success data were collected on a daily basis and 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 × 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 × time interaction. Random effects were aquarium within treatment and the residual. Post hoc mean comparisons were made using Tukey's HSD method. Means are reported ± standard error (SEM) and $n = 5$, unless otherwise noted. In all tests, the level of significance used was 0.05. To better illustrate the effects of depuration on some response variables, we made comparisons between the depuration and spawning exposure experiments. However, these comparisons should be viewed with caution because the results were from separate experiments.

Results

Spawning exposure experiment

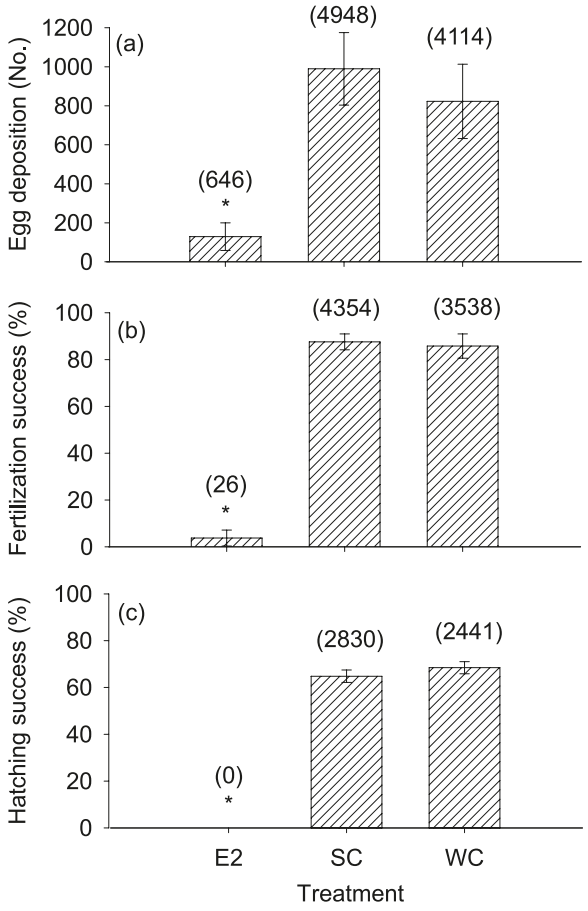
Fish survival was 100% for all treatments during the chemical exposure period. There were no significant differences among treatments in weight, total length, or condition factor of males and of females at the beginning or conclusion of the study (see Supplemental Table S1[3]). Waterborne concentrations of estradiol on all four sample dates ranged from 34 to 160 ng·L^{-1} (mean = 70 ng·L^{-1}, standard deviation (SD) = 60 ng·L^{-1}, $n = 4$, mean 58% of 120 ng·L^{-1} nominal value) 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. There were no significant differences between SC and WC treatment groups in all endpoints, unless otherwise specified.

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 ($p = 0.0168$, $F = 6.07$; Fig. 2*a*). Percent fertilization success was significantly lower in the E2 treatment than in the WC and SC treatments ($p = 0.0398$, $\chi^2 = 6.45$; Fig. 2*b*). Of the eggs fertilized, the percentage that survived to hatching was significantly lower in the E2 treatment than in the SC and WC treatments ($p = 0.0328$, $\chi^2 = 6.83$; Fig. 2*c*). None of the eggs fertilized in the E2 treatment survived to hatching. Because only three of the five E2 treatment aquaria deposited eggs, $n = 3$ compared with $n = 5$ in the SC and WC treatments with regard to fertilization and hatching success.

Prespawning courtship behaviors were suppressed in males from the E2 treatment (Fig. 3*a*). The frequencies of dances ($p = 0.0078$, $\chi^2 = 9.70$), courts ($p = 0.0183$, $\chi^2 = 7.98$), and quivers ($p = 0.0196$, $\chi^2 = 7.87$) in the E2 treatment were significantly lower compared with those of both controls.

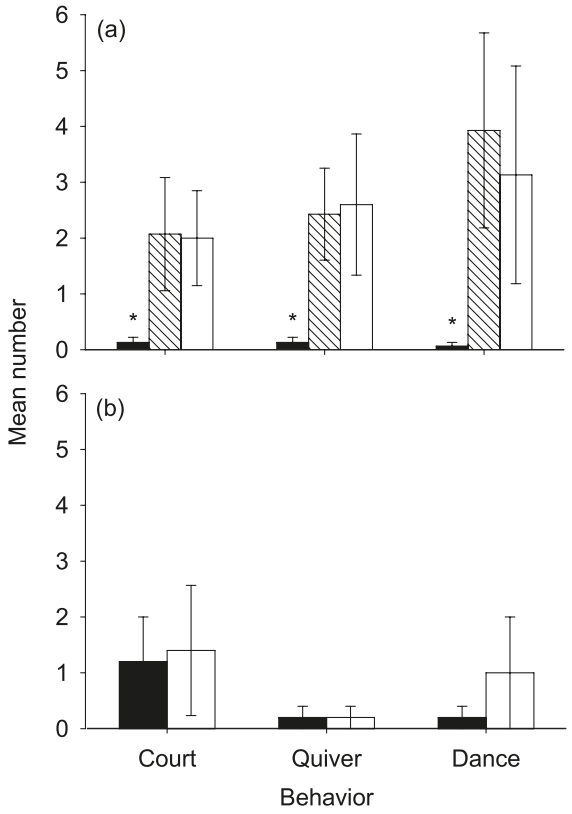
Estradiol had an effect on the secondary sexual characteristics of male fish. The number of tubercles on E2 treatment fish was lower than on SC and WC treatment fish ($p = 0.0083$, $\chi^2 = 9.58$; Fig. 4*a*). Tubercle developmental stage was significantly lower in the E2 treatment than in the SC and WC treatments ($p < 0.001$, Fisher's exact test; Fig. 4*c*). Tubercles seen on E2 treatment fish reached a developmental stage of 1 or below, while SC and WC treatment fish exhibited tubercles at stages 3 or 4. Visually, males showed obvious differences between treatments (Fig. 5). The coloration of E2 treatment males was different than that of SC and WC fish in the pectoral fin and caudal fin regions (Table 1). The pectoral fin of E2 treatment males had higher mean blue ($p = 0.0002$, $F = 19.40$) and lower mean saturation ($p < 0.0001$, $F = 32.41$) than those of SC and WC males, mean red was lower than that in WC males, but not SC males ($p = 0.0532$, $F = 3.78$), and green was higher than the SC treatment, but not the WC treatment ($p = 0.0311$, $F = 4.70$). The caudal fin of E2 treatment males had higher

Fig. 2. Mean (± standard error, SEM) egg deposition (*a*), fertilization success (*b*), and hatching success (*c*) of the red shiner (*Cyprinella lutrensis*) after exposure to 17β-estradiol (E2), solvent control (SC), and water control (WC) treatments. Asterisks indicate statistically significant differences from the water control ($p < 0.01$; analysis of variance (ANOVA) and Kruskal–Wallis tests; $n = 5$ for SC and WC treatments and $n = 5$ (egg deposition) and $n = 3$ (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.



[3] Supplementary data for this article are available on the journal Web site (http://cjfas.nrc.ca).

Fig. 3. Mean (± standard error, SEM) prespawning courtship behaviors of male red shiners (*Cyprinella lutrensis*) in the spawning exposure experiment (*a*), after males were exposed to 17β-estradiol (E2; solid), solvent control (SC; hatched), and water control (WC; open) treatments (n = 15 per treatment) and in the depuration experiment (*b*), after males were previously exposed to 17β-estradiol (E2WC; solid) and water control (WCWC; open) treatments (n = 5 per treatment). Asterisks indicate statistically significant differences from the water control ($p < 0.05$; Kruskal–Wallis tests).

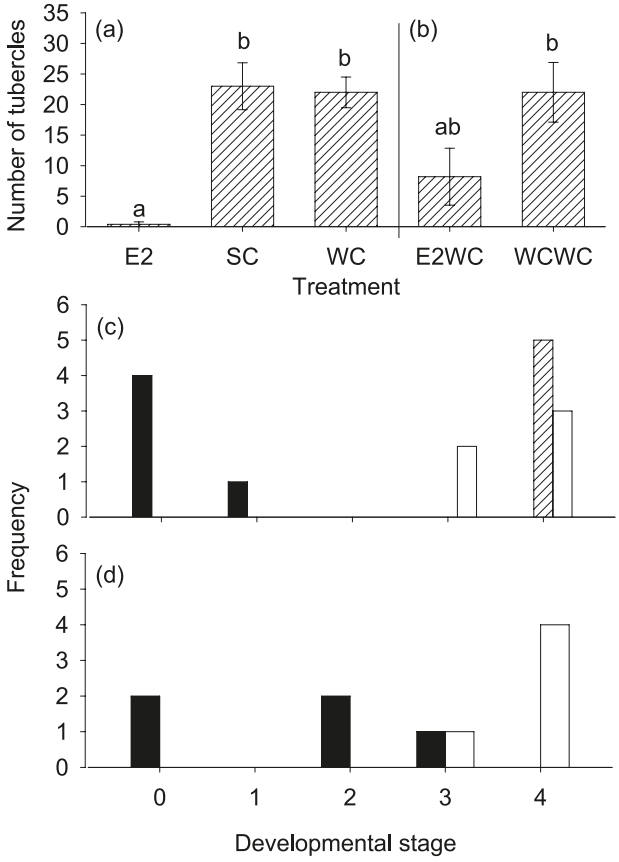


mean blue (p = 0.0003, F = 17.15) and lower mean saturation (p = 0.0012, F = 12.35) than those of SC and WC males, higher mean green than the SC treatment, but not WC treatment (p = 0.0087, F = 7.22), and red did not differ between treatments (p = 0.5239, F = 0.68); WC and SC did not significantly differ in any test.

Male plasma vitellogenin concentration was significantly higher in the E2 treatment (p = 0.0090, χ^2 = 9.42; Fig. 6). Male gonadal stage was significantly reduced in the E2 treatment when compared with SC and WC treatments (p < 0.0001, Fisher's exact test). All males in the E2 treatment exhibited stage 0 (sperm absent) gonad development, and all males in the SC and WC treatments exhibited stage 1 (sperm present). Histology also confirmed that exposure to estradiol blocked spermatogenesis and caused regression of seminiferous tubules (Figs. 7*a*, 7*b*).

Plasma vitellogenin concentration was significantly different among female fish that were in aquaria with E2, SC, and WC treatment males (p = 0.0042, χ^2 = 10.92); it was lower in females held in E2 aquaria. Female gonadal stage (p = 0.5615) and amount of previtellogenic oocytes (p = 0.1567) did not significantly differ between treatments (Fisher's exact test). Females in aquaria with males that were exposed

Fig. 4. Male red shiner (*Cyprinella lutrensis*) tubercle number and developmental stage in the spawning exposure and depuration experiments. Fish were exposed for 83 days to 70 ng·L^{-1} 17β-estradiol (E2), solvent control (SC), or water control (WC) treatments in the spawning exposure experiment and for 91 days to 70 ng·L^{-1} 17β-estradiol followed by 4 weeks of depuration (E2WC) and water control (WCWC) treatments in the depuration experiment. Mean (± standard error, SEM) tubercle numbers in the spawning exposure (*a*) and depuration (*b*) experiments are shown. Values with different lowercase letters indicate statistical significance ($p < 0.05$; Kruskal–Wallis test; n = 5 per treatment). Frequency of tubercle developmental stage from the spawning exposure (*c*: E2, solid; SC, hatched; WC, open) and depuration (*d*: E2WC, solid; WCWC, open) experiments are shown. 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. Fisher's exact test, $p < 0.0001$, n = 5 per treatment.



to estradiol (E2 treatment) exhibited developmental 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 had female gonads in categories A, B, and C, with the females exposed to estradiol having slightly more category C, previtellogenic 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 = 0.5524, F = 0.62). 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 = 0.0394, F = 4.29). The GSI of females exposed to estradiol in aquaria

Fig. 5. Coloration of adult male red shiner (*Cyprinella lutrensis*) after 83 days exposure to 70 ng·L^{-1} 17β-estradiol (*a*) or water control (*b*) treatments. Solvent control treatment males (not pictured) were visually similar to water control treatment males.

Table 1. Mean (± standard error, SEM) pectoral fin and caudal fin coloration of male red shiners (*Cyprinella lutrensis*) in the spawning exposure experiment and depuration experiment.

Area	Variable	E2	SC	WC	E2WC	WCWC
Pectoral fin	Red	176.10±9.02a	190.36±3.52ab	200.29±4.84b	175.52±5.54	184.22±3.19
	Blue	128.37±19.70a	42.73±4.68b	66.5±5.94b	91.83±18.81*	33.95±2.65
	Green	156.52±13.44a	114.49±6.12b	135.15±7.98ab	134.83±7.28*	101.32±4.05
	Saturation	52.69±14.55a	170.70±8.02b	135.94±8.02b	92.18±26.88*	184.29±5.11
Caudal fin	Red	186.91±6.45a	186.33±3.47a	194.96±6.96a	189.06±1.64	188.37±3.07
	Blue	133.59±7.75a	73.90±7.62b	96.99±6.33b	110.47±16.02	70.12±8.44
	Green	167.88±7.12a	140.51±4.51b	150.92±2.72ab	159.89±7.17*	135.48±3.28
	Saturation	56.05±3.03a	127.01±12.39b	98.71±12.13b	82.39±18.03	127.71±10.89

Note: In the spawning exposure experiment, fish were exposed for 83 days to 70 ng·L^{-1} 17β-estradiol (E2), solvent control (SC), and water control (WC) treatments (values with different letters indicate statistical significance). In the depuration experiment, fish were exposed for 91 days to 70 ng·L^{-1} 17β-estradiol followed by 4 weeks of depuration (E2WC) or water control (WCWC) treatments (asterisks denote significant differences from the control). Analysis of variance (ANOVA) tests, $\alpha = 0.05$, $n = 5$ per treatment.

with E2 treatment males (7.051 ± 1.875) was not significantly lower than the GSI of females in aquaria with SC (11.732 ± 0.711) and WC treatment males (10.096 ± 0.589) ($p = 0.0898$, $\chi^2 = 4.82$). The HSI of females in E2 treatment aquaria (0.741 ± 0.087) was not significantly different than the HSI of females in WC aquaria (0.833 ± 0.056) or SC aquaria (1.270 ± 0.205) ($p = 0.0539$, $\chi^2 = 4.54$).

Depuration experiment

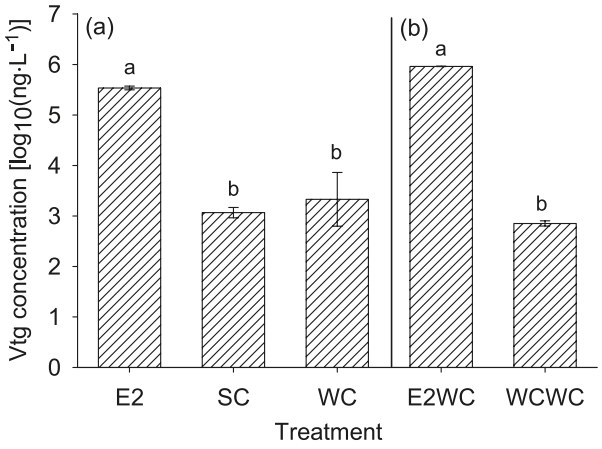
Fish survival was 100% for all treatments. There were no significant differences among treatments in weight, total length, or condition factor of males and of females at the beginning or conclusion of the study (see Supplemental Table S1[3]). Waterborne concentrations of estradiol on all sample dates were below detection limits of the assay. Water chemistry was similar to the spawning exposure experiment and remained constant among treatments over the duration of the experiment.

The mean number of eggs deposited by females differed between the E2WC and WCWC treatments (Fig. 8*a*), especially in the first 2 weeks of the depuration experiment, but overall differences were not statistically significantly different between treatments ($p = 0.0571$) or across the 4 weeks ($p = 0.0585$) (Proc Mixed).

Percent fertilization success was 0% in the E2WC treatment compared with 98% ± 1.1% and 99% ± 0.3% in the WCWC treatment for weeks one and two of the study, respectively (Fig. 8*b*). Fertilization success between treatments was significantly different ($p = 0.0385$, $F = 6.46$); there was not a significant week effect ($p = 0.2825$, $F = 1.45$), and the interaction was significant ($p \leq 0.0006$, $F = 58.78$; Proc Glimmix). Since there was no fertilization or hatching success for the first 2 weeks in the E2WC treatment, the logistic model could not produce exact parameter estimates and the statistics are based on comparisons of the last 2 weeks.

Eggs were not available to hatch until week three in the E2WC treatment, while the WCWC treatment had hatching success of 87% ± 0.1% and 82% ± 0.2% during week one and week two of the study, respectively (Fig. 8*b*). Of the eggs fertilized, the percentage that survived to hatching was not significantly different between treatments ($p = 0.0951$, $F = 4.22$) or weeks ($p = 0.2253$, $F = 24.22$) during weeks three or four (Proc Glimmix; Fig. 8*b*).

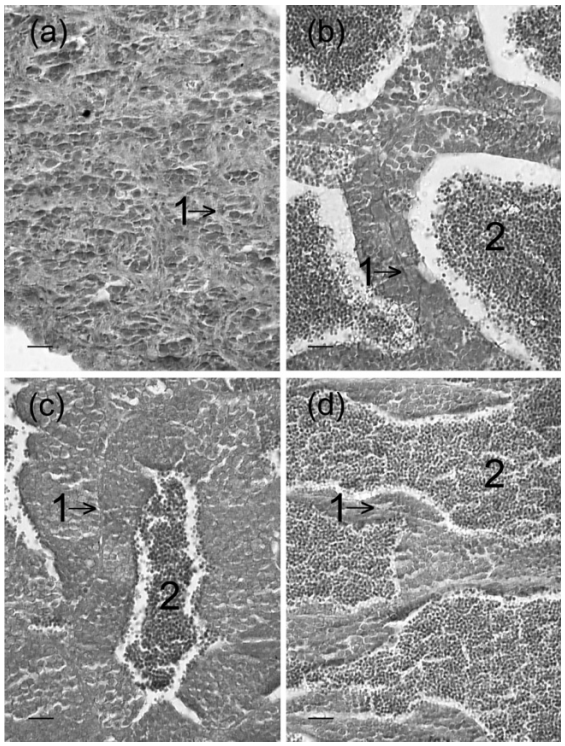
Fig. 6. Mean (± standard error, SEM) vitellogenin (Vtg) plasma concentrations in adult male red shiners (*Cyprinella lutrensis*) at the conclusion of the spawning exposure (*a*) and depuration (*b*) experiments. Fish were exposed to 70 ng·L^{-1} 17β-estradiol (E2), solvent control (SC), or water control (WC) treatments for 83 days (*a*) or exposed to 70 ng·L^{-1} 17β-estradiol followed by a 4-week depuration (E2WC) or water control (WCWC) treatment for 91 days (*b*) before measurements. Values with different lowercase letters indicate statistical significance (analysis of variance (ANOVA) test, $p < 0.05$). The low error associated with the E2WC treatment is not visible in this figure.



Courtship behaviors appeared to recover in males of the E2WC treatment. Frequencies of dancing ($p = 0.8815$, $\chi^2 = 0.02$), courting ($p = 1.0000$, $\chi^2 = 0.00$), and quivering ($p = 1.0000$, $\chi^2 = 0.00$) in the E2WC treatment were not significantly different than in the WCWC treatment (Fig. 3*b*). Secondary sexual characteristics of male fish also showed some recovery. The mean number of tubercles on E2WC fish was not significantly different than those on 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. 4*b*). Tubercles seen on E2WC fish exhibited developmental stages between 0 and 3, while WCWC treatment fish exhibited tubercles at stages 3 or 4 (Fig. 4*d*). The E2WC fish had a significantly lower developmental stage than the WCWC treatment ($p = 0.0476$, Fisher's exact test; Fig. 4*d*). The coloration of E2WC fish was different than WCWC fish (Table 1). The pectoral fin of E2WC treatment males had significantly higher mean blue, higher mean green, and lower mean saturation than fins of WCWC treatment males, with no significant difference in mean red. The caudal fin of E2WC treatment males had significantly higher mean green than those of 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.

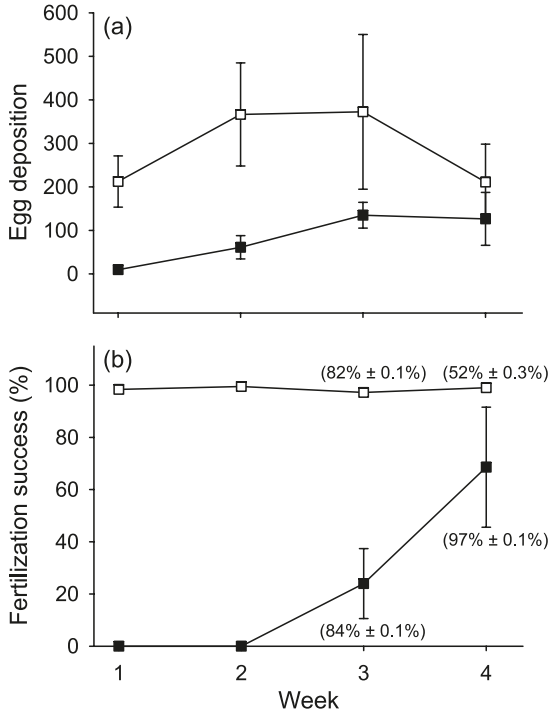
Male plasma vitellogenin concentration in the E2WC treatment was significantly higher than that in the WCWC treatment ($p = 0.0090$, $\chi^2 = 6.82$; Fig. 6*b*). The E2WC treatment was not significantly different than the E2 treatment of the spawning exposure experiment ($p = 0.7292$, $\chi^2 = 0.01$), and the E2 and E2WC treatments were statistically different from the controls in both experiments ($p < 0.0001$, all comparisons; Kruskal–Wallis test). Male gonadal stage was similar between the E2WC and WCWC treatments ($p = 0.4444$, Fisher's exact test), with two E2WC treatment fish in stage 0 and three fish in stage 1 and all WCWC treatment fish in stage 1. Histological examination indicated recovery of spermatogenesis in estradiol-exposed males (Figs. 7*c*, 7*d*). Female gonadal stage ($p = 1.0000$, Fisher's exact test) and plasma vitellogenin concentration ($p = 0.3642$, $\chi^2 = 0.82$) were similar between groups.

Fig. 7. Photomicrographs of representative testicular sections using hematoxylin and eosin staining. Panels (*a*) and (*b*): male red shiners (*Cyprinella lutrensis*) exposed for 83 days to 70 ng·L^{-1} 17β-estradiol (E2, *a*) or solvent control (SC, *b*) treatments. Whereas seminiferous tubules in SC fish were replete with sperm, seminiferous tubules were regressed and spermatogenesis was not evident in E2-exposed males. Panels (*c*) and (*d*): males exposed to 70 ng·L^{-1} 17β-estradiol for 91 days followed by 28 days depuration (E2WC, *c*) or water control (WCWC, *d*). 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. Scale bar: 15 μm.



The GSI of E2WC treatment males (1.398 ± 0.278) was not significantly different than the GSI of WCWC males (1.890 ± 0.155) ($p = 0.1606$, $F = 2.39$). The HSI of E2WC treatment males (0.850 ± 0.231) was not significantly different than the HSI of WCWC males (0.494 ± 0.057) ($p = 0.1770$, $F = 2.19$). The GSI of females in aquaria with E2WC treatment males (9.948 ± 1.381) was not significantly different than the GSI of females with WCWC treatment males (9.771 ± 1.417) ($p = 0.9311$, $F = 0.01$). The HSI of females in aquaria with E2WC treatment males (0.949 ± 0.060) was not significantly different than the HSI of females in aquaria with WCWC treatment males (1.007 ± 0.211) ($p = 0.7989$, $F = 0.07$).

Fig. 8. Mean (± standard error, SEM) egg deposition (*a*), percent fertilization success (*b*), and hatching success (shown in parentheses above and below the treatments, respectively, for weeks three and four) of red shiners (*Cyprinella lutrensis*) in the depuration experiment. Fish were exposed to 70 ng·L^{-1} 17β-estradiol followed by a 4-week depuration (E2WC; solid squares) or water control (WCWC; open squares) treatment for 91 days.



Discussion

Our study clearly demonstrated that estradiol, at an environmentally relevant concentration of estrogenicity, can adversely impact reproductive success of adult red shiners and that reproductive success improved markedly when males were removed from estradiol exposure. Although the effects of EDCs on individual reproductive traits have been well documented, relatively few studies have examined the ultimate reproductive consequences of EDC exposure on fishes (Mills and Chichester 2005), and fewer still documented the recovery of reproduction when exposure ceased (e.g., Nash et al. 2004; Fenske et al. 2005). Our results are a necessary first step in beginning to understand the potential effects of EDCs on population dynamics and persistence, especially in Great Plains ecosystems. Many plains streams are dominated by wastewater discharge and agricultural runoff known to contain EDCs (Woodling et al. 2006). Despite this high potential for exposure, few studies have been conducted to examine individual and population responses of native plains fishes to these compounds. Beyond our study on the red shiner, the only other native plains fish investigated as comprehensively is the fathead minnow (Bringolf et al. 2004; Kidd et al. 2007; Miller et al. 2007). The reproductive decline seen in our study suggests that chronic exposure to EDCs could have severe population repercussions for the red shiner and perhaps other Great Plains fishes but that these impacts could be reversible.

The inability of exposed male red shiners to fertilize eggs could be attributable to impaired spawning behavior, impaired spermatogenesis, decreased milt quality or abundance, or other reproductive disruption resulting from estrogen exposure. Androgens are necessary in spermatogenesis and spawning in fishes (Pandey 1969; Demski and Hornby 1982; Schulz and Miura 2002); exposure to exogenous estrogens can suppress endogenous androgen function through interactions with the hypothalamo–pituitary–gonadal axis (Martinović et al. 2007). 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). GSI did not differ between treatments in either of our experiments. It appears that exposed males were developing gonadal tissue but, based on histological examination and reproductive success, were not producing functional gametes. Although frequently used as an indicator of EDC exposure, GSI may not be as indicative of an individual's reproductive capability or ultimate reproductive success.

The failure of eggs to hatch when the male parent was exposed to estradiol was likely due to low fertilization success from weakened or dead sperm (Gale 1986). 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). By 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. The potential for EDCs to interfere with hatching success and thus to reduce recruitment in wild fish populations clearly needs further exploration. Our study suggests that a single estrogenic chemical, at concentrations similar to the total estrogenicity of WWTP effluent, may reduce fecundity of red shiner populations.

Estradiol exposure reduced the number of eggs deposited by females. Reduced egg deposition by females may be due to insufficient stimulatory cues such as decreased mating behavior by the males, reduced secondary sexual traits, or a combination of both. Exposed male red shiners showed fewer courting, dancing, and quivering behaviors than those in control treatments. Other studies that exposed fishes to estrogenic compounds at concentrations comparable to ours have correlated reduced spawning activity and courtship behavior with decreased egg deposition and reproductive success (Bell 2001; Majewski et al. 2002; Martinović et al. 2007). Both coloration and tubercles are thought to be important for male red shiner reproductive success (Matthews 1995). Male red shiners exposed to estradiol had significantly fewer and less developed tubercles and reduced spawning coloration compared with controls. Studies with other fish species also show that males exposed to estradiol had fewer tubercles (Bjerselius et al. 2001; Bringolf et al. 2004) and reduced coloration (Toft and Baatrup 2003; Brion et al. 2004; Kristensen et al. 2005). Despite evidence that es-

trogenic exposure reduces mating behavior, male appearance, and reproductive success, little is known about the repercussions of exposure in wild populations. Our data suggest these changes could have major effects on reproductive output that ultimately may translate to population-level impacts.

Since female fish were exposed to estradiol for a short period of time (39 days) in our spawning exposure experiment, decreased egg deposition could have been due to changes in female reproductive condition. Plasma vitellogenin concentration was significantly reduced in exposed females; however, oocyte stage and vitellogenic oocyte abundance were not, suggesting that exposed females were recruiting oocytes normally. Previous studies have found that female fecundity can be increased with low levels of estrogenic exposure similar to those used in our experiment (Imai et al. 2005; Kristensen et al. 2005). Decreased egg production did not occur until higher concentrations were reached (Imai et al. 2005). Since females in our study were exposed as adults for a short period of time, showed no differences in oocyte numbers from unexposed females, and were exposed at a concentration lower than that found to be detrimental to egg production in other studies (Imai et al. 2005), decreased egg deposition in our experiment was more likely a response to alterations in male spawning cues.

We found that estrogenic effects on most individual male reproductive traits were reversible when male red shiners were removed from estradiol exposure. Mating behaviors and number of tubercles were similar to males that were never exposed. Sperm development did not statistically differ between treatments because three of five previously exposed males developed viable sperm and ultimately reproduced. Exposed males continued to exhibit elevated levels of vitellogenin, and tubercle development remained lower than control males. Egg deposition declined in the control treatments in week four of the depuration experiment, and male reproductive behaviors were lower in this experiment compared with the spawning exposure experiment; however, these results may have been due to declining reproductive activity or capacity. The red shiner is known to have distinct periods of successful reproduction and periods with no evident reproductive success (Durham and Wilde 2005).

Despite the lack of complete recovery in individual reproductive traits and potential declining reproductive activity, the improvement in measures of reproductive success during the depuration experiment was dramatic. Egg deposition in previously exposed treatments increased from 0 in the first week of the experiment to approximately 100 eggs in each tank by weeks three and four. Mean fertilization success increased to 69% after 4 weeks, and mean hatching success increased to 84% in week three and exceeded the control value at 97% in week four. Our depuration study suggests that for red shiners and other plains fish populations that spawn for prolonged periods, an entire reproductive season may not be lost if exposure to EDCs is temporary.

The potential to reverse the effects of chronic exposure suggests potential mitigation or management strategies for fishes during critical reproductive periods. Management strategies could focus on reducing the concentrations of EDCs in aquatic systems through removal of these compounds at WWTP facilities or temporary dilution of effluent by addition of fresh water. Currently, most urban WWTPs are ill-equipped to remove these chemicals and would require changes in treatment processes that presently may not be practical or feasible (Kolpin et al. 2002). Many potential agricultural sources of EDCs are non-point sources that enter streams and rivers from runoff after land application of chemicals and animal waste or seepage and overflow from feedlot operations (Tyler and Routledge 1998; Kolpin et al. 2002). Removal of EDCs in most agricultural settings will require substantial changes in land-use practices to protect surface water and groundwater from agricultural seepage and run-off. Dilution of effluent concentrations could be accomplished by increasing stream flows or reducing effluent discharge. This alternative may improve reproductive success of fish (Robinson et al. 2003), but the efficacy of this strategy depends on the composition of EDCs in the environment (Mills and Chichester 2005) and also on the availability of water, a limited and contentious resource in western Great Plains river basins. Dilution during critical life history stages such as reproduction or development may represent a first step, especially in light of our findings that endocrine disruption in fish exposed as adults may be reversible.

It is important to note that the influence of EDCs on fish reproduction and potential reversibility of exposure depends on EDC concentration and duration, as well as the developmental time period during which the fish are exposed, and may be species-specific (Nash et al. 2004; Fenske et al. 2005; Schafers et al. 2007). To determine how exposure to EDCs could affect particular plains fish populations, knowledge of the normal reproductive biology of each species is critical. The red shiner was intended to serve as a surrogate for other native plains species, especially for native cyprinids or species with similar reproductive strategies, but depending on the interspecific degrees of reproductive complexity, comparisons are not always suitable. Negative effects caused by exposure to estradiol may be intensified in more reproductively complex species, such as those with strong site fidelity for nests or mating territories (e.g., central stoneroller, *Campostoma anomalum*; or centrarchid species). Conversely, effects of EDCs may be reduced in species with less complex mating systems or secondary sexual characteristics. The interaction of life history characteristics with EDC exposure needs to be further explored before predictions can be made about the long-term effects of EDCs on population and community dynamics of Great Plains fishes.

Our study clearly indicates that exposure to estrogenic compounds could dramatically reduce red shiner reproduction, which could have negative implications for recruitment in wild populations. In situ field studies in Colorado suggest that reproductive potential may be compromised because of estrogenic compounds in WWTP effluent. Downstream of WWTPs on the South Platte River and Boulder Creek, white suckers were found with abnormal gonadal development and altered sex ratios with predominantly females in the population (Woodling et al. 2006; Vajda et al. 2008). Adult male fathead minnow experimentally exposed to wastewater from the Boulder Creek facility showed similar changes in secondary sexual characteristics that we observed, including fewer and less prominent nuptial tubercles, less prominent fat

pads, and decreased sperm abundance within the testes than reference fish within 14 days of 50% or 100% effluent. Vitellogenin induction was seen within 14 days in 25%, 50%, and 100% effluent (Vajda 2006). Our findings on how estrogenic chemicals affect the red shiner, a native cyprinid of the Great Plains, complement and validate results of the studies on white suckers (catostomid) and fathead minnow (cyprinid), which are also native.

Many streams and rivers in the Great Plains are dominated by urban and agriculturally derived water (Strange et al. 1999; Woodling et al. 2006) that include compounds known to disrupt reproduction in fishes (Woodling et al. 2006). The problem is not unique to the Great Plains, and it is becoming increasingly evident that many ecosystems worldwide have similar problems associated with human water use and EDCs (Mills and Chichester 2005). Management of these ecosystems will require an understanding of the effects of EDCs on fish populations and communities, as well as mitigation strategies to conserve and protect fishes. Our study is a first step in understanding population-level effects on a common native fish of the Great Plains, shows that red shiner reproductive success is substantially reduced at relevant concentrations of estrogenicity, and suggests that these effects may also occur in wild populations living at or above these concentrations.

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