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Reproductive Disruption in Fish Downstream from an Estrogenic Wastewater Effluent

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To assess the impact of an estrogenic wastewater treatment plant (WWTP) effluent on fish reproduction, white suckers (*Catostomus commersoni*) were collected from immediately upstream and downstream (effluent site) of the city of Boulder, CO, WWTP outfall. Gonadal intersex, altered sex ratios, reduced gonad size, disrupted ovarian and testicular histopathology, and vitellogenin induction consistent with exposure to estrogenic wastewater contaminants were identified in white suckers downstream from the WWTP outfall and not at the upstream site. The sex ratio was female-biased at the effluent site in both the fall of 2003 and the spring of 2004; the frequency of males at the effluent site (17–21%) was half that of the upstream site (36–46%). Intersex white suckers comprised 18–22% of the population at the effluent site. Intersex fish were not found at the upstream site. Chemical analyses determined that the WWTP effluent contained a complex mixture of endocrine-active chemicals, including 17 β -estradiol (E₂) 17 α -ethynylestradiol, alkylphenols, and bisphenol A resulting in an estimated total estrogen equivalence of up to 31 ng E₂ L⁻¹. These results indicate that the reproductive potential of native fishes may be compromised in wastewater-dominated streams.

Introduction

Chemical signaling through estrogen-like pathways is an evolutionarily ancient means of communication within and between organisms (1–3) and is central to the development and regulation of reproduction in all vertebrates (4). Endocrine-active chemicals (EACs) of dietary and environmental origin are capable of interacting with estrogen signaling pathways at very low doses (5). It is firmly established that exposure to exogenous EACs during critical life stages can disrupt organismal development and function (1, 4).

Human settlements often discharge EACs continuously into surface waters along with their treated and untreated wastewater effluents. Many surface waters that receive municipal wastewater treatment plant (WWTP) effluent have detectable levels of steroid and nonsteroidal estrogens (6–8), androgens (9, 10), and nonestrogenic neuroactive pharma-

ceuticals (7, 11). Other EACs, including degradation products of alkylphenolpolyethoxylate nonionic surfactants, also have widespread occurrence in WWTP effluents and surface waters (7, 12) and have been shown to have endocrine-disrupting properties in fishes (13–16). Fishes and other organisms downstream from WWTP effluent outfalls are chronically exposed to complex mixtures of synthetic and biogenic EACs, and inappropriate sexual differentiation or development may result from exposures during early life stages (1, 4).

Gonadal malformations, including gonadal intersex, have been identified in fishes in association with estrogenic WWTP effluents (17, 18). Relatively arid areas may be particularly susceptible to the effects of contamination by estrogenic compounds because low base-flows in streams are unable to adequately dilute WWTP effluents and their EACs to below threshold levels. Rapidly increasing human populations and water demands increase the relative contribution of wastewater to total surface water flow. Gonadal intersex and altered sex ratios in native fishes has been reported (19) below the WWTP outfalls of three Colorado cities (Denver, serving 1,500,000 people; Colorado Springs, serving 177,000 people; and Boulder, serving 136,000 people), suggesting a link to WWTP effluents. Elevated concentrations of EACs have been reported previously in the Boulder WWTP effluent and downstream in Boulder Creek (20, 21) and may account for the reproductive disruption observed in downstream fish.

The occurrence of reproductive disruption in fish below the WWTP outfall of Boulder, CO, has implications for many similar WWTP effluent-impacted streams in arid and semiarid environments. The objective of this study was to relate the extent and magnitude of reproductive disruption in fish below the Boulder WWTP to the occurrence and concentrations of wastewater EACs.

Materials and Methods

Study Sites. Boulder Creek, a tributary of the South Platte River, was selected for this investigation because effluent discharged from the city of Boulder WWTP comprises a large fraction of stream flow. This WWTP has an average discharge of 0.74 m³ s⁻¹ (17 million gallons/day) and can contribute from <10% of stream flow during high-flow conditions (April–July) to >75% of stream flow during low-flow conditions (August–March) (22). The Boulder WWTP uses a combined trickling filter/activated sludge treatment process with nitrification/denitrification and chlorination/dechlorination. The mean annual concentrations (January 2003 to December 2004) of NH₃-N, NO₃-N, biological oxygen demand, and total suspended solids in the WWTP effluent were 7, 12, 15, and 6 mg L⁻¹, respectively; 3.7 km downstream of the WWTP outfall NH₃-N and NO₃-N were 1 and 4 mg L⁻¹, respectively (23).

Study Species. The white sucker was selected as the study species because of its occurrence upstream and downstream from the Boulder WWTP outfall. Widely distributed in North America east of the continental divide, the white sucker is a long-lived, iteroparous, benthic, eurythermal, native species (24). White sucker gametogenesis begins in the late summer and early fall, proceeds through the winter and early spring, and culminates in a late-spring spawn. Sexual differentiation of white sucker occurs during the first 2–3 months post-hatching, coinciding with low-flow conditions in Boulder Creek. Lack of dilution results in maximal concentrations of wastewater derived compounds downstream from the WWTP

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outfall during this period of potentially heightened developmental sensitivity (21, 22).

Field Protocols. Fish were collected from Boulder Creek at a site approximately 200 m downstream from the WWTP outfall (effluent site) and from a site approximately 2 km upstream (upstream site). A low-head dam 100 m upstream from the WWTP outfall likely prevents upstream migration of fish but does not impede downstream movement. To investigate impacts at different stages of the reproductive cycle, fish were collected during low-flow conditions (gametogenesis) in the fall of 2003 (September 24; $n = 27$ upstream site, $n = 24$ effluent site), and during low-flow conditions (early spawning) in the spring of 2004 (June 18; $n = 16$ upstream site, $n = 40$ effluent site). During the fall sampling, water temperature in Boulder Creek was 12 °C at the upstream site and 18 °C at the effluent site. During the spring sampling, the upstream site was 12 °C and the effluent site was 17 °C.

White suckers were sampled by electroshocking with a pulsed direct current. Fish were anesthetized by immersion in tricaine methanesulfonate, and weight (g) and length (mm) were recorded. The condition factor was calculated as [body weight (g)/total length (mm³) × 100]. Blood was collected from the caudal vein into heparinized capillary tubes and kept on ice until centrifuged for 5 min at 4500 rpm (within 3 h of collection). Hematocrit was recorded, and aliquots of plasma were frozen and stored at -40 °C until assayed for vitellogenin. Anesthetized fish were sacrificed by rapid decapitation. Gonads were dissected, gross abnormalities noted, weighed, and preserved in 10% neutral-buffered formalin until prepared for histology. Gonadosomatic index (GSI) was calculated as [gonad weight (g)/body weight (g) × 100].

Biological Analysis. Gonads were prepared for histology using standard procedures (25). For larger gonads, separate portions from the anterior, middle, and posterior gonad were embedded. For smaller gonads, the entire gonad was prepared. At least 10 cross-sections were evaluated from each segment (≥ 30 sections evaluated per gonad) for determination of sex, sperm abundance, ovarian staging, intersex status, and any abnormalities (26, 27). Gonads were immature and sexually undifferentiated in 5 of 27 upstream-site fish and 1 of 24 effluent-site fish in the fall sampling, and in 6 of 40 effluent-site fish in the spring sampling. Fish were designated as intersex if microscopic evaluation determined the simultaneous occurrence of both ovarian and testicular tissue. The degree of intersexuality was assessed on a 1–3 scale (17): 1 = mostly testicular tissue with interspersed oocytes, 2 = approximately equal mix of ovarian and testicular tissue, and 3 = mostly ovarian tissue with interspersed testicular tissue. Sperm abundance within seminiferous tubules of mature males was assessed on a 0–4 scale (28): 0 = sperm absent, 1 = sperm in <25% of tubules, 2 = sperm in 25–50% of tubules, 3 = sperm in 50–75% of tubules, and 4 = sperm in >75% of tubules. Ovarian development was assessed on the basis of the degree and mode of oocyte development (27): 1 = chromatin nucleolar, 2 = chromatin peri-nucleolar, 3 = corpora alveolar, 4 = early vitellogenic, 5 = midvitellogenic, and 6 = late vitellogenic. Ovaries were staged by identifying the most mature oocytes present in the histological section (29).

Plasma vitellogenin was measured by enzyme-linked immunosorbent assay using an anticarp kit (Biosense; Bergen, Norway). Reactivity with white sucker vitellogenin was verified by the manufacturer and via parallelism experiments with diluted plasma (data not shown). Male plasma was diluted 1:10 and female plasma was diluted 1:1000 or 1:10000 on the basis of the maturation stage. All samples were run in duplicate. Non-intersex status of male and

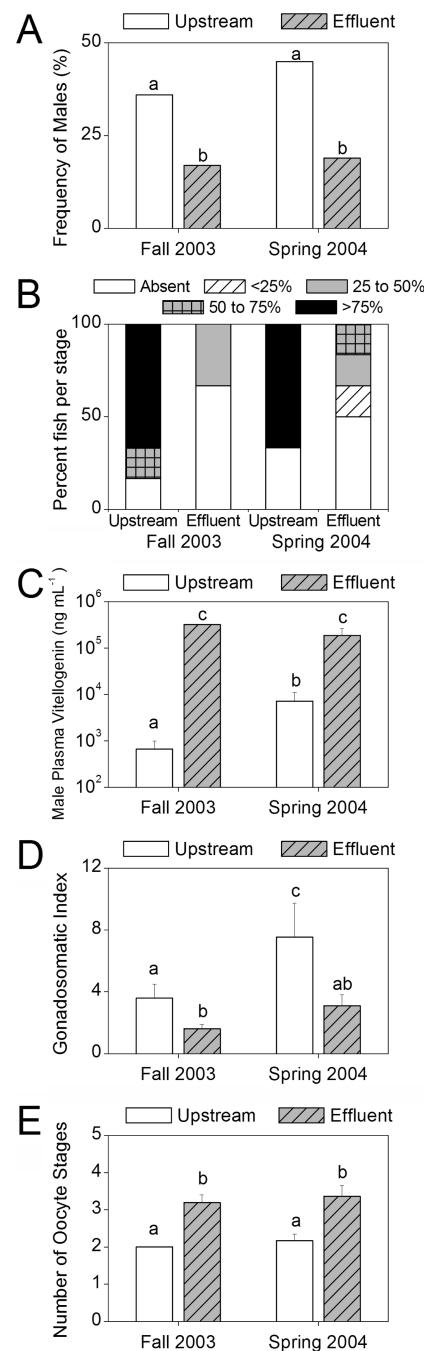


FIGURE 1. Evidence of reproductive impairment in white suckers collected from the Boulder Creek upstream and effluent sites during the fall and the spring. (A) Female-biased population sex ratio in effluent-exposed white suckers, calculated as frequency of males [males/(males + females + intersex) × 100]. Fall upstream site, $n = 22$; effluent site, $n = 23$. Spring upstream site, $n = 16$; effluent site, $n = 34$. (B) Sperm abundance in male white suckers. (C) Plasma vitellogenin concentrations in male white suckers (mean \pm SEM). Fall upstream site, $n = 5$; effluent site, $n = 1$. Spring upstream site, $n = 5$; effluent site, $n = 5$. Bars with different superscript letters differ significantly ($p < 0.05$). (D) Gonadosomatic index in female white suckers (mean \pm SEM). Fall upstream site, $n = 10$; effluent site, $n = 14$. Spring upstream site, $n = 6$; effluent site, $n = 19$. (E) Number of oocyte stages identified in the ovaries of adult female white sucker (mean \pm SEM). Fall upstream site, $n = 8$; effluent site, $n = 6$. Spring upstream site, $n = 10$; effluent site, $n = 11$.

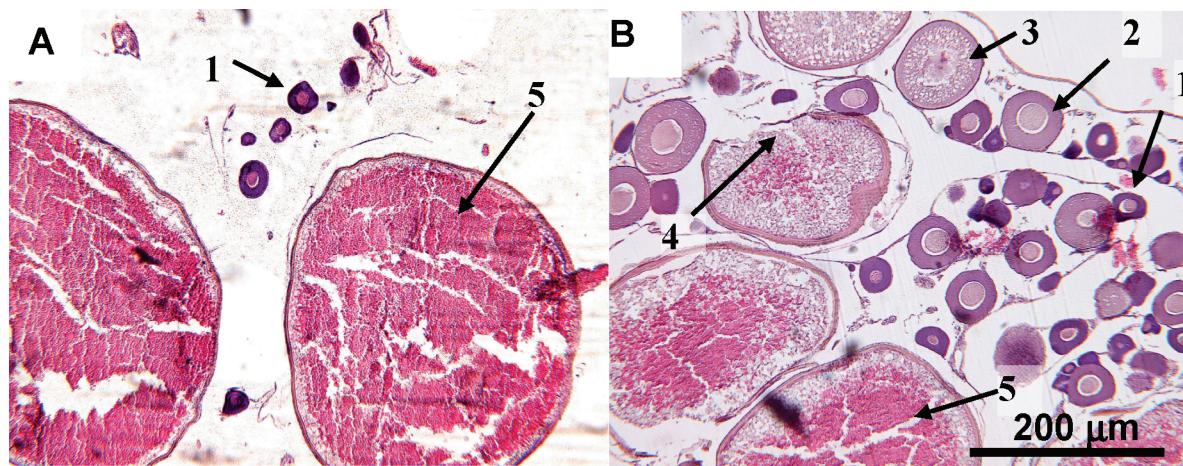


FIGURE 2. Ovarian asynchrony in effluent-site female white suckers. (A) Synchronous ovarian development in upstream females with only two stages of oocyte simultaneously present (stages 1 and 5). (B) Asynchronous ovarian development in effluent-site fish with five stages (1, 2, 3, 4, and 5) of oocyte present. Photomicrographs A and B are presented at the same magnification.

female plasma samples was verified by examination of gonadal histology.

Statistical Analysis. Proportional sex-ratio data were analyzed using Fischer's exact χ^2 test to compare each site with a predicted 1:1 male to female ratio. Nonnormal scores for the degree of sperm abundance and ovarian stage were analyzed by Kruskall-Wallis tests followed by Dunn's posthoc comparisons. Remaining data were tested for homoscedasticity and analyzed by two-way ANOVA for effects of the site and sampling period, followed by Fischer's posthoc comparisons (30). Vitellogenin concentration data were log-transformed prior to analysis. Statistical significance was accepted at $p < 0.05$. Biological results are expressed as mean \pm standard error of the mean (SEM) of the back-transformed data where necessary.

Water Sampling and Chemical Analysis. Water samples were collected for analysis of EACs in September 2003 and April 2005 using depth- and width-integrated compositing techniques at sites 0.1 km upstream from the WWTP outfall, the WWTP outfall, and 3.7 km downstream from the WWTP. Steroidal hormones and other wastewater EACs were measured by solid-phase extraction (SPE) and continuous liquid/liquid extraction (CLLE) and gas chromatography/mass spectrometry (GC/MS) analysis (12, 20). In all methods surrogate standards were added to unfiltered water samples prior to extraction. Steroids were isolated by C₁₈ SPE disks, the disks were rinsed with 20% methanol/water and eluted with 95% methanol/water, and the residue was dried under N₂. Methoxime and trimethylsilyl derivatives were formed by reaction with O-methoxyamine in pyridine followed by bis(trimethylsilyl)trifluoroacetamide with 10% trimethylchlorosilane. The derivatives were analyzed by gas chromatography/tandem mass spectrometry (GC/MS/MS) in splitless mode. The limits-of-quantification (LOQ), based on a peak-to-peak signal-to-noise ratio of 10, ranged from 0.2 to 2 ng L⁻¹ or less for all the estrogens except estrone (40 ng L⁻¹). Nonsteroidal EACs were isolated by CLLE. Ionic strength was increased by adding sodium chloride, pH was adjusted to <2 with H₂SO₄, and the water was extracted with methylene chloride for 6 h. The extracts were analyzed by GC/MS in the full scan and selected ion monitoring (SIM) modes. Compound identification was based on matching retention time (± 0.05 min) and ion ratios (3 ions \pm 20%) against authentic standards. Quantitation was based on an external calibration curve. Acidic nonylphenolethoxycarboxylates were determined by evaporation to dryness, reaction with acetyl chloride/propanol to form the propyl esters of the carboxylic acids, and analysis by SIM GC/MS.

Results and Discussion

Reproductive disruption at multiple loci, including skewed sex ratio (Figure 1A), decreased sperm abundance (Figure 1B), elevated vitellogenin in male plasma (Figure 1C), disrupted ovarian development (Figure 1D,E; Figure 2A,B), and gonadal intersex (Figure 3A–C) was observed in Boulder Creek effluent-site white suckers. No evidence of reproductive disruption was observed at the upstream site. These findings are consistent with exposure to estrogenic wastewater constituents (17, 31, 32) such as those identified in the WWTP effluent and the Boulder Creek effluent site (Table 1). Similar trends in reproductive disruption in white sucker previously have been observed in this same reach of Boulder Creek and other urban-impacted streams of Colorado's Front Range (19, 33).

Female-Biased Sex Ratio. Exposure to EACs can disrupt gonadal patterning and yield partial or complete sex reversal, skewing population sex ratios in teleost fish (34). The male to female sex ratio of white suckers at the effluent site was significantly biased toward females (χ^2 , $p < 0.05$), and a significantly higher frequency (2 \times) of male white suckers was observed at the upstream site relative to the effluent site (Figure 1A) in both the fall and spring samplings (χ^2 , $p < 0.05$). Mean length, weight, condition factor, and hematocrit of white suckers did not differ between the upstream and effluent site regardless of sex in either the fall or spring ($p > 0.05$) (33). Mean fish length in the fall was 205 ± 15 mm (range of 57–450 mm) and the mean length in the spring was 237 ± 11 mm (range of 75–365 mm).

Impaired Testicular Histopathology. Disrupted testicular function is a commonly reported outcome in estrogen-exposed male fishes (35). Sperm abundance among effluent-site males was significantly reduced relative to upstream males in both the fall and spring samplings (Figure 1B, $p < 0.05$), but there was no significant difference in GSI ($p > 0.05$, data not shown), suggesting that sperm abundance is more sensitive to disruption than GSI. Impairment of gonadal histopathology in effluent-site adult male white suckers is consistent with the activational effects of exposure to exogenous EACs (4).

Elevated Vitellogenin in Effluent-Site Males. Induction of vitellogenin synthesis in male fish is estrogen-dependent and is a reliable biomarker of exposure to exogenous estrogens (31). Plasma vitellogenin concentrations in effluent-site males was significantly ($p < 0.05$) elevated (Figure 1C), further implicating exposure to estrogenic EACs as underlying the observed reproductive disruption. In the spring sampling, effluent-site males ($n = 5$) had

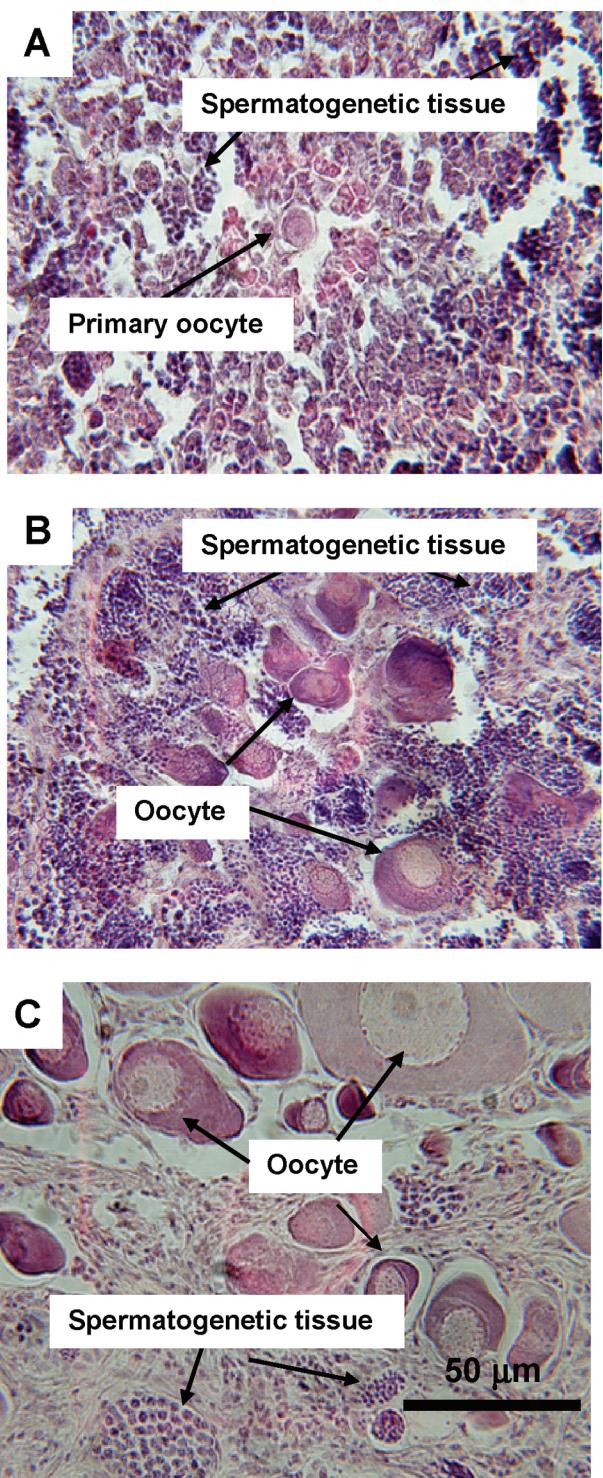


FIGURE 3. Gonadal intersex in effluent-exposed white suckers. (A) Gonad with mostly testicular tissue with interspersed oocyte, (B) gonad with an approximately equal mix of ovarian and testicular tissue, and (C) gonad with mostly organized ovarian tissue with interspersed testicular tissue. All photomicrographs are presented at the same magnification.

approximately 25 times more vitellogenin than upstream males ($n = 5$, $p < 0.05$). In the fall sampling, a single effluent-site male (only one of three males was available for measurement) had almost 500 times more vitellogenin than upstream males ($n = 5$). Site and sampling period did not affect female plasma vitellogenin concentrations ($p > 0.05$; $n = 24$).

Impaired Ovarian Histopathology. In upstream fish, gonadal development was consistent with previously reported patterns of natural white sucker reproduction (24). In effluent-site females, GSI (Figure 1D) was reduced by 50% compared to upstream females ($p < 0.05$). While upstream females had greater GSI values in the spring than in the fall ($p < 0.05$), consistent with a seasonal pattern of reproductive development, no such sampling period effect was observed in effluent-site females. Ovarian development at the upstream site was consistent with the group-synchronous pattern previously reported for white suckers (24). Among adult females (≥ 200 mm total length), there was no difference between sites in maximal oocyte stage in either the fall or spring sampling ($p > 0.05$). However, there was a significant effect of site on the number of oocyte stages present within the ovary ($p < 0.05$). Upstream females had synchronously developing ovaries with only two oocyte stages [one previtellogenic (stage 1, 2, or 3) and one vitellogenic (stage 4, 5, or 6)], while downstream females had asynchronously developing ovaries with up to five oocyte stages (Figures 1E and 2A,B). Contrasts in major patterns of ovarian development have been reported between temperate and tropical species (27, 36) but not between adjacent populations of the same species.

Intersex Fish at Effluent Site. Gonadal intersex can be induced in fish through exposure to steroid estrogens, androgens, antiandrogens, endocrine-active phytochemicals, and alkylphenols (34). Although dosages of these compounds used to experimentally induce gonadal intersex are typically higher than levels encountered in the environment, their effects can be additive or even synergistic (37), and complex mixtures of EACs are present in WWTP effluents. Intersex white suckers were identified at the effluent site and were approximately as abundant as males, comprising 22% of the population during the fall (5 of 23) and 18% in the spring (6 of 34). Only a single case of intersex previously has been reported for white suckers (38), suggesting a low natural incidence of this phenomenon. Unlike other studies reporting intersex fish (17), no intersex fish were observed at the upstream site during either collection period.

The relative proportion of ovarian and testicular tissue and the degree of tissue organization varied between intersex fish. Likewise, the intersex observed differs from the testis–ova reported in other studies (17, 18) where primary oocytes were interspersed in an otherwise normal testis. Only 27% of the intersex white suckers in Boulder Creek fit this testis–ova pattern (Figure 3A). The more common observation indicates different levels of tissue organization ranging from randomly interspersed ovarian and testicular tissue (27%; Figure 3B) to highly organized ovarian tissue occurring adjacent to testicular tissue (45%; Figure 3C). Whether different patterns of ovarian and testicular tissue organization within intersex gonads reflect differences in mode or severity of reproductive disruption is unknown. Gonadal intersex in gonochoristic teleosts is recognized as a biomarker of early life-stage exposure to exogenous EACs that is related to the timing, dosage, and duration of exposure (39). Reproductive fitness also may be impaired in intersex fish (40, 41). Sperm were not expressed by any of the intersex white suckers collected in the fall and were weakly present in only one of the intersex fish collected in the spring. Vitellogenic oocytes (stage 4) were found in one of the five intersex fish collected in the fall, and no vitellogenic oocytes were observed in intersex fish collected in the spring. Enlarged previtellogenic oocytes (stage 2 and stage 3) were found in four of the five intersex fish collected in the spring.

Occurrence of EACs. Of the eight steroid estrogens monitored in this study, 17β -estradiol (E_2), estrone, and 17α -ethynodiol were detected in the WWTP effluent and at the effluent site (Table 1) at concentrations ranging from <2

TABLE 1. Concentrations of Endocrine-Active Chemicals Detected in the Boulder Creek Upstream Site, the Boulder WWTP Effluent, and the Boulder Creek Effluent Site on September 3, 2003 and April 19, 2008^a

compd	av ^b in vitro EEF	max ^c in vitro or in vivo EEF	data source ^d	upstream site 09/03 (ng L ⁻¹)	WWTP effluent 09/03 (ng L ⁻¹)	effluent site 09/03 (ng L ⁻¹)	upstream site 04/05 (ng L ⁻¹)	WWTP effluent 04/05 (ng L ⁻¹)	effluent site 04/05 (ng L ⁻¹)
steroids									
17 α -ethynodiol	1.2	33	13, 14, 13–15, 52–56	<2 <0.8	<2	<2	<0.8	2.1	0.7
17 β -estradiol	1.0	1.0	n/a	2.9	2.1	<0.2	3.2	1.6	36
estrone	0.2	0.8	13, 53	n/a	n/a	<5	110	110	36
other endocrine-active compounds									
bisphenol A	7.0 \times 10 ⁻⁵	1.6 \times 10 ⁻⁴	14, 55, 57	3.1	2.7	2.5	27	85	35
1,2-dichlorobenzene	1.0 \times 10 ⁻⁹	1.0 \times 10 ⁻⁹	56	<5	<5	<10	20	20	<10
1,4-dichlorobenzene	1.0 \times 10 ⁻⁷	1.0 \times 10 ⁻⁷	56	<5	<5	5.5	<10	290	120
4-nonylphenolmonoethoxycarboxylate	2.0 \times 10 ⁻⁵	2.0 \times 10 ⁻⁵	54	700	120000	47000	300	83000	44000
4-nonylphenoldiethoxycarboxylate	2.0 \times 10 ^{-5e}	2.0 \times 10 ^{-5e}	54	<100	39000	16000	200	75000	39000
4-nonylphenoltriethoxycarboxylate	2.0 \times 10 ^{-5e}	2.0 \times 10 ^{-5e}	54	<100	1100	400	<100	1500	700
4-nonylphenoltetraethoxycarboxylate	2.0 \times 10 ^{-5e}	2.0 \times 10 ^{-5e}	54	<100	600	200	<100	800	200
4-nonylphenol	5.3 \times 10 ⁻⁵	3.6 \times 10 ⁻³	13, 14, 16	<33	46	39	120	720	340
4-nonylphenolmonoethoxyacetate	1.0 \times 10 ^{-6f}	1.0 \times 10 ^{-6f}	52, 54	>20	1800	29	60	4700	480
4-nonylphenoldiethoxyacetate	1.0 \times 10 ⁻⁶	6.0 \times 10 ⁻⁶	52, 54	>20	330	21	150	1300	220
4-nonylphenoltriethoxyacetate	1.0 \times 10 ^{-6f}	1.0 \times 10 ^{-6f}	52, 54	<50	170	<20	<20	540	>50
4- <i>tert</i> -octyphenol	3.6 \times 10 ⁻⁴	5.0 \times 10 ⁻⁴	52, 54	>5	6.7	5.2	18	120	81
4- <i>tert</i> -octyphenolmonoethoxyacetate	3.6 \times 10 ^{-4g}	3.6 \times 10 ^{-4g}	54	>5	29	<5	<5	130	17
4- <i>tert</i> -octyphenoldiethoxyacetate	3.6 \times 10 ^{-4g}	3.6 \times 10 ^{-4g}	54	>5	63	<5	<5	300	<5
EEq ^h av	0.02	6.2	3.4	0.03	31	11	11	11	11
EEq ^h max	0.02	6.2	3.4	0.03	54	19	19	19	19

^a Estradiol equivalency quotients (EEQ) in ng E₂ L⁻¹ are presented for each site based on the estrogen equivalence factor (EEF) of each compound.^b Average in vitro estrogenic EEF relative to 17 β -estradiol. ^c Maximum reported in vitro or in vivo EEF relative to 17 β -estradiol. ^d See references section for data sources. ^e EEF is assumed to be equal to 4-nonylphenolmonoethoxyacetate. ^f EEF is assumed to be equal to 4-nonylphenoldiethoxyacetate. ^g EEF is assumed to be equal to 4-*tert*-octyphenol. ^h EEF is calculated by multiplying aqueous concentration of each compound by the EEF (average or maximum) of the compound and summing for all detected compounds.

to >100 ng L $^{-1}$. In 2003, recovery E₂ averaged $123 \pm 12\%$ ($n = 4$). In 2005, recovery of eight steroidal estrogens averaged $85 \pm 21\%$ and average deviation of duplicate analyses was 15%. Steroidal estrogens were not detected in blank QA samples in this study. In 2003, recovery of octylphenol, nonylphenol, and their ethoxylates in natural water matrix spikes averaged $23.6 \pm 9.1\%$ ($n = 3$). Reported results are not recovery corrected so potentially represent an underestimate of concentration during this sampling period. In 2005, recovery averaged $103 \pm 16\%$ ($n = 4$). Several compounds were sporadically detected at trace levels in blank QA samples (orders of magnitude lower than observed in environmental samples).

The concentrations of steroidal estrogens, particularly estrone, were consistent with concentrations reported for trickling filter (TF), or TF combined with activated sludge effluents (6, 8, 42). The relatively high levels of estrogens, especially estrone and 17 α -ethynodiol, are driven by both source water quality and efficiency of removal by the WWTP process. The nonsteroidal estrogens measured in the WWTP effluent included 4-*tert*-octylphenol, 4-*tert*-octylphenolethoxylates, 4-nonylphenol, 4-nonylphenolethoxylates, 4-nonylphenolethoxycarboxylates, and bisphenol A (Table 1). The 4-nonylphenolethoxycarboxylates were the most abundant alkylphenolic compounds detected with concentrations orders of magnitude greater than the other EACs. Although alkylphenolic compounds are less estrogenic than steroidal estrogens, because of their higher concentrations, they also contribute to estrogenic effects (43, 44).

Steroidal estrogens, alkylphenolic EACs, and mixtures thereof can interact additively with the estrogen receptor in fishes to stimulate vitellogenesis and other estrogen-dependent end points (14, 15, 37). The measured concentration data were used to estimate total estrogen equivalency (EEq; in ng E₂ L $^{-1}$) of the water samples. Stream and effluent EEq was determined by multiplying measured concentrations by compound-specific estrogen equivalence factors (EEF) (45) obtained from published studies on compound-specific in vitro or in vivo estrogenic activity (Table 1). The fall 2003 data indicate that nonsteroidal estrogens account for 53% of measured EEq of the WWTP effluent, whereas the spring 2005 data indicate that they account for 11% of the EEq. This is likely the result of improved analytical techniques allowing for lower level detection of 17 α -ethynodiol and estrone in spring 2005, because levels of other analytes were comparable between the two sampling periods. High values of EEq at the effluent and downstream sites were derived primarily from the presence of the steroidal estrogens; however, the estimated effluent EEq resulting from nonsteroidal EACs (>3 ng E₂ L $^{-1}$) was potentially sufficient to induce biological effects (13).

Implications for Fish Populations. Fish inhabiting WWTP effluent-impacted streams in arid environments may be particularly vulnerable to reproductive disruption due to the large volumes of WWTP effluent discharged into relatively small streams. Decreased concentrations of individual compounds and overall EEq downstream results primarily from dilution by upstream water (21). In spring 2005, EEq decreased from 31 ng E₂ L $^{-1}$ at the effluent site to 11 ng E₂ L $^{-1}$ at the downstream site where wastewater comprised 40% of the flow. A similar trend was evident in the fall when wastewater comprised 37% of flow. Because it appears that EAC removal is slow in Boulder Creek, and there are no further downstream inputs of water not affected by municipal WWTP discharge, it is likely that EAC concentrations remain elevated for a considerable distance. During low-flow conditions WWTP effluent can comprise nearly all of the stream flow resulting in even higher instream EAC concentrations.

Nonestrogenic environmental factors characteristic of WWTP effluents (increased stream temperatures and decreased oxygenation), social factors (skewed sex ratio), or wastewater nutrients could account for some individual effects in effluent-site white suckers (46–49). However, these factors are not likely to account for the full and widespread suite of disrupted reproductive end points observed in this investigation. For example, increased temperatures at the effluent site would be expected to accelerate and not inhibit reproductive development (34). On the other hand, numerous studies have demonstrated that the concentrations of E₂, 17 α -ethynodiol, alkylphenols, and bisphenol A found in Boulder Creek are in the range reported to induce female-biased sex ratios and gonadal intersex (34), impaired spermatogenesis (36), and male vitellogenesis (17, 32, 50) observed at the Boulder Creek effluent site. Although the population-level consequences of this observed reproductive disruption are unclear, wild fish populations have been shown to collapse upon prolonged residency in a lake with a similar EEq (51).

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