Aquatic Toxicology 103 (2011) 213–221

Contents lists available at ScienceDirect

Aquatic Toxicology

journal homepage: www.elsevier.com/locate/aquatox

Demasculinization of male fish by wastewater treatment plant effluent

Alan M. Vajda[a,*], Larry B. Barber[b], James L. Gray[b], Elena M. Lopez[a], Ashley M. Bolden[a], Heiko L. Schoenfuss[c], David O. Norris[a]

[a] Department of Integrative Physiology, University of Colorado, UCB 354, Boulder, CO 80309, United States
[b] U.S. Geological Survey, 3215 Marine Street, Boulder, CO 80303, United States
[c] St. Cloud St. University, 720 Fourth Avenue South, Aquatic Toxicology Laboratory, St. Cloud, MN 56301, United States

ARTICLE INFO

Article history:
Received 15 July 2010
Received in revised form 31 January 2011
Accepted 12 February 2011

Keywords:
Endocrine disruption
Estrogen
Nonylphenol
Wastewater
Fish

ABSTRACT

Adult male fathead minnows (*Pimephales promelas*) were exposed to effluent from the City of Boulder, Colorado wastewater treatment plant (WWTP) under controlled conditions in the field to determine if the effluent induced reproductive disruption in fish. Gonadal intersex and other evidence of reproductive disruption were previously identified in white suckers (*Catostomus commersoni*) in Boulder Creek downstream from this WWTP effluent outfall. Fish were exposed within a mobile flow-through exposure laboratory in July 2005 and August 2006 to WWTP effluent (EFF), Boulder Creek water (REF), or mixtures of EFF and REF for up to 28 days. Primary (sperm abundance) and secondary (nuptial tubercles and dorsal fat pads) sex characteristics were demasculinized within 14 days of exposure to 50% and 100% EFF. Vitellogenin was maximally elevated in both 50% and 100% EFF treatments within 7 days and significantly elevated by 25% EFF within 14 days. The steroidal estrogens 17β-estradiol, estrone, estriol, and 17α-ethynylestradiol, as well as estrogenic alkylphenols and bisphenol A were identified within the EFF treatments and not in the REF treatment. These results support the hypothesis that the reproductive disruption observed in this watershed is due to endocrine-active chemicals in the WWTP effluent.

1. Introduction

Exogenous endocrine-active chemicals (EACs) can interact directly with endocrine regulatory systems (Tabb and Blumberg, 2006), leading to the disruption of reproductive development and function in exposed organisms (McLachlan, 2001). Anthropogenic EACs enter aquatic environments through diverse human sources including military (Bordeleau et al., 2008), medical (Thornton et al., 1996), industrial (Parrot et al., 2006), agricultural (Horrigan et al., 2002), energy (Lye, 2000), and wastewater (Aerni et al., 2004; Barber et al., 2006; Johnson et al., 2005; Schultz et al., 2010; Vajda et al., 2008) activities. The resulting presence of EACs in the environment can elicit adverse effects in exposed organisms, including plant–microbe symbioses (Fox et al., 2001), invertebrates (Oberdörstor et al., 2001), and vertebrates (Mills and Chichester, 2005).

Effluent discharges from wastewater treatment plants (WWTPs) contain a complex mixture of synthetic and biogenic EACs (Aerni et al., 2004; Barber et al., 2006; Johnson et al., 2005). Many surface waters that receive WWTP effluent have detectable levels of steroidal and non-steroidal estrogens (Kolpin et al., 2002; Johnson et al., 2005), androgens (Kolodziej et al., 2003) and neuroactive pharmaceuticals (Schultz et al., 2010). In some cases, as with 17β-estradiol, exogenous EACs are structurally and functionally identical to endogenous steroid hormones. Their addition to endogenous hormone loads can destabilize vertebrate endocrine signaling (Vajda and Norris, 2006).

Reproductive disruption has been observed in fishes inhabiting estrogen-contaminated waters (Jobling et al., 1998; Mills and Chichester, 2005). Recently, gonadal intersex, reduced sperm abundance, and elevated plasma vitellogenin levels in male fish were identified in white sucker (*Catostomus commersoni*) in a WWTP effluent-dominated reach of Boulder Creek, Colorado (Vajda et al., 2008; Woodling et al., 2006). Effluent discharged from the Boulder WWTP comprises up to 80% of streamflow during low-flow conditions, and rarely contributes less than 35% (Murphy et al., 2003). As a result, native fish are exposed to a complex mixture of EACs at all stages of their life cycle (Barber et al., 2006; Vajda et al., 2008).

The objective of this study was to determine whether the reproductive disruption previously observed in wild fish could be induced by the Boulder WWTP effluent. Likewise, the lack of reproductive disruption in the upstream water was evaluated. The biological results were then compared to the predicted relative estrogenicity of the two waters based on measured EAC concentrations. This study improves upon previous work that involves *in situ* exposure of caged fish by controlling for parameters known to effect endocrine status (i.e. temperature, oxygen, biotic environment)

* Corresponding author. Current address: Department of Integrative Biology, University of Colorado, CB171, Denver, CO 80217, United States. Tel.: +1 303 556 6765.
E-mail address: alan.vajda@ucdenver.edu (A.M. Vajda).

0166-445X/$ – see front matter © 2011 Elsevier B.V. All rights reserved.
doi:10.1016/j.aquatox.2011.02.007

(Aerni et al., 2004; Giesy et al., 2003; Harries et al., 1997; Purdom et al., 1994). Conversely, previously published controlled studies that expose fish to transported effluent that may not accurately reflect in-stream EAC concentrations due to chemical transformation after sample collection (Robinson et al., 2003). In the present study, controlled on-site, continuous-flow fish exposure experiments were conducted in a bioassay laboratory (Barber et al., 2007; Harries et al., 1999; Rodgers-Gray et al., 2001). Because EACs and other organic contaminants are known to leach from plastic materials (Soto et al., 1991), the system was constructed using only glass, stainless steel, and Teflon®. We evaluate primary and secondary sex characteristics of adult male fathead minnows (*Pimephales promelas*) exposed to WWTP effluent and reference water from upstream of the WWTP outfall under controlled conditions in the field. Concentrations of EACs were measured in the WWTP effluent and upstream reference water to evaluate their occurrence and concentrations relative to observed biological effects.

2. Materials and methods

2.1. Study site

The WWTP for the City of Boulder, Colorado was selected because reproductive disruption was observed among free-living white suckers in Boulder Creek below the WWTP outfall (Vajda et al., 2008; Woodling et al., 2006) and the hydrogeochemistry of Boulder Creek (including estrogenic EACs) is well characterized (Barber et al., 2006; Murphy et al., 2003). This WWTP has an average discharge of 0.74 $m^3 s^{-1}$ (17 million gallons a day) and can contribute from <40% of stream flow during spring runoff conditions to >75% of the flow during base-flow conditions in late summer. Sewage from the City of Boulder was treated using a combined trickling filter/activated sludge process with nitrification/denitrification and chlorination/dechlorination, and hydraulic retention time of ~12 h. The mean annual concentrations (January 2005–December 2007) of ammonia, nitrate, biological oxygen demand, and total suspended solids in the WWTP effluent were 6.6, 11.8, 14.5, and 6 mg L^{-1}, respectively (City of Boulder). Two source waters were used for the fish exposure experiments, WWTP effluent (EFF), and Boulder Creek water from approximately 500 m upstream from the WWTP outfall (REF).

2.2. On-site fish bioassay laboratory

Fish exposure experiments were conducted during July 2005 and August 2006 in a mobile laboratory deployed at the Boulder WWTP. During all exposure experiments, temperature (22 ± 1 °C), lighting, diet, aeration (>85% saturation), and flow (200 mL min^{-1}, six daily volume replacements) were controlled. Contaminant concentrations in WWTP effluents and surface water can fluctuate on various time scales, and this design reflects these fluctuations rather than maintaining a constant exposure concentration. All surfaces in contact with test solutions were made of glass, stainless steel, or Teflon®. Water from the EFF and REF sites was continuously pumped through Teflon® tubing to the lab using stainless steel pumps (Grundfos SQE; Geotech Environmental Equipment, Denver, CO) into separate 200-L stainless steel holding tanks positioned above the laboratory. The two water sources were thermally equilibrated, then flowed by gravity to stainless steel splitter tanks that distributed the water to 10-L glass aquaria housing the fish.

2.3. Experimental design

Reproductively stimulated 12-month-old adult male fathead minnows were obtained from the U.S. Environmental Protection Agency (Cincinnati, OH) for experiments in 2005 and from Aquatic Biosystems (Ft. Collins, CO) for experiments in 2006. For both experiments, 10 randomly collected fish were processed upon arrival as initial controls. The remaining fish were weighed, measured, and randomly distributed to exposure treatments. In 2005, the fish were exposed to 100% EFF, 50:50 EFF:REF, and 100% REF treatments with four exposure durations (4, 7, 14, and 28 days) yielding 12 treatment groups. In 2006, the fish were exposed to 100% EFF, 50:50 EFF:REF, 25:75 EFF:REF, 10:90 EFF:REF, 5:95 EFF:REF, and 100% REF treatments with two exposure durations (14 and 28 days) yielding 12 treatment groups. In both experiments, 5–6 fish were placed into each of the 10-L aquarium. Mean body weight and total length [mean ± standard deviation (SD)] of the fish at the start of each study did not differ between treatment groups.

Fish were fed frozen brine shrimp twice daily at 08:00 and 20:00 h. The feeding rate was 2% of body weight per day, based on mean body weight on day-0 and adjusted for any deaths and fish sampled during the studies. Surplus food and feces were removed daily. Animal care and handling was in accordance with the Institutional Animal Care and Use Committee of the University of Colorado.

2.4. Tissue sampling and analysis

In 2005, two tanks of fish (n = 6–10) were sampled from each treatment at 4, 7, 14, and 28 days. In 2006, one tank of fish (n = 5–6) was sampled from each treatment at day 14 and day 28. Fish wet weight (0.01 g) and total length (mm) were recorded and used to calculate the condition factor [(body weight (g)/total lengths^3 (mm)) × 100]. Nuptial tubercle prominence (Smith, 1974; Smith, 1978) was scored as: 1 = tubercles not visible, 2 = tubercles visible as white discs, 3 = tubercles prominent, and 4 = tubercles prominent and protruding sharply. Dorsal fat pad prominence was assessed as 1 = not visible, 2 = soft discolored tissue, 3 = spongy thickened tissue, and 4 = dorsal hump with spongy tissue. Blood was collected into heparinized capillary tubes from the caudal vein, kept on ice, and centrifuged for 5 min at 3000 × g within 3 h. Hematocrit was recorded and aliquots of plasma were frozen and stored at −40 °C until analyzed for vitellogenin by homologous enzyme-linked immunosorbent assay (ELISA) using an anti-fathead minnow kit (Biosense, Bergen, Norway).

Gonads, liver, and spleen were dissected and weighed (0.001 g), and the gonadosomatic index (GSI) [(gonad weight (g)/body weight (g)) × 100], hepatosomatic index (HSI) [(liver weight (g)/body weight (g)) × 100], and splenosomatic index (SSI) [(spleen weight (g)/body weight (g)) × 100] were calculated. Freshly dissected gonads were preserved in 10% neutral-buffered formalin until processed for histology (Presnell and Schreibman, 1997), at which time a whole testis from each fish was prepared. At least 10 whole cross-sections from each testis were evaluated on a 1–5 scale by light microscopy for the amount of mature sperm present (Pawlowski et al., 2004a,b): 1 = sperm absent, 2 = weak, sperm prominent in <25% of tubules, 3 = moderate, sperm prominent in 25–50% of tubules, 4 = strong, sperm prominent in 50–75% of tubules, and 5 = very strong, sperm prominent in >75% of tubules.

2.5. Water sampling and chemical analysis

Weekly water grab samples were collected starting at day-0 from the EFF and REF inflows into the on-site laboratory. The EFF and REF samples were analyzed for EACs by solid phase extraction (SPE) and continuous liquid/liquid extraction (CLLE), followed by gas chromatography/mass spectrometry (GC/MS) and gas chromatography/tandem mass spectrometry (GC/MS/MS) analysis (Barber et al., 2000). Surrogate standards were added to unfiltered water samples prior to extraction to evaluate method perfor-

Table 1
Average concentrations (±SD) of endocrine active chemicals detected in the Boulder wastewater treatment plant (WWTP) effluent (EFF) and Boulder Creek upstream from the WWTP outfall (REF). Also shown are chemical specific *in vitro* and *in vivo* 17β-estradiol equivalency factors used to calculate 17β-estradiol equivalency quotients.

Compound	*In vitro* estradiol equivalency factor[a]	*In vivo* estradiol equivalency factor[a]	Source[b]	2005 REF, $n=6$ ($ng\,L^{-1}$)	SD	2005 EFF, $n=7$ ($ng\,L^{-1}$)	SD	2006 REF, $n=6$ ($ng\,L^{-1}$)	SD	2006 EFF, $n=6$ ($ng\,L^{-1}$)	SD
Steroids											
17β-Estradiol	1	1	1–3	<0.2		2.1	1.0	<0.2		3.2	3.2
17α-Ethynylestradiol	1.2	25	2,3	<0.2		1.2	0.7	<0.2		<0.2	
Estrone	0.2	0.4	2,3	<0.2		75	28	<0.2		60	75
Estriol				<0.2		3.4	3.2	<0.2		<0.2	
17α-Estradiol				<0.2		0.1	0.3	<0.2		<0.2	
Other estrogens											
4-Nonylphenol	5.3E−5	0.003	3,4	210	230	2000	1300	73	34	240	100
Bisphenol A				33	46	42	44	18	27	10	5.2
1,2-Dichlorobenzene				<10		<10		<10		120	79
1,4-Dichlorobenzene				*81*	140	2100	3000	<10		1200	360
4-*tert*-Butylphenol				<10		68	73	<10		47	43
4-*tert*-Pentylphenol				<10		65	82	<10		1.9	2.2
4-Nonylphenolmonoethoxylate				480	1200	9800	5800	68	71	1500	770
4-Nonylphenoldiethoxylate				150	400	5000	4000	89	140	440	380
4-Nonylphenoltriethoxylate				62	160	2000	1800	<50		280	240
4-Nonylphenoltetraethoxylate				<50		450	730	<50		39	31
4-Nonylphenolmonoethoxycarboxylate				270	330	31600	9400	370	220	50000	6800
4-Nonylphenoldiethoxycarboxylate				300	510	42000	7300	200	110	34000	13000
4-Nonylphenoltriethoxycarboxylate				<50		1500	690	<50		510	240
4-Nonylphenoltetraethoxycarboxylate				<50		890	740	<50		210	72
4-*t*-Octylphenol				17	29	200	120	<10		30	14
4-*t*-Octylphenolmonoethoxylate				17	42	290	260	<10		22	11
4-*t*-Octylphenoldiethoxylate				82	220	2100	2700	8.7	11	53	67
4-*t*-Octylphenoltriethoxylate				<10		260	350	13	33	<10	
4-*t*-Octylphenoltetraethoxylate				<10		<10		<10		<10	
Estradiol equivalency quotient[c]	*In vitro*			<1		13.7	10.5	<1		12.6	10.8
Estradiol equivalency quotient[c]	*In vivo*			<1		50	34	<1		23	20

[a] *In vitro* and *in vivo* estrogen equivalency factors expressed as $ng\,L^{-1}$ relative to 17β-estradiol.

[b] References for estrogen equivalency factors: (1) Jobling and Sumpter (1993); (2) Soto et al. (1995); (3) Van Den Belt et al. (2004); (4) Preuss et al. (2006).

[c] Estrogen equivalency quotient in ng 17β-estradiol L^{-1} calculated by multiplying concentration of compound by the estradiol equivalency factor of the compound and summing. Concentrations reported are the mean ± SD from 6 to 10 samples collected throughout the experiment.

mance. Steroid hormones were isolated by C_{18} SPE, and eluted with 95:5 methanol:water. The residue was dried under N_2 and the methoxime:trimethylsilyl derivatives were formed by reaction with *O*-methoxyamine in pyridine followed by bis(trimethylsilyl)-trifluoroacetamide with 10% trimethylchlorosilane. The derivatives were analyzed by GC/MS/MS in splitless mode. The limits-of-quantification (LOQ), based on a signal-to-noise ratio of 10, ranged from 0.2 to 2 ng L^{-1}. Non-steroid EACs were isolated by CLLE from 1-L unfiltered samples using methylene chloride following ionic strength and pH adjustment. 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 ± 20%) against authentic standards. Quantitation was based on an external calibration curve. Acidic nonylphenolethoxycarboxylates were determined by evaporation to dryness, reaction with 10:90 acetyl chloride/propanol to form the propyl esters of the carboxylic acids, and analysis by SIM GC/MS (Barber et al., 2000).

2.6. Statistical analysis and calculations

Proportional, non-normal scores for degree of nuptial tubercle prominence, dorsal fat pad prominence, and sperm abundance were analyzed by Kruskal–Wallis tests. Remaining data were tested for homoscedasticity and analyzed by two-way ANOVA. Concentrations of vitellogenin were log transformed prior to statistical analysis. All computations were performed with PRISM 4.0 (GraphPad Software, Inc., San Diego, CA). Significance was accepted at the 5% level. Biological results are expressed as mean ± standard error of the mean (SEM). Estrogen equivalency factors (EEFs) and estrogen equivalency quotients (EEqs) were calculated as described previously (Vajda et al., 2008).

3. Results

3.1. Environmental and chemical measurements

Wastewater discharge from the City of Boulder WWTP during the 2005 and 2006 experiments averaged 0.74 m^3 s^{-1} (17 million gallons a day). The mean annual concentrations (January 2005–December 2006) of ammonia, nitrate, biological oxygen demand, dissolved organic carbon, and total suspended solids in the WWTP effluent were 6.6, 11.8, 14.5, 10.0 and 6 mg L^{-1}, respectively (City of Boulder, 2008).

The City of Boulder WWTP effluent contained a mixture of steroidal- and non-steroidal EACs that varied in composition, concentration, and potency throughout each experiment (Table 1 and Fig. 1). 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} for the steroids and 10 to 100 ng L^{-1} for non-steroidal EACs. Matrix spikes yielded acceptable recoveries (70–120%), and none of the compounds were detected in the blanks. Estrone and 17β-estradiol were detected in the WWTP effluent during both experiments (Table 1) at concentrations ranging from <2.0 to >75 ng L^{-1}. Estriol (1.2 ± 0.7 ng L^{-1}, $n = 7$) and the synthetic estrogen 17α-ethynylestradiol (3.4 ± 3.2 ng L^{-1}, $n = 7$) were detected in the WWTP effluent in 2005 (1.2 ± 0.7 ng L^{-1}, $n = 7$) but not 2006. Steroids were not detected in the REF samples in either experiment. Alkylphenolethoxylates and alkylphenolethoxycarboxylates were detected in all 2005 and 2006 EFF samples. Alkylphenolethoxylates concentrations ranged from 450 to 9800 ng L^{-1} and alkylphenolethoxycarboxylates concentrations ranged from 890 to 50,000 ng L^{-1}). EFF concentrations of 4-nonylphenol declined significantly from 2000 ± 1300 ng L^{-1} ($n = 7$) in 2005 to 240 ± 100 ng L^{-1} ($n = 6$) in 2006 ($p < 0.05$). When calculated using *in vivo* values, there was a significant ($p < 0.05$), 50% decline in the maximum predicted EFF EEqs from 2005 (50 ± 34 ng L^{-1}, $n = 7$) to 2006 (23 ± 20 ng L^{-1}, $n = 6$). No such difference in EFF EEq was detected when calculated with *in vitro* EEFs.

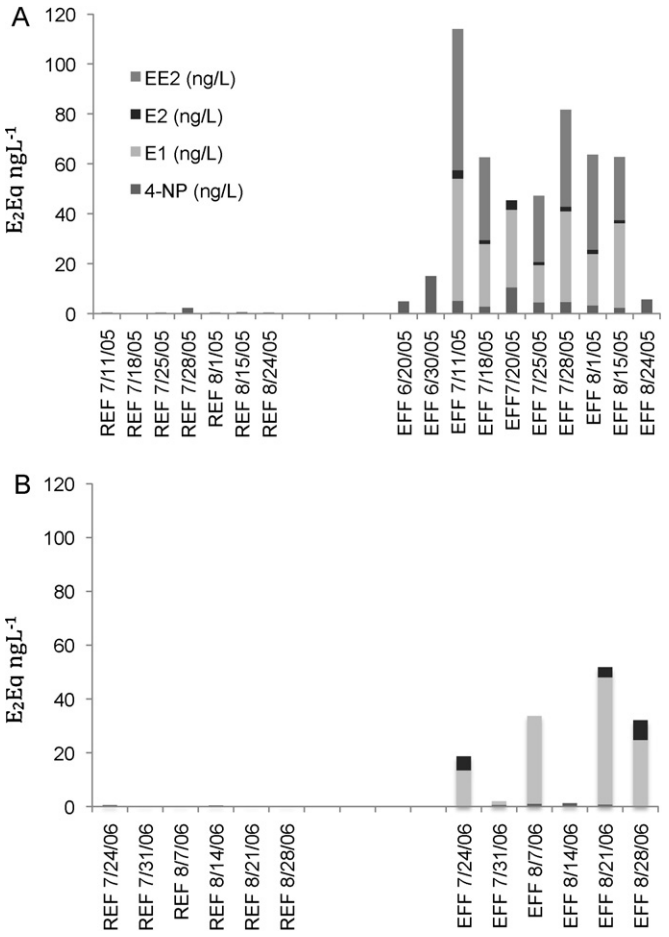

Fig. 1. Estradiol equivalency quotients for wastewater treatment plant (WWTP) effluent (EFF) in (A) 2005 and (B) 2006 calculated from measured concentrations of individual estrogenic endocrine-active chemicals [17α-ethynylestradiol (EE_2), 17β-estradiol (E_2), estrone (E_1) and 4-nonylphenol (4-NP)] multiplied by *in vivo* fathead minnow 17β-estradiol equivalency factors. Mean estrogen equivalency quotients were significantly greater in 2005 than 2006, as were the contributions of 17α-ethynylestradiol and 4-nonylphenol ($p < 0.05$).

3.2. Biological measurements

There was no significant effect of exposure or exposure duration on survivorship or condition in 2005 or 2006 ($p < 0.05$). The response of primary and secondary sex characteristic expression to EFF-exposure differed between experiments. EFF-exposure significantly suppressed the expression of secondary sex characteristics in both experiments. EFF-exposed fish had fewer ($p < 0.05$) and less prominent (Kruskal–Wallis, $p < 0.05$) nuptial tubercles (Fig. 2A–D) and significantly less prominent dorsal fat pads (Kruskal–Wallis, $p < 0.05$) (Fig. 2E and F). Primary sex characteristics were significantly affected by exposure in 2005, but not in 2006. In 2005, GSI was significantly reduced in the 100% EFF-exposed fish on day-14 and day-28 ($p < 0.05$). On day-14, reference male GSI was significantly greater than initial controls ($p < 0.05$). Histological examination of testes revealed that EFF-exposure significantly reduced the relative abundance of sperm (Kruskal–Wallis, $p < 0.05$) (Fig. 3A and B). Beginning on day-4 of the 2005 experiment, sperm were 'strongly' or 'very strongly' expressed in all REF fish at all sam-

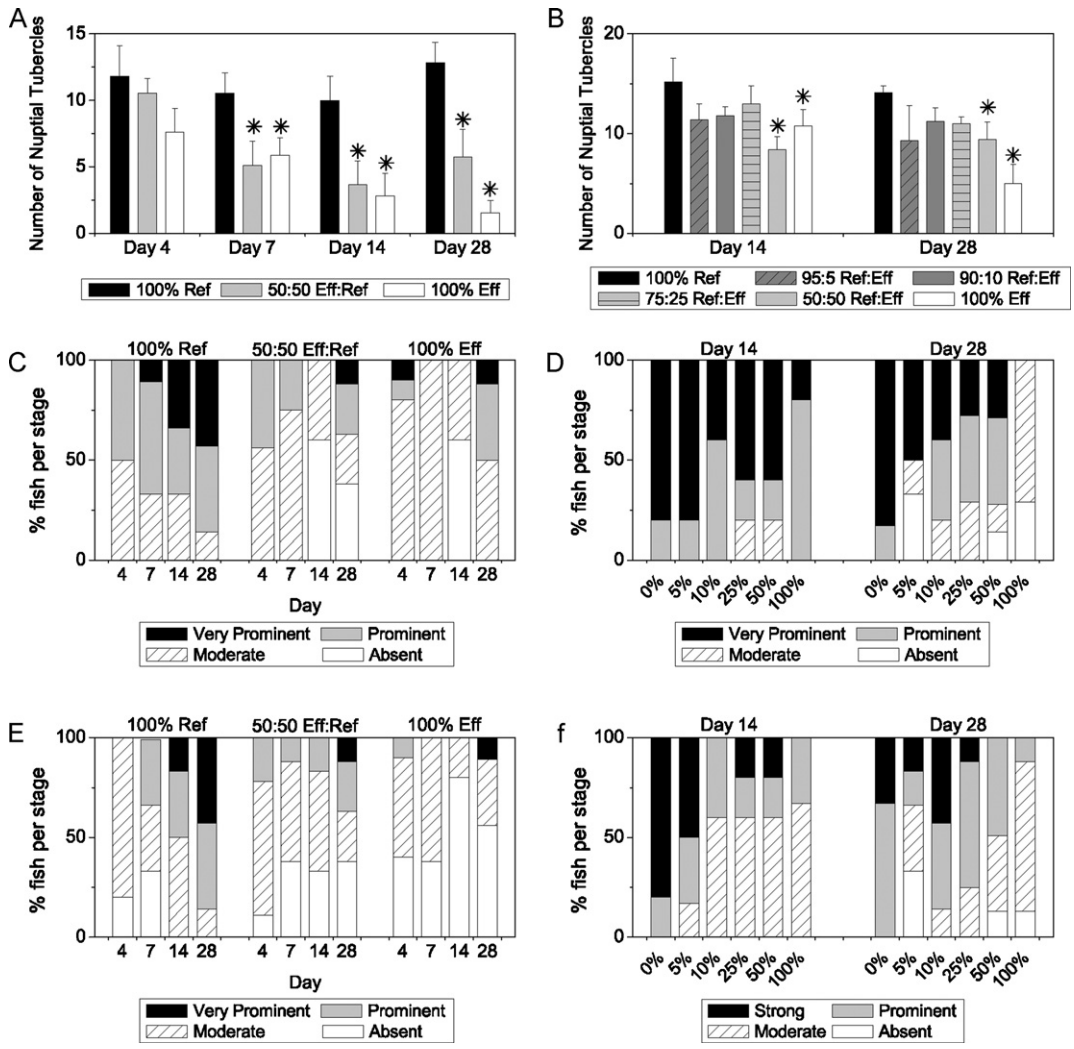

Fig. 2. (A) Number of nuptial tubercles expressed by male fathead minnows exposed to wastewater treatment plant (WWTP) effluent (100% EFF), Boulder Creek water upstream from the WWTP outfall (100% REF), and a mixture of the two during the 2005 male fathead minnow exposure experiment. (B) Number of nuptial tubercles expressed by male fathead minnows exposed to 100% EFF, 100% REF, and mixtures of the two during 2006. (C) Prominence of nuptial tubercles expressed by male fathead minnows exposed to 100% EFF, 100% REF, and a mixture of the two during 2005. (D) Prominence of nuptial tubercles expressed by male fathead minnows exposed to 100% EFF, 100% REF, and mixtures of the two during 2006. (E) Dorsal fat pad prominence expressed by male fathead minnows exposed to 100% EFF, 100% REF, and a mixture of the two during 2005. (F) Dorsal fat pad prominence expressed by male fathead minnows exposed to 100% EFF, 100% REF, and mixtures of the two during 2006 [bars with different superscript letters or * differ significantly ($p < 0.05$)].

pling dates. Sperm were never 'very strongly' expressed in 100% EFF or 50:50 EFF:REF exposed fish from day-4 forward. By day-14, sperm were 'weakly' expressed or absent in 100% EFF-exposed fish. However, expression of testicular sperm abundance was not always predictive of secondary sex characteristics. For example, in 2005 after 28-days of exposure to 100% EFF, the only male fish to express both nuptial tubercles and a dorsal fat pad exposure also had an absence of sperm.

Plasma vitellogenin concentrations were significantly elevated by EFF-exposure in both experiments (Fig. 4A and B). In the 2005 experiment, concentration dependence was not observed, as vitellogenin concentrations peaked after 7 days among both 50:50 EFF:REF and 100% EFF exposed males ($p < 0.05$). The 2006 experiment included more dilute exposures, and concentration dependence was observed. In 2006, plasma vitellogenin was significantly elevated by effluent concentrations of 25% and higher ($p < 0.05$). Reference male secondary sex characteristics and plasma vitellogenin did not differ from initial controls at any sampling period in 2005 or 2006 ($p > 0.05$). HSI was significantly elevated by EFF-exposure in 2005 and 2006; on day-28, HSI was significantly elevated in the 100% treatment than in REF or 50:50 EFF:REF treatments. There was no effect of exposure or duration on the splenosomatic index in 2005 or 2006 ($p < 0.05$). In general, organ indices were less sensitive to effluent exposure than secondary sex characteristics, sperm abundance, or plasma vitellogenin.

4. Discussion

4.1. Environmental measurements and complex mixtures

The Boulder WWTP effluent contained a complex and dynamic mixture of EACs that varied within and between experiments (Table 1). This estrogenic effluent is discharged to Boulder Creek where it accounts for up to 75% of stream flow, which provides habitat to numerous species and supplies water to downstream users. Fish were exposed to dilutions of the WWTP effluent with upstream Boulder Creek water under controlled conditions to determine whether the reproductive disruption observed in free-living Boulder Creek fish (Vajda et al., 2008; Woodling et al., 2006) could be attributed to the WWTP effluent chemistry. Dilutions of EFF

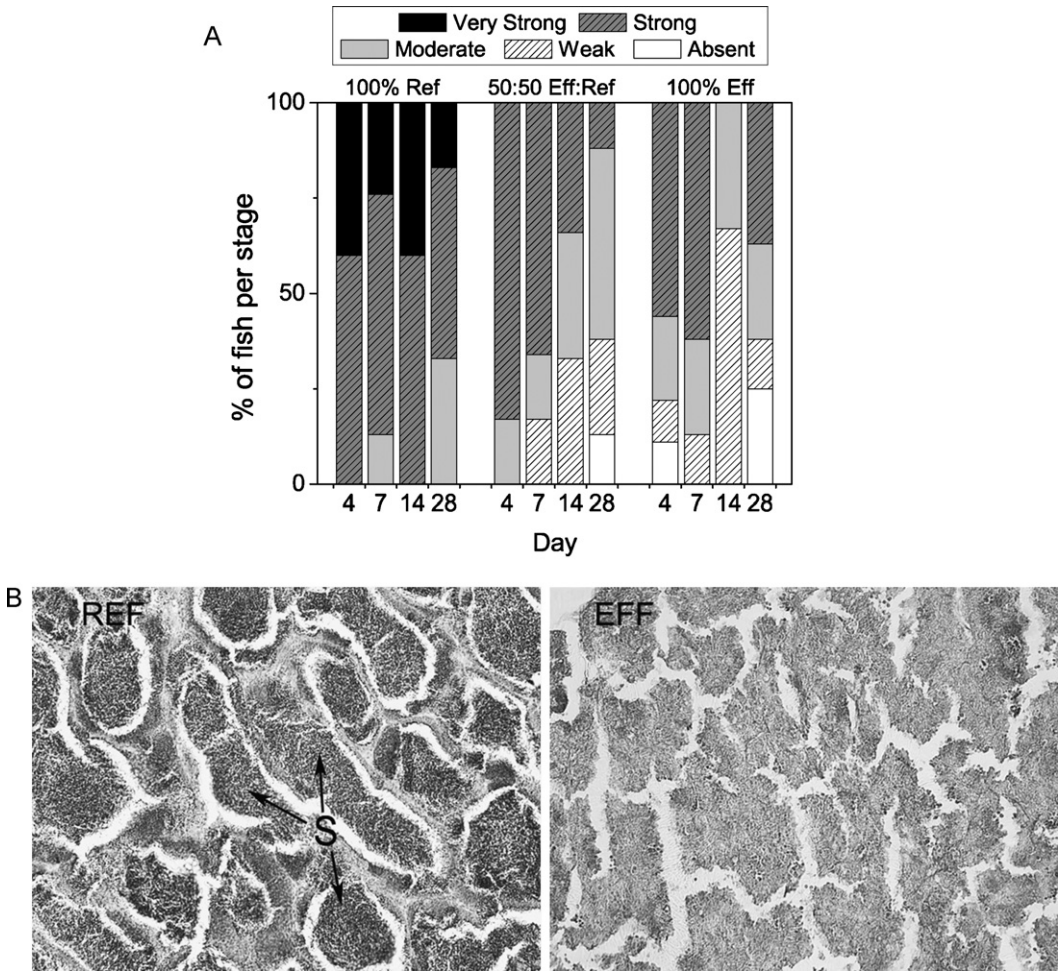

Fig. 3. (A) Relative sperm abundance in male fathead minnows exposed to wastewater treatment plant (WWTP) effluent (100% EFF), Boulder Creek water upstream from the WWTP outfall (100% REF), and a mixture of the two during the 2005 experiment. (B) Histology of REF exposed fish did not differ from initial controls in sperm (S) abundance, whereas EFF exposed fish had sperm that was weakly expressed or absent [both photomicrographs are presented at the same magnification].

with REF approximated seasonal base-flow conditions and attenuated downstream concentrations. In Boulder Creek, as with many streams in the Arid West, reproduction in most fishes follows a prolonged period (August–April) of low in-stream base flows when WWTP effluent discharges are most concentrated.

During the exposure experiments, the WWTP effluent contained a complex mixture of steroidal estrogens, non-steroidal estrogens, and other wastewater contaminants known to modulate endocrine function in vertebrates. Estrone and 17β-estradiol were detected in the WWTP effluent during both experiments (Table 1); estrone and

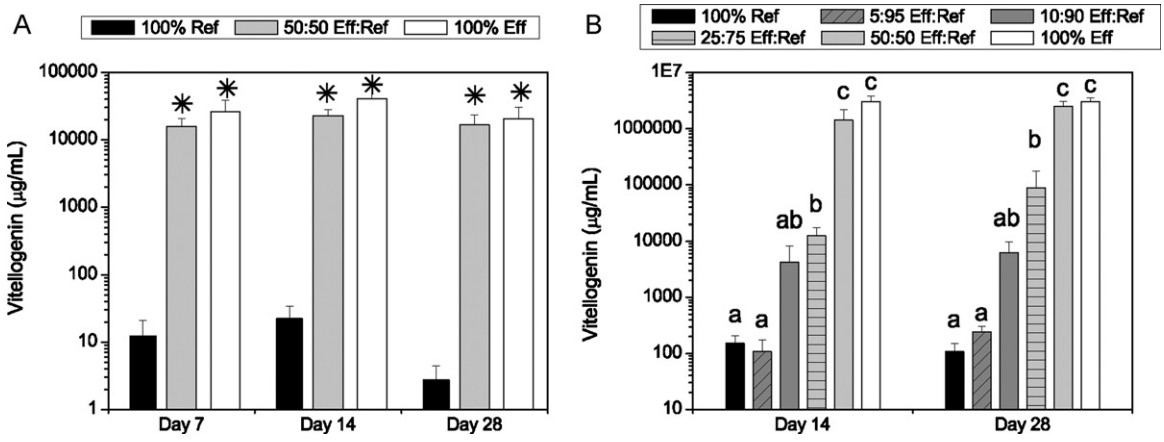

Fig. 4. (A) Plasma vitellogenin concentrations in male fathead minnows exposed to wastewater treatment plant (WWTP) effluent (100% EFF), Boulder Creek water upstream from the WWTP outfall (100% REF), and a mixture of the two during the 2005 experiment. (B) Plasma vitellogenin concentrations in male fathead minnows exposed to 100% EFF, 100% REF, and mixtures of the two during 2006 [REF-exposed males did not differ from initial controls; bars with different superscript letters differ significantly ($p < 0.05$)].

the synthetic estrogen 17α-ethynylestradiol were detected in the WWTP effluent only in 2005, but not 2006. The concentrations of steroidal estrogens were consistent with concentrations reported for WWTP effluent from trickling filter, or trickling filter combined with activated sludge treatment (Johnson et al., 2005).

Estrogen equivalency quotients (EEqs) (Körner et al., 1999) provide a means to consider the chemical mixture effects of the EACs (Vajda et al., 2008) and were summed after multiplying the aqueous concentration of 17α-ethynylestradiol, 17β-estradiol, estrone, and 4-nonylphenol by the estrogen equivalency factor (EEF) for each compound (Jobling and Sumpter, 1993; Preuss et al., 2006; Soto et al., 1995; Van Den Belt et al., 2004) using methodology described in a previous paper from this group (Vajda et al., 2008). EEq values for the WWTP effluent fluctuated over the course of each experiment, as did the contributions of individual chemicals (Table 1 and Fig. 1A and B). Similar short-term changes in WWTP effluent composition and estrogen concentration have been reported at other sites (Barber et al., 2007; Martinovic et al., 2008), and are captured in these on-site, flow-through, *in vivo* exposure experiments. It is important that the experimental design captures the variability of EAC concentration because equivalent maximal vitellogenin induction can be attained through either intermittent or sustained exposure to exogenous estrogens (Panter et al., 2000).

When calculated using *in vivo* values, there was a 50% reduction in the maximum predicted EEqs in 2006, paralleling observations of reduced impacts on fish reproduction, largely due to the absence of 17α-ethynylestradiol. The WWTP effluent characteristics were complex and the steroidal estrogens were not the only EACs that contribute to reproductive effects. In the 2006 experiment, following the publication of the US EPA 4-nonylphenol aquatic water quality criteria (U.S. Environmental Protection Agency), there was a significant decrease (up to 85%) in concentrations of 4-nonylphenol, which also contributes to the reductions in the calculated EEq. Similar reductions in 4-nonylphenol concentrations and estrogenic effects following regulatory action were observed in the United Kingdom (Sheahan et al., 2002). Estimation of EEq integrates independent, complex analytical chemistry results into a biologically relevant index to allow comparisons between locations or sampling dates. Although useful in evaluating impacts on reproductive endpoints, EEqs have limitations when examining complex endpoints such as regulation of behavior where estrogens signal with compound and signaling pathway specific potency (Tabb and Blumberg, 2006). This critical complexity is lost when the chemical composition and concentration of the WWTP effluent are reduced to the single dimension of estrogen equivalency quotients.

4.2. Vitellogen induction, suppression of primary and secondary sex characteristics

Evidence of reproductive disruption consistent with exposure to EACs was found for each reproductive endpoint examined. Male fish began each exposure experiment in stimulated reproductive condition with mature gonads and prominent secondary sex-characteristics, but were rapidly demasculinized after exposure to EFF and its dilutions. In contrast, male fish in the REF treatment remained reproductively stimulated with no significant reduction from initial conditions. The observed reproductive responses occurred in the absence of overt toxicity and morbidity previously reported for fishes downstream of WWTP effluents (Tsai, 1973). These results are consistent with exposure to exogenous estrogenic chemicals (Arukwe and Goksøyr, 2003; Brian et al., 2005; Kidd et al., 2007; Panter et al., 1998; Parrott and Blunt, 2005).

Nuptial tubercles and dorsal fat pads play key roles in male fathead minnow competitive, territorial, and spawning behaviors (Smith, 1978). Disruption of these secondary sex characteristics may have adverse fitness consequences if it alters the outcome of territorial contests, decreases access to mates, or alters the quality of parental care (Grist et al., 2003; Lange et al., 2001; Scott and Sloman, 2004). Secondary sex characteristics were demasculinized upon exposure to dilutions of wastewater in both experiments, as evidenced by a significant reduction in the number and prominence of nuptial tubercles (Fig. 2A–D), and in a reduced prominence of the dorsal fat pad (Fig. 2E and F). Expression of these secondary sex characteristics in male fathead minnows is androgen-dependent (Panter et al., 2004; Pawlowski et al., 2004a,b; Smith, 1974) and their suppression here is consistent with antagonism by exposure to exogenous estrogens (Parrott and Blunt, 2005; Pawlowski et al., 2004a,b). Although the suppression of secondary sex characteristics observed here might reverse upon depuration, even the transient suppression of reproductive traits can have adverse fitness consequences if it coincides with a restricted breeding season.

Paralleling observations of decreased relative sperm abundance in free-living white suckers (Vajda et al., 2008), sperm abundance declined rapidly and severely in males exposed to 50:50 EFF:REF and 100% EFF. While effects on testicular morphology and spermatogenesis are commonly reported in estrogen-exposed male fathead minnows (Leino et al., 2005; Miles-Richardson et al., 1999a, b; Pawlowski et al., 2004a,b), the rapid induction of sperm loss is not commonly reported and may be an effect of the complex mixture characteristics of the WWTP effluent. Adverse effects of effluent-exposure on primary sex characteristic expression was restricted to the 2005 experiments, as there was no significant effect on GSI or sperm abundance in 2006.

Induction of vitellogenin is estrogen-dependent (Arukwe and Goksøyr, 2003) and elevated levels in male fish are a reliable biomarker of exposure to exogenous estrogens (Sumpter and Jobling, 1995). Plasma vitellogenin was significantly elevated by environmentally relevant mixtures of the WWTP effluent typical of in-stream dilutions in both 2005 and 2006 (Fig. 4). Increased male plasma vitellogenin concentrations have been correlated with increased mortality and decreased fitness in male fathead minnows (Thorpe et al., 2007). The elevation in plasma vitellogenin in EFF-exposed fish supports the hypothesis that estrogenic contaminants are responsible for the reproductive disruption in free-living fish downstream from the WWTP outfall (Vajda et al., 2008).

Reproductive disruption in EFF-exposed male fathead minnows was less severe in 2006 than in 2005, but significant effects of exposure were observed for all biological endpoints with the exception that in 2006 there was no significant effect on GSI or sperm abundance. Although reduced biological affects parallels reductions in effluent EAC concentrations, the use of different fish stocks the two experiments prevented statistically appropriate comparisons of the relative reproductive disruption.

4.3. Implications

The rapid and severe demasculinization of male fathead minnows exposed to the WWTP effluent parallels previous observations of free-living male white suckers in Boulder Creek (Vajda et al., 2008; Woodling et al., 2006) and is consistent with exposure to exogenous estrogens. The effects of exposure to dilutions of the WWTP effluent did not manifest overt toxicity and mortality, but showed rapid disruption of multiple endpoints in fish exposed to dilutions that corresponds to the prevalent in-stream effluent contribution downstream from the WWTP (Barber et al., 2006). This integrated application of field and laboratory, biological, chemical and hydrological investigative methods to a complex environmental system has demonstrated definitive adverse effects of WWTP effluent exposure on multiple fish species.

Acknowledgements

This research was partially funded by the U.S. Environmental Protection Agency (USEPA), the U.S. Geological Survey (USGS) National Research Program, and the USGS Toxic Substances Hydrology Program. We thank Steve Brinkman and Nicole Vieira (Colorado Division of Wildlife), Ted Noyes, Greg Brown, and Steffanie Keefe (USGS), Jim Lazorchak (USEPA), and the City of Boulder for their assistance. Use of trade names is for identification purposes only and does not imply endorsement by the U.S. Government.

References

Aerni, H.R., Kobler, B., Rutishauser, B.V., Wettstein, F.E., Fisher, R., Giger, W., Hungerbuhler, A., Marazuela, M.D., Peter, A., Schoneberger, R., Vogeli, A.C., Sutter, M.J.F., Eggen, R.I.L., 2004. Combined biological and chemical assessment of estrogenic activities in wastewater treatment plant effluents. Anal. Bioanal. Chem. 378, 688–696.

Arukwe, A., Goksøyr, A., 2003. Eggshell and egg yolk proteins in fish: hepatic proteins for the next generation: oogenetic, population, and evolutionary implications of endocrine disruption. Comp. Hepatol. 2, 1–21.

Barber, L.B., Brown, G.K., Zaugg, S.D., 2000. Potential endocrine disrupting organic chemicals in treated municipal wastewater and river water. In: Keith, L.H., Jones-Lepp, T.L., Needham, L.L. (Eds.), Analysis of Environmental Endocrine Disruptors, Am. Chem. Soc. Symposium Series 747, Washington, DC, pp. 97–123.

Barber, L.B., Lee, K.E., Swackhamer, D.L., Schoenfuss, H.L., 2007. Reproductive responses of male fathead minnows exposed to wastewater treatment plant effluent, effluent treated with XAD8 resin, and an environmentally relevant mixture of alkylphenol compounds. Aquat. Toxicol. 82, 36–46.

Barber, L.B., Murphy, S.F., Verplanck, P.L., Sandstrom, M.W., Taylor, H.E., Furlong, E.T., 2006. Chemical loading into surface water along a hydrological, biogeochemical, and land use gradient—a holistic watershed approach. Environ. Sci. Technol. 40, 475–486.

Bordeleau, G., Martel, R., Ampleman, G., Thinoutot, S., 2008. Environmental impacts of training activities at an air weapons range. J. Environ. Qual. 37, 308–317.

Brian, J.V., Harris, C.A., Scholze, M., Backhaus, T., Booy, P., Lamoree, M., Pojana, G., Jonkers, N., Runnalls, T., Bonfa, A., Marcomini, A., Sumpter, J.P., 2005. Accurate prediction of the response of freshwater fish to a mixture of estrogenic chemicals. Environ. Health Perspect. 113, 721–728.

City of Boulder, 2008 [http://www.ci.boulder.co.us/publicworks/depts/utilities/].

Fox, J.E., Starcevic, M., Kow, K.Y., Burrow, M.E., McLachlan, J.A., 2001. Endocrine disrupters and flavonoid signaling. Nature 413, 128–129.

Giesy, J.P., Snyder, E.M., Nichols, K.M., Snyder, S.A., Villalobos, S.A., Jones, P.D., Fitzgerald, S.D., 2003. Examination of reproductive endpoints in goldfish (*Carrassius auratus*) exposed in situ to municipal sewage treatment plant effluent discharges in Michigan, USA. Environ. Toxicol. Chem. 22, pp. 2416–2413.

Grist, E.P.M., Wells, N.C., Whitehouse, P., Brighty, G., Crane, M., 2003. Estimating the effects of 17α-ethinylestradiol on populations of the fathead minnow *Pimephales promelas*: are conventional toxicological endpoints adequate? Environ. Sci. Technol. 37, 1609–1616.

Harries, J.E., Janbakhsh, A., Jobling, S., Mattthiessen, P., Sumpter, J.P., Tyler, C.R., 1999. Estrogenic potency of effluent from two sewage treatment works in the United Kingdom. Environ. Toxicol. Chem. 18, 932–937.

Harries, J.E., Sheahan, D.A., Jobling, S., Matthiessen, P., Neall, P., Sumpter, J.P., Tylor, T., Zaman, N., 1997. Estrogenic activity in five United Kingdom rivers detected by measurement of vitellogenesis in caged male trout. Environ. Toxicol. Chem. 16, 534–542.

Horrigan, L., Lawrence, R.S., Walker, P., 2002. How sustainable agriculture can address the environmental and human health harms of industrial agriculture. Environ. Health Perspect. 110, 445–456.

Jobling, S., Nolan, M., Tyler, C.R., Brighty, G., Sumpter, J.P., 1998. Widespread sexual disruption in wild fish. Environ. Sci. Technol. 32, 2498–2506.

Jobling, S., Sumpter, J.P., 1993. Detergent components in sewage effluent are weakly oestrogenic to fish: an *in vitro* study using rainbow trout (*Oncorhynchus* mykis) hepatocytes. Aquat. Toxicol. 27, 361–372.

Johnson, A.C., Aerni, H.R., Gerritsen, A., Gibert, M., Giger, W., Hylland, K., Jürgens, M., Nakari, T., Pickering, A., Suter, M.J.F., Svenson, A., Wettstein, F.E., 2005. Comparing steroid estrogen, and nonylphenol content across a range of European sewage plants with different treatment and management practices. Water Res. 39, 47–58.

Kidd, K.A., Blanchfield, P.J., Mills, K.H., Palace, V.P., Evans, R.E., Lazorchak, J.M., Flick, R.W., 2007. Collapse of a fish population after exposure to a synthetic estrogen. Proc. Natl. Acad. Sci. U.S.A. 104, 8897–8901.

Kolodziej, E.P., Gray, J.L., Sedlak, D.L., 2003. Quantification of steroidal hormones with pheromonal properties in municipal wastewater effluent. Environ. Toxicol. Chem. 22, 2622–2629.

Kolpin, D.W., Furlong, E.T., Meyer, M.T., Thurman, E.M., Zaugg, S.D., Barber, L.B., Buxton, H.T., 2002. Pharmaceuticals, hormones, and other organic wastewater contaminants in U.S. Streams, 1999–2000: a national reconnaissance. Environ. Sci. Technol. 36, 1202–1211.

Körner, W., Hanf, V., Schuller, W., Kempter, C., Metzger, J., Hagenmaier, H., 1999. Development of a sensitive E-screen assay for quantitative analysis of estrogenic activity in municipal sewage plant effluents. Sci. Tot. Environ. 225, 33–48.

Lange, R., Hutchinson, T.H., Croudace, C.P., Siegmund, F., Schweinfurth, H., Hampe, P., Panter, G.H., Sumpter, J.P., 2001. Effects of the synthetic estrogen 17α-ethinylestradiol on the life-cycle of the fathead minnow (*Pimephales promelas*). Environ. Toxicol. Chem. 20, 1216–1227.

Leino, R.L., Jensen, K.M., Ankley, G.T., 2005. Gonad histology and characteristic histopathology associated with endocrine disruption in the adult fathead minnow (*Pimephales promelas*). Environ. Toxicol. Pharmacol. 19, 85–98.

Lye, C.M., 2000. Impact of oestrogenic substances from oil production at sea. Toxicol. Lett. 112, 265–272.

Martinovic, D., Denny, J.S., Schmieder, P.K., Ankley, G.T., Sorensen, P.W., 2008. Temporal variation in the estrogenicity of a sewage treatment plant effluent and its biological significance. Environ. Sci. Technol. 42, 3421–3427.

McLachlan, J.A., 2001. Environmental signaling: what embryos and evolution teach us about endocrine disrupting chemicals. Endocr. Rev. 22, 319–341.

Miles-Richardson, S.R., Kramer, V.J., Fitzgerald, S.D., Render, J.A., Yamini, B., Barbee, S.J., Giesy, J.P., 1999a. Effects of waterborne exposure of 17β-estradiol on secondary sex characteristics and gonads of fathead minnows (*Pimephales promelas*). Aquat. Toxicol. 47, 129–145.

Miles-Richardson, S.R., Pierens, S.L., Nichols, K.M., Kramer, V.J., Snyder, E.M., Snyder, S.A., Render, J.A., Fitzgerald, S.D., Giesy, J.P., 1999b. Effects of waterborne exposure to 4-nonylphenol and nonylphenol-ethoxylate on secondary sex characteristics and gonads of fathead minnows (*Pimephales promelas*). Aquat. Toxicol. 47, 129–145.

Mills, L.J., Chichester, C., 2005. Review of evidence: are endocrine-disrupting chemicals in the aquatic environment impacting fish populations? Sci. Tot. Environ. 343, 1–34.

In: Murphy, S.F., Verplanck, P.L., Barber, L.B. (Eds.), 2003. Comprehensive water quality of the Boulder Creek Watershed, Colorado, during high-flow and low-flow Conditions, 2000. U.S. Geol. Survey Water Res. Invest. Rept. 03-4045, 198 p.

Oberdörstor, E., Clay, M.A., Cottam, D.M., Wilmot, F.A., McLachlan, J.A., Milner, M.J., 2001. Common phytochemicals are ecdysteroid agonists and antagonists: a possible evolutionary link between vertebrate and invertebrate steroid hormones. J. Steroid Biochem. Mol. Biol. 77, 229–238.

Panter, G.H., Hutchinson, T.H., Hurd, K.S., Sherren, A., Stanley, R.D., Tyler, C.R., 2004. Successful detection of (anti-)androgenic and aromatase inhibitors in prespawning adult fathead minnows (*Pimephales promelas*) using easily measured endpoints of sexual development. Aquat. Toxicol. 70, 11–21.

Panter, G.H., Thompson, R.S., Sumpter, J.P., 1998. Adverse reproductive effects in male fathead minnows (*Pimephales promelas*) exposed to environmentally relevant concentrations of the natural oestrogens oestradiol and oestrone. Aquat. Toxicol. 42, 243–253.

Panter, G.H., Thompson, R.S., Sumpter, J.P., 2000. Intermittent exposure of fish to estradiol. Environ. Sci. Technol. 34, 2756–2760.

Parrot, J.L., McMaster, M.E., Hewitt, L.M., 2006. A decade of research on the environmental impacts of pulp and paper mill effluents in Canada: development and application of fish bioassays. J. Toxicol. Environ. Health B Crit. Rev. 9, 297–317.

Parrott, J.L., Blunt, B.R., 2005. Life-cycle exposure of fathead minnows (*Pimephales promelas*) to an ethinylestradiol concentration below 1 ng/L reduces egg fertilization success and demasculinizes males. Environ. Toxicol. 20, 131–141.

Pawlowski, S., Sauer, A., Shears, J.A., Tyler, C.R., Braunbeck, T., 2004a. Androgenic and estrogenic effects of the synthetic androgen 17α-methyltestosterone on sexual development and reproductive performance in the fathead minnow (*Pimephales promelas*). Aquat. Toxicol. 68, 277–291.

Pawlowski, S., van Aerle, R., Tyler, C.R., Braunbeck, T., 2004b. Effects of 17α-ethinylestradiol in a fathead minnow (*Pimephales promelas*) gonadal recrudescence assay. Ecotoxicol. Environ. Saf. 57, 215–223.

Presnell, J.K., Schreibman, M.P., 1997. Humason's Animal Tissue Techniques. Johns Hopkins University Press, Baltimore, MD.

Preuss, T.G., Gerhardt, J., Schirmer, K., Coors, A., Rubach, M., Russ, A., Jones, P.D., Giesy, J.P., Ratte, H.T., 2006. Nonylphenol isomers differ in estrogenic activity. Environ. Sci. Technol. 40, 5147–5153.

Purdom, C.E., Hardiman, P.A., Bye, V.J., Eno, N.C., Tyler, C.R., Sumpter, J.P., 1994. Estrogenic effects of effluents from sewage treatment works. Chem. Ecol. 9, 275–285.

Robinson, C.D., Brown, E., Craft, J.A., Davies, I.M., Moffat, C.F., Pirie, D., Robertson, F., Stagg, R.M., Struthers, S., 2003. Effects of sewage effluent and ethynyl oestradiol upon molecular markers of oestrogenic exposure, maturation and reproductive success in the sand goby (*Pomatoschistus minutus*, Pallus). Aquat. Toxicol. 62, 119–134.

Rodgers-Gray, T.P., Jobling, S., Kelly, C., Morris, M., Brighty, G., Waldock, M., Sumpter, J.P., Tyler, C.R., 2001. Exposure of juvenile roach (*Rutilus rutilus*) to treated sewage effluent induces dose-dependent and persistent disruption in duct development. Environ. Sci. Technol. 35, 462–470.

Schultz, M.M., Furlong, E.T., Kolpin, D.W., Werner, S.L., Schoenfuss, H.L., Barber, Blazer, V.S., Norris, D.O., Vajda, A.M., 2010. Antidepressant pharmaceuticals in two U.S. effluent-impacted streams: occurrence and fate in water and sediment, and selective uptake in fish neural tissue. Environ. Sci. Technol. 44, 1918–1925.

Scott, G.R., Sloman, K.A., 2004. The effects of environmental pollutants on complex fish. behaviour: integrating behavioural and physiological indicators of toxicity. Aquat. Toxicol. 68, 369–392.

Sheahan, D.A., Brighty, G.C., Daniel, M., Jobling, S., Harries, J.E., Hurst, M.R., Kennedy, J., Kirby, S.J., Morris, S., Routledge, E.J., Sumpter, J.P., Waldock, M.J., 2002. Reduc-

tion in the estrogenic activity of a treated sewage effluent discharge to an English river as a result of a decrease in the concentration of industrially derived surfactants. Environ. Toxicol. Chem. 21, 515–519.
Smith, R.J.F., 1974. Effects of 17α-methyltestosterone on the dorsal pad and tubercles of fathead minnows (*Pimephales promelas*). Can. J. Zool. 52, 1031–1038.
Smith, R.J.F., 1978. Seasonal changes in the histology of the gonads and dorsal skin of the fathead minnow, *Pimephales promelas*. Can. J. Zool. 56, 2103–2109.
Soto, A.M., Justicia, H., Wray, J.W., Sonnenschein, C., 1991. p-Nonyl-phenol—an estrogenic xenobiotic released from "modified" polystyrene. Environ. Health Perspect. 92, 167–173.
Soto, A.M., Sonnenschein, C., Chung, K.L., Fernandez, M.F., Olea, N., Olea Serrano, F., 1995. The E-Screen assay as a tool to identify estrogens: an update on estrogenic environmental pollutants. Environ. Health Perspect. 103, 113–122.
Sumpter, J.P., Jobling, S., 1995. Vitellogenesis as a biomarker for estrogenic contamination of the aquatic environment. Environ. Health Perspect. 103, 173–178.
Tabb, M.M., Blumberg, B., 2006. New modes of action for endocrine-disrupting chemicals. Mol. Endocrinol. 20, 475–482.
Thornton, J., McCally, M., Orris, P., Weinberg, J., 1996. Hospitals and plastics. Dioxin prevention and medical waste incinerators. Public Health. Rep. 111, 473–475.
Thorpe, K.L., Benstead, R., Hutchinson, T.H., Tyler, C.R., 2007. Associations between altered vitellogenin concentrations and adverse health effects in fathead minnow (*Pimephales promelas*). Aquat. Toxicol. 85, 176–183.
Tsai, C.-F., 1973. Water quality and fish life below sewage outfalls. Trans. Am. Fish. Soc. 102, 281–292.
U.S. Environmental Protection and Agency, 2005. Aquatic Life Ambient Water Quality Criteria—Nonylphenol FINAL. U.S. Environ. Protect. Agency, EPA 822-R-05-005.
Vajda, A.M., Lopez, E.M., Woodling, J.D., Barber, L.B., Norris, D.O., 2008. Reproductive disruption of fish by an estrogenic wastewater effluent. Environ. Sci. Technol. 42, 3407–3414.
Vajda, A.M., Norris, D.O., 2006. Endocrine active phytochemicals: signaling context and mechanisms. In: Norris, D.O., Carr, J.A. (Eds.), Endocrine Disruption, Biological Bases for Health Effects in Wildlife and Humans. Oxford University Press, pp. 390–423.
Van Den Belt, K., Berckmans, P., Vangenechten, C., Verheyen, R., Witters, H., 2004. Comparative study on the *in vitro/in vivo* estrogenic potencies of 17β-estradiol, estrone, 17α-ethinyl estradiol, and nonylphenol. Aquat. Toxicol. 66, 183–195.
Woodling, J.D., Lopez, E.M., Maldonado, T.M., Norris, D.O., Vajda, A.M., 2006. Intersex and other reproductive disruption of fish in wastewater effluent dominated Colorado streams. Comp. Biochem. Physiol. C Toxicol. Pharmacol. 144, 10–15.