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Occurrence and biological effect of exogenous steroids in the Elkhorn River, Nebraska, USA

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Abstract

Recent studies of surface waters in North America, Japan, and Europe have reported the presence of steroidogenic agents as contaminants. The current study has three objectives: 1) to determine if steroidogenic compounds are present in the Elkhorn River, 2) to determine if sediments collected from the Elkhorn River can act as a source of steroidogenic compounds to aquatic organisms, and 3) to determine if site-specific biological effects are apparent in the hepatic gene expression of fathead minnows. Evidence was obtained using three approaches: 1) deployment of polar organic chemical integrative samplers (POCIS), 2) deployment of caged fathead minnows, and 3) a laboratory experiment in which POCIS and fish were exposed to sediments from the deployment sites. Deployment sites included: the Elkhorn River immediately downstream from a Nebraska wastewater treatment plant, two waterways (Fisher Creek and Sand Creek) likely to be impacted by runoff from cattle feeding operations, and a reference site unlikely to be impacted by waste water inputs. The POCIS extracts were analyzed for a number of natural steroids and metabolites, as well as four different synthetic steroids: ethinylestradiol, zearalonol, 17β-trenbolone and melengestrol acetate. Estrogenic and androgenic metabolites, as well as progesterone and trace levels of melengestrol acetate were detected in POCIS deployed at each site. POCIS deployed in tanks containing field sediments from the four sites did not accumulate the synthetic steroids except for ethinylestradiol, which was detected in the aquarium containing sediments collected near the wastewater treatment plant. Fish deployed in Sand Creek and at the wastewater treatment plant experienced significantly elevated levels of gene expression for two genes (StAR and P450scc) relative to those deployed in Fisher Creek. Fish exposed to the sediments collected from Sand Creek had significantly higher levels of hepatic StAR and P450scc gene expression than did fish exposed to sediments from the two other field sites, as well as the no-sediment control tank. In conclusion: 1) detectable levels of steroidogenic compounds were detected in passive samplers deployed in the Elkhorn River, 2) sediments do not appear to be a significant source for steroidogenic compounds, and 3) site-specific differences were found in mRNA expression among the different treatment groups of fish; however, a functional explanation for these differences is not readily forthcoming.

Keywords: caged fish, passive chemical samplers, gene expression, agricultural runoff, cattle feedlot, steroid hormones

1. Introduction

The Elkhorn River, Nebraska, USA is a 4000-mi^2 watershed with a high density of livestock feeding operations. Approximately 1.2 million head of cattle are maintained in this area compared to a human population of approximately 200,000. Recent studies have suggested that the Elkhorn River could be contaminated with endocrine-disrupting compounds. Water samples collected from six sites throughout the basin, were found to contain androgenic and estrogenic activity (as determined using the A-screen and E-screen bioassays and a transected cell assay: Soto et al., 2004; Orlando et al., 2004). Furthermore, resident fathead minnows (*Pimephales promelas*) collected at two sites within the watershed were found to exhibit demasculinization of the males and defeminization of the females relative to a within-basin reference site (Orlando et al., 2004).

A potential source for steroidogenic compounds in the Elkhorn River is runoff associated with waste from beef cattle concentrated animal feeding operations. In 2004, Soto et al. and Orlando et al. found the highest levels of androgenic activity in water samples collected from a cattle feedlot runoff retention pond. The implication from these findings was that metabolites of synthetic androgenic growth-promoting steroids used by the cattle industry, such as trenbolone acetate (TBA), were responsible for the androgenic activity as reported by the A-screen assay and an androgen receptor transfected cell assay. Indeed, trace levels of trenbolone (TB) metabolites were detected in the retention pond water, although the concentrations measured were not large enough to account for the androgenic activity as indicated by the A-screen test. A more recent study (Durhan et al., 2006) found similar results to that of Soto et al. (2004). Androgenic activity (measured using CV-1 assays), and detectable levels of TB metabolites were found in runoff collected at the discharge drain of a cattle feeding operation in southwest Ohio. However, no clear association could be established between the androgenic activity at the discharge site, and the presence of TB metabolites.

Synthetic anabolic steroids may not be consistently detected in the vicinity of feedlot operations in part because these compounds can strongly sorb to sediments. Related research has found that 17β-estradiol (17β-E2) and ethinylestradiol (EE2) sorb to the sediments collected from a range of English rivers (Holthaus et al., 2002). Furthermore, it has also been found that the estrogenic activity of sediments downstream from wastewater treatment plants (WWTP) in the United Kingdom can be 530–748 times higher than that in the overlying water (Peck et al., 2004). Metabolites of anabolic steroids used as growth-promoting compounds can sorb to sediment particles, and this can influence bioavailability. For example, soil fertilized with solid dung from cattle treated with TBA and melengestrol acetate (MGA, a growth-promoting gestagin) contained detectable levels of these steroids for 58 and 195 days post-fertilization (Schiffer et al., 2001).

Although steroidogenic compounds sorb to soil and sediment particles, there is also evidence that these compounds can be released from the sediments and reenter the water. Estrogenic compounds, such as 17β-E2 have been shown to be mobile, and have been found in runoff from manured lands (Nichols et al., 1997), and in mantled karst aquifers (Peterson et al., 2000). Trenbolone metabolites have also been shown to be mobile, as dung piles released small amounts of TB metabolites in runoff following rain storms (Schiffer et al., 2001). Regardless of whether soils and sediments act primarily as a sink or a source for steroidal metabolites, it is likely that they will have an impact on the bioavailability of these compounds to aquatic organisms.

In this study, polar organic chemical integrative samplers (POCIS) and caged fish were employed as bio-monitoring tools. POCIS provide information on time-weighted concentrations of water-soluble contaminants in environments where the concentration of the contaminants can vary considerably over time (Alvarez et al., 2004, 2005; Petty et al., 2004; Vermeirssen et al., 2005; Matthiessen et al., 2006). Caged fish can also be used as bio-monitoring tools, and two traits commonly measured are: concentration of xenobiotics in fish tissues (Gallassi et al., 1996; Otto et al., 1996) and inappropriate mRNA or protein expression (Sheahan et al., 2002; Vermeirssen et al., 2005; Roberts et al., 2005; Burki et al., 2006).

This study focused on the hepatic mRNA expression of six genes as potential biomarkers of exposure to steroidogenic compounds: androgen receptor (AR), estrogen receptor α (ER-α), vitellogenin (vg), cytochrome P450 3A (Cyp3A), cytochrome P450 side chain cleavage ($P450_{SCC}$), and steroidogenic acute regulatory protein (StAR). These genes were selected for analysis either because of their responsiveness to exogenous steroids, or because of their importance in steroidogensis or steroid metabolism. For example, hepatic ER-α and vg mRNA expression has been shown to be responsive to exogenous estrogens (Yadatie et al., 1999; Yamaguchi et al., 2005; Miracle et al., 2006), whereas hepatic expression of AR has been shown to be responsive to estrogens (Hook et al., 2006) and the anti-androgen, flutamide (Filby et al., 2007). Cyp3A enzymes are involved

in the metabolism of numerous compounds, including toxins, therapeutic drugs and hormones (Hegelund and Celander, 2003), and are therefore likely to be differentially expressed in the presence of exogenous steroids. StAR and P450scc genes encode proteins that are involved in the initiation of steroidogenesis (Garcia-Reyero et al., 2006; Aluru et al., 2005; Aluru and Vijayan, 2006), although it is currently unclear how exogenous steroids will influence the hepatic expression of these genes.

This study is a follow-up study to recently published studies on the Elkhorn River (Orlando et al., 2004; Soto et al., 2004). This current study has three objectives: 1) to determine if steroidogenic compounds are present in the Elkhorn River, 2) to determine if sediments collected from the Elkhorn River can act as a source of steroidogenic compounds to aquatic organisms and 3) to determine if site-specific biological effects are apparent in the hepatic mRNA expression of fathead minnows.

2. Materials and methods

2.1. Field sites

During late spring and early summer of 2005, caged fathead minnows and POCIS were deployed at four locations in the Elkhorn River. May is the month with the greatest river flow, whereas June is the month with the greatest rainfall, and consequently the month that the river will receive the most runoff from nearby agricultural operations. Two of the locations were at the confluence between two small tributaries (Sand Creek and Fisher Creek) and the Elkhorn River. Large beef cattle feeding operations are located upstream from the Fisher Creek and Sand Creek locations. A third location was in the Elkhorn River immediately downstream from the Norfolk WWTP discharge structure, whereas the fourth site was another tributary of the Elkhorn River (Battle Creek). The Battle Creek site has been used as a reference site in other studies (Orlando et al., 2004; Soto et al., 2004).

The first deployment was initiated on May 11, and POCIS were deployed at all four field sites. POCIS were recovered 3 weeks later on June 1 from three of the four field sites: Fisher Creek, Sand Creek and Battle Creek. The POCIS at the Norfolk WWTP site was lost presumably to vandalism. The second deployment was initiated on June 6, with POCIS and caged minnows being deployed at the four field sites. POCIS and living fish were recovered 3 weeks later on June 27 from the Fisher Creek, Sand Creek and Norfolk WWTP sites; however, the Battle Creek site was lost in a flood event. The other three sites were not dramatically affected by the flood, as they were located in areas with much greater baseline flow, or were located more than 40 miles downstream. Water temperature and pH at the beginning of the deployment period at each location is given in Table 1.

Table 1. Water quality measurements at the four field sites during the spring and summer 2005

Site	Deployment dates	Temp.	pH	Retrieved (P = POCIS)
Battle Creek	May 11–June 1	15.7	7.1	P
	June 6–June 27	21.6	7.4	NA
Fisher Creek	May 11–June 1	17.1	7.2	P
	June 6–June 27	20.0	7.6	P, Fish
Sand Creek	May 11–June 1	14.5	7.4	P
	June 6–June 27	17.6	7.8	P, Fish
Norfolk WWTP	May 11–June 1	19.8	7.2	NA
	June 6–June 27	23.9	7.8	P, Fish

Water quality values were taken on the first day of deployment of the POCIS and fish. NA = samples were not recovered, see text for details.

Following the second deployment, sediments were collected at each of the four field sites and brought back to the laboratory. Sediments were held at room temperature for 4 days, then used in laboratory sediment exposure experiments.

2.2. POCIS deployment and analysis of steroid hormones

POCIS suitable for monitoring levels of steroid hormones were purchased from Environmental Sampling Technologies, Inc. (St. Joseph, MO). POCIS assemblies (Aquasense-PÔ) were deployed in triplicate in stainless steel canisters, and were attached adjacent to the minnow cages at each site. Deployment canisters were retrieved and immediately transported to the University of Nebraska Water Sciences Laboratory for processing and analysis.

Processing and extraction of the POCIS followed procedures described by Alvarez et al. (2004). Each individual POCIS device was removed from its deployment canister, briefly rinsed with water if needed to remove any debris, and opened. The contents of the POCIS were transferred into silanized glass chromatography columns using high purity methanol, and target compounds eluted by passing 50 mL of 1:1:8 methanol: toluene:dichloromethane through the sorbent at a flow rate near 1 mL/min. Labeled internal standards (d2-estradiol and d3-testosterone) equivalent to 100 ng were

added to the eluate and used for quantification. Extracts were evaporated under nitrogen to approximately 1 mL, and quantitatively transferred to silanized autosampler vials, evaporated to dryness and re-dissolved into a 50:50 mixture of acetonitrile and water. References standards were prepared in methanol and stored at-20 °C until analysis. Extracts were analyzed for the following steroidogenic compounds: α-zearalanol, 17β-E2, estriol (E3), estrone (E1), EE2, 11-ketotestosterone (11-kT), 17β-trenbolone (17β-TbOH), testosterone (T), 4-androstenedione (4-A), MGA and progesterone (P).

POCIS extracts were analyzed using a Waters 2695 quaternary pump and autosampler connected to a Micromass Quattro Micro triple-quadrupole mass spectrometer (LC/MS/MS) with electrospray ionization operated in both positive and negative modes. Detection and quantification of steroids utilized multiple reaction monitoring (MRM) with argon collision gas during two separate runs. A Thermo (Bellefonte, PA) Betabasic-18 column (250 × 2.1 mm, 5 µm, 50 °C) was used for separation at a flow rate of 0.2 mL/min with each ionization mode using slightly different gradients of methanol, 0.001% formic acid in water, and acetonitrile. Mass spectrometer parameters are: capillary 3.5 kV, extractor 2 V, RF lens 0.1 V, source temp 70 °C, desolvation temp 425 °C, cone gas flow at 120 L/h, and desolvation gas flow at 650 L/h. Compound retention times, ionization modes and MRM transitions are listed in Table 2. Positive identification of target compounds in extracts is made if the MRM retention time is within 0.2 min of the compound in the calibration standard.

Isotope dilution for estrogens and androgens will correct for incomplete recovery of non-labeled analogues, and help reduce uncertainty resulting from electrospray ionization variability caused by co-extracted matrix (Ferguson et al., 2001; Reddy et al., 2005). Recovery of target compounds was checked by extraction and analysis of unexposed POCIS sorbent spiked with known amounts of each compound, and averaged between 67% for 11-kT and 132% for E3. Two additional laboratory fortified blanks and two additional reagent blanks were carried through the sample preparation procedures. Based on the variability of the lowest standard (5 pg/µL), the estimated detection limits are near 100 pg on-column, corresponding to 1.0 ng recovered from the POCIS. Levels of steroid hormones in laboratory reagent blanks were below the estimated detection limit. The use of three POCIS per site effectively provides triplicate analysis for each sampling event, provides greater confidence in a compound's detection, and indicates the variability in trapping efficiency.

2.3. Field fish

Fathead minnows were produced by the University of Nebraska at Omaha colony according to the protocols outlined in Peake et al. (2004). Fish were initially raised from eggs at 26 °C under a 16:8 photo period. Two weeks prior to deployment, the temperature of the water was reduced to room temperature (21 °C). On June 2, the fish were divided into groups of 30 individuals. The fish were juveniles at the time of deployment and were too small for visual determination of sex. The mean mass of the fish in each group was approximately 1.2 g, and there were no significant differences in body mass among the four groups (ANOVA, $P < 0.05$). On June 6, four groups of fish were transported, in 30-L aerated coolers, to the four field sites. Fish were deployed at the sites, alongside the POCIS, in standard minnow cages with the openings of the cages permanently wired shut.

Table 2. Retention times, preferred ionization modes, and MRM transitions currently used for detection of free steroid hormones

Compound	Molecular weight	Retention time (min)	Ionization mode	Precursor ion	Product ion
E3	288.38	5.1	Negative	287	171
α-zearalanol	320.38	11.8	Negative	321	277
d2-17β estradiol	274.39	12.0	Negative	273	147
17β-E2	272.38	12.0	Negative	271	145
EE2	296.40	13.2	Negative	295	145
E1	270.37	13.7	Negative	269	145
11-KT	302.41	10.4	Positive	303	121
17β-TBOH	270.37	11.2	Positive	271	199
d3-testosterone	291.44	12.1	Positive	292	97
T	288.42	12.1	Positive	289	97
4-A	286.41	12.6	Positive	287	97
MGA	396.52	14.8	Positive	397	337
P	314.46	14.9	Positive	315	97

Fathead minnows deployed at each location were returned to the laboratory 21 days after deployment, on June 27. Immediately upon arrival to the laboratory, the fish were measured for body mass, length, liver mass and gonad mass. Hepatosomatic (HSI) and gonadosomatic (GSI) indices were generated by dividing the mass of the tissues into the body mass of the fish then multiplying by 100. Immediately upon dissection, livers were snap frozen in liquid nitrogen and stored in a − 80 °C freezer until analysis.

2.4. Laboratory sediment exposures

Field sediments from Battle Creek, Fisher Creek, Sand Creek and the Elkhorn River were placed into aquaria, covering the bottom with approximately 10 cm of sediment. A fifth containing fish but no sediment acted as a negative control or the blank. The tanks were continuously aerated, and clean, laboratory water entered each tank such that they received approximately two water turnovers per day. The day after the sediments were added to each tank, an individual POCIS device and 10 adult male (mean body mass = 1.6 g ± 0.05 SE) and 10 adult female (mean body mass 1.2 g ± 0.03 SE) fathead minnows were added to each of the five tanks. There were no significant differences in the body mass of the males or the females among the five groups at the beginning of the exposure (ANOVA, $P < 0.05$). Fish and POCIS were maintained in each of the five tanks for 21 days. Fish were fed commercially available flake food each day during the 21-day experiment.

Water quality was monitored in each tank during the 21-day field sediment experiment. Mean values (± SE) were: temperature 21.6 ± 0.1 °C, pH 7.6 ± 0.04, conductivity 631 ± 12 μS and dissolved oxygen 6.5 ± 0.2 mg/L, and in no case were there significant differences in any of these variables among the five different tanks (ANOVA, $P > 0.05$ in all cases). Ammonia was monitored in each tank, and was not detectable at any time. Sediments from Fisher Creek, Sand Creek and Battle Creek were primarily silt and clay, with an organic carbon concentration that varied from 15.8 to 17.3 mg/g (dry weight). In contrast, the sediments from the Norfolk WWTP were sandy, with a much lower (1.1 mg/g) organic carbon content.

2.5. RNA isolation

Total RNA was isolated from each sample using the SV Total RNA Isolation System (Promega Corp. Madison, WI). Samples were quantified spectrophotometrically at 260 nm, and only samples with absorbance$_{260}$ to absorbance$_{280}$ ratios greater than 1.7 were used in the subsequent analyses. The integrity of purified RNA was evaluated using denaturing agarose gel electrophoresis. The ratios of 28S to 18S ribosomal RNA were approximately 2:1 by ethidium bromide staining, indicating that no gross degradation of RNA occurred. Only samples that corresponded to expected high RNA integrity were analyzed for mRNA gene expression. RNA was stored at − 80 °C until analysis.

2.6. Molecular cloning of Cyp3A

Total RNA (5 μg) was reverse-transcribed in 20 μl of reaction mixture with super script III (Invitrogen, Carlsbad, CA) and random 6-mer primers (Invitrogen). The products of reverse transcription (RT) were used for polymerase chain reactions (PCR) as the template DNA with degenerate primers. The degenerate primers for Cyp3A were based on amino acid sequences of teleost Cyp3A, whose species and Genebank accession numbers were *Danio rerio* (AAS09920; AAH72702), *Oncorhynchus mykiss* (O42563; AAK58569), *Fundulus heteroclitus* (Q9PVE8; Q8AXY5) and *Oryzias latipes* (Q98T91; AAG35209). Amino acid sequences were aligned by using Clustal X computer program (Jeanmougin et al., 1998), and aligned sequences were divided into homologous segments by the Blocks Multiple Alignment Processor: http://blocks.fhcrc.org/blocks/process_blocks.html. Segmented and aligned sequences were used in the CODEHOP program (Rose et al., 1998) to design degenerate primers. PCR products amplified with degenerate primers were run on agarose gel and were stained with ethidium bromide. PCR bands, which were of the expected size, were cut out and purified by Wizard SV Gel and PCR clean-up system (Promega, Madison, WI). Purified PCR product was ligated into pGEM-T easy vector (Promega), and the ligation reaction (ligated PCR product into pGEM-T-Easy vector) was transected into *E. coli*, JM109 (Promega). The plasmid DNA was isolated by Wizard *Plus* SV minipreps DNA purification system (Promega). The sequencing reactions were carried out using the BigDye terminator cycle sequencing kit (Applied Biosystems, Foster City, CA), and sequencing reactions were analyzed using a ABI Prism 3100 genetic analyzer (Applied Biosystems).

2.7. Gene expression analyses

The relative expression of six genes was quantified in hepatic tissue: AR, ER-α, vg1, Cyp3A, P450$_{SCC}$ and StAR. In addition to these genes, a "housekeeping" gene (ribosomal L8) was also quantified and used as a nor-

malizing standard (see below). Gene sequences for AR, ERα, vg1 and L8 were obtained from GenBank. Primers for quantitative real-time PCR analysis are shown in Table 3 and were developed from fathead minnow sequences using commercially available software Primer-Express (Applied Biosystems).

Gene expression was determined in hepatic tissue from males and females. Males and females have differing gene expression patterns, and as such were analyzed separately. mRNA expression was evaluated for five fish of each sex from each experimental group. Fish were chosen for analysis based on their body mass, GSI and HSI; outliers were removed from consideration, and analysis was conducted on five individuals chosen randomly from the remaining pool of fish from each treatment group.

For quantitative RT-PCR, first-strand cDNA was synthesized from 1 µg total RNA in 20 µl of reaction using an iScript cDNA Synthesis Kit (Bio-Rad Inc, Hercules, CA). Q-PCR was carried out using a Bio-Rad iCycler equipped with a real-time PCR detection system (MyiQ). Real-time PCR reactions were performed using iQ SYBR-Green supermix (Bio-Rad) as per the manufacturer's protocol. Hepatic gene expression was evaluated using a Bio-Rad MyiQ Real-Time Polymerase Chain Reaction (PCR) Detection System managed by Optical System Software version 1.0. Data were quantified by the standard curve method using series diluted cDNA samples as a standard. The expression of each target gene mRNA was normalized by the expression of ribosomal protein L8 mRNA, and expressed in relative terms.

2.8. Statistical analysis

For many of the variables, within-group variability tended to be greatest in those groups with the largest mean values. Consequently, pairwise comparisons were analyzed with the nonparametric Mann–Whitney *U*, whereas single factor ANOVA was conducted using the nonparametric Kruskal–Wallis ANOVA followed by nonparametric multiple comparison tests.

3. Results

3.1. Field studies – POCIS

The compounds detected in POCIS extracts are reported in Table 4, and include E1, 17β-E2, E3, T, 4-A, P, and MGA. Neither EE2, 11-kT, α-zearalonol, nor 17β-TbOH were detected in POCIS retrieved from any of the three sites successfully sampled in May and June. There were no significant differences in the mass of any of the detected compounds among the field sites during May or June (Table 4, Kruskal–Wallis ANOVA $P > 0.05$ in all cases). Likewise, between the months of May and June there were no significant differences in any of the compounds detected in the POCIS deployed at Sand Creek (Mann–Whitney *U*, $P > 0.05$ in all cases). At Fisher Creek, the amount of 4-A collected by the POCIS was significantly greater during the June deployment period that it was during May (Table 4, Mann–Whitney *U*, $P < 0.05$).

3.2. Laboratory study – POCIS

Detectable levels of 17β-E2, T, 11-kT and EE2 (Table 5) were detected in POCIS extracts from the aquaria containing fish and river sediments. Ethinylestradiol was the only synthetic steroid found in POCIS extracts from the aquarium containing sediment collected downstream from the Norfolk WWTP.

Table 3. Primer sequences for real-time reverse transcriptase-polymerase chain reaction

	DNA sequence	Temp[a]	Accession #
Ribosomal Protein L8	FW 5′-GCC CAT GTC AAG CAC AGA AAA-3′ RV 5′-ACG GAA AAC CAC CTT AGC CAG-3′	63.8	AY919670
P450scc	FW 5′-CGA CAC CCG GAC TTG CA-3′ RV 5′-CAC GTC TCC TTT AGA GGT GAT ACG-3′	63.8	DQ360498-
StAR	FW 5′-CTT GAA CAG CAA ACA GAT GAC CTT-3′ RV 5′-CTC CCC CAT TTG TTC CAT GT-3′	62.5	DQ360497
AR	FW 5′-GTT TCC GTA ACC TGC ATG TGG-3′ RV 5′-CGC GCA TTA GCG TTC TTG TA-3′	64.5	AY727529
Vg1	FW 5′-TAT GCA CGA GAA AAT CGC CAC-3′ RV 5′-AGC ATG ACG ACT TCA CGC AG-3′	65	AF130354
ER-α	FW 5′-AGT GAG CAG TCA AGC CGT GTT-3′ RV 5′-GGT CAG GTG GCA TGC ATA AAG-3′	60.0	AY727528
CYP3A	FW 5′-TCA GCA AGG CGA GGA ACA C-3′ RV 5′-CTG GGT TGG TTG CGA GAT TG-3′	64.5	EF524201

[a] Temperature (°C) for PCR annealing and elongation.

Table 4. Nanograms of steroidogenic compounds (mean ± standard error (SE), $n = 3$) recovered in POCIS after they had been deployed at four sites in the Elkhorn River during May and June 2005

Site	Estrogens			Androgens		Progestogens	
	E2	E3	E1	T	4-A	P	MGA
May							
Battle Cr.	ND	3.3 (3.3)	2.6 (1.1)	0.6 (0.3)	ND	2.0 (1.1)	1.7 (1.7)
Fisher Cr.	ND	ND	12.9 (2.6)	ND	3.5 (2.2)	4.2 (2.1)	2.6 (1.3)
Sand Cr.	5.8 (3.1)	ND	3.3 (0.4)	1.3 (1.3)	4.9 (1.7)	3.5 (1.1)	1.5 (1.5)
Norfolk WWTP	–	–	–	–	–	–	–
June							
Battle Cr.	–	–	–	–	–	–	–
Fisher Cr.	8.8 (3.4)	ND	9.0 (0.8)	1.8 (1.7)	21 (0.8)	2.3 (0.2)	1.1 (0.2)
Sand Cr.	5.5 (3.8)	2.1 (1.3)	1.4 (0.7)	1.6 (0.2)	4.3 (0.6)	1.4 (0.03)	0.7 (0.2)
Norfolk WWTP	1.7 (0.3)	ND	7.4 (2.2)	0.9 (0.3)	5.3 (0.4)	2.1 (0.7)	1.2 (0.6)

Asterisks denote significant differences between values at one field site between May and June. Note that detection limit is near 1 ng.

3.3. Molecular cloning of Cyp3A

Using a PCR approach with degenerate primers, we obtained a 787 base nucleotide sequence that encoded 262 amino acid for fathead minnow hepatic Cyp3A (Genbank accession number EF524201). The deduced amino acid sequence of fathead minnow Cyp3A showed 90%, 69–80% or 57–59% sequence identity when compared with *Ctenopharyngodon idella* (grass carp), other teleosts or mammals, respectively. This cDNA sequence was used to design PCR primers for quantitative real-time PCR analysis of fathead minnow Cyp3A.

3.4. Field studies – fish

Upon recovery, the fish deployed at the three different field sites (Sand Creek, Fisher Creek, Norfolk WWTP) were different in size, and had experienced different levels of mortality. Mortality of the fish deployed at Fisher Creek was 7%, 20% at Sand Creek and 63% at the Norfolk WWTP. Mortality at the Norfolk WWTP was significantly greater than that at either Sand or Fisher Creek (Chi-square, $P > 0.01$ in both cases).

Fish deployed in the field were initially size matched prior to deployment. Upon retrieval, no significant differences among body mass, HSI or GSI were apparent among the female minnows (Kruskal–Wallis ANOVA, $P > 0.05$, data not shown). On average, the female minnows retrieved from the field had a body mass of 0.95 ± 0.03 g, a GSI of 4.1 ± 0.7, and an HSI of 2.0 ± 0.3. In males, there were no significant differences among GSI or HSI (Kruskal–Wallis ANOVA, $P > 0.05$). On average, the male minnows had a GSI of 0.9 ± 0.1 and an HSI of 1.8 ± 0.1. There were significant differences in the body mass of recovered males from the three field sites (Kruskal–Wallis ANOVA, $P < 0.05$). Males retrieved from below the Norfolk WWTP were significantly (nonparametric multiple comparisons, $P < 0.05$) larger (1.5 ± 0.1 g) than the males retrieved from the other two field sites (Fisher Creek, 1.1 ± 0.1 g; Sand Creek 1.1 ± 0.1 g).

In females, the relative expression of AR, ER-α, Cyp3A, vg1, and P450scc mRNA was not significantly different from among the three sites (data not shown). However, StAR mRNA expression was significantly

Table 5. Nanograms of steroidogenic compounds ($n = 1$) recovered in POCIS deployed for 21 days in aquaria containing either no sediment or sediment from one of the four field sites

Site	Estrogens			Androgens	
	E2	E1	EE2	T	11-KT
No sediment	54	10.	ND	1.2	28.
Battle Cr.	ND	11.	ND	2.4	20.
Fisher Cr.	ND	7.8	ND	2.4	16.
Sand Cr.	4.9	21.	ND	3.5	68
Norfolk WWTP	ND	12.	9.3	2.0	45

Note that detection limit is near 1 ng.

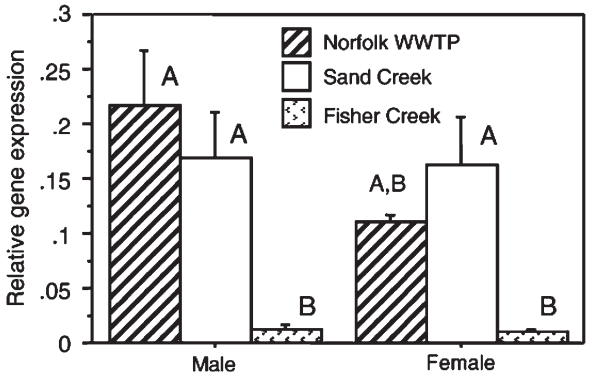

Figure 1. The mean (± SE) relative hepatic mRNA expression of StAR for fathead minnows deployed at three locations during June 2005. For both males and females, StAR expression was significantly greater at Sand Creek than it was at Fisher Creek.

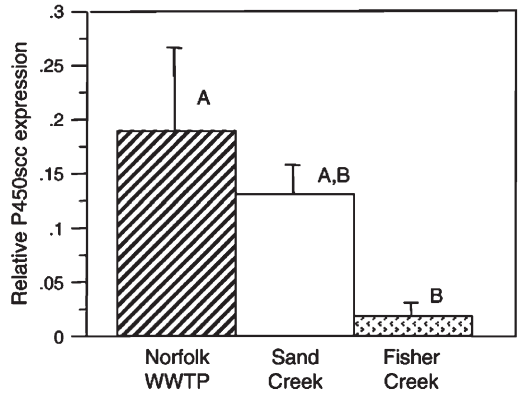

Figure 2. The mean (± SE) relative hepatic mRNA expression of P450scc for male fathead minnows deployed at three field sites for 21 days. Males deployed at Fisher Creek had lower levels of expression than did the fish deployed below the Norfolk WWTP.

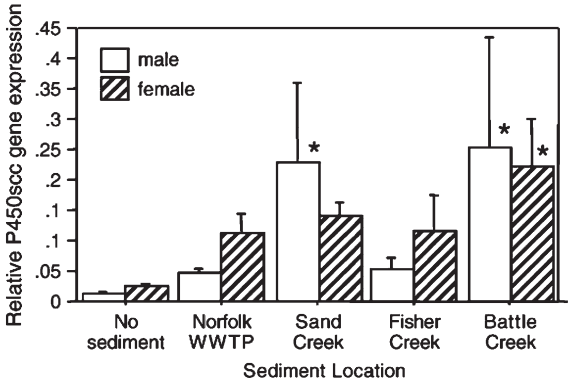

Figure 3. The mean (± SE) relative hepatic mRNA expression of P450scc in male and female fathead minnows exposed to sediments from four field sites throughout the Elkhorn River watershed. Exposure to a no-sediment aquaria acted as a negative control. An asterisk represents a significant difference in expression between the fish in the no-sediment aquaria and the fish exposed to field sediments.

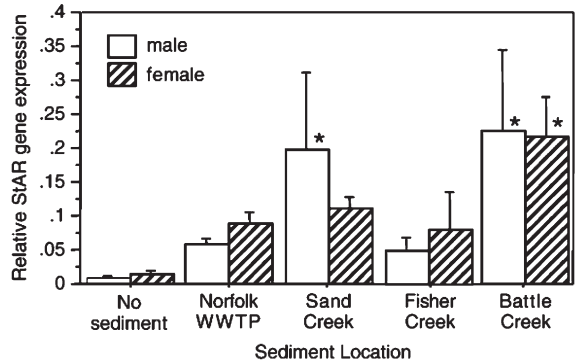

Figure 4. The mean (± SE) relative hepatic mRNA expression of StAR in male and female fathead minnows exposed to sediments from four locations throughout the Elkhorn River watershed. Exposure to a no-sediment aquaria acted as a negative control. An asterisk represents a significant difference in expression between the fish in the no-sediment aquaria and the fish exposed to field sediments.

different among the three field sites (Kruskal–Wallis, $P < 0.05$). Females deployed at Sand Creek had significantly higher levels (nonparametric multiple comparisons test, $P < 0.05$) of StAR mRNA expression than did fish deployed at Fisher Creek (Figure 1).

For male minnows, there were no significant differences in the relative expression of AR, Cyp3A or vg1 mRNA among the three sample sites (Kruskal–Wallis, $P > 0.05$ in all cases). With respect to ER-α mRNA expression, there were significant differences among the three groups of fish; however, post-hoc pairwise comparisons between the groups did not yield significant differences among the pairs. Males from the three field sites were significantly different from each other with respect to mRNA expression of hepatic StAR and P450cc (Kruskal–Wallis ANOVA, $P < 0.05$). For both StAR and P450scc mRNA (Figure 1 and Figure 2), the fish deployed at Fisher Creek had significantly lower levels of mRNA expression than did the males deployed at the other two sites (nonparametric multiple comparisons, $P < 0.05$).

3.5. Laboratory study – fish

There were no significant differences in mean body mass, HSI or GSI for females used in the sediment experiments. On average, at the end of the sediment exposures, female minnows had a body mass of 1.1 ± 0.04 g, a GSI of 12.4 ± 0.8, and an HSI of 2.7 ± 0.2. In males, there were no significant differences in body mass or GSI. At the end of the sediment exposures, male minnows had a mean body mass of 1.9 ± 0.05 g, and a mean GSI

of 1.9 ± 0.2. The males in the five treatment groups had significantly different HSIs (Kruskal–Wallis $P > 0.05$). Males deployed in the no-sediment aquarium had significantly greater HSI (2.8 ± 0.3) than did fish that were held in an aquaria containing sediments from the Norfolk WWTP (1.8 ± 0.2) (nonparametric multiple comparisons, $P < 0.05$). In fish exposed to the other sediments, HSI ranged from 2.2 to 2.7 and was not significantly different from any of the other exposure groups.

For both males and females, there were no significant differences in the relative expression of hepatic AR, ERα, vg1 and Cyp3A mRNA in the groups held in the five different treatment aquaria (Kruskal–Wallis, $P < 0.05$).

For males and females, there were significant differences in hepatic StAR and P450scc mRNA expression among the groups held in aquaria containing sediments from the different locations. Male fish held in aquaria containing sediments from Battle Creek and Sand Creek had greater hepatic expression of StAR and P450scc mRNA than males held in the no-sediment aquaria (Figure 3 and Figure 4). For female minnows, hepatic expression of StAR and P450scc mRNA was greatest in the fish held in tanks containing sediments collected at Battle Creek relative to the no-sediment aquaria (Figure 3 and Figure 4).

4. Discussion

The objectives of this study were to: 1) determine if steroidogenic compounds were present in the Elkhorn River, 2) determine if sediments collected from the Elkhorn River could act as a source of steroidogenic compounds to aquatic organisms and 3) determine if site-specific biological effects were apparent in the hepatic gene expression of fathead minnows. Results from our experiments suggest that: 1) steroidogenic compounds are found in the Elkhorn River, 2) sediment-bound steroids may serve as a source for exposure of these compounds, and that 3) fish deployed at different locations in the Elkhorn River (or fish maintained on sediments from different location in the Elkhorn River) do experience different levels of hepatic gene expression.

4.1. Field studies – POCIS

Contaminants recovered from POCIS are intended to indicate relative levels of substances that aquatic organisms may be exposed to. While it is possible to calibrate POCIS by exposing the devices to known contaminant concentrations and determine the potential for recovery, there are many variables that can affect actual recovery in the field (Alvarez et al., 2004). Nevertheless, these devices have proven to be useful in evaluating the occurrence and potential for exposure in other studies (Alvarez et al., 2005; Matthiessen et al., 2006).

The POCIS extracts contained a number of estrogen and androgen metabolites, as well as the synthetic progestogen, MGA (Table 4). The POCIS were never found to contain α-zearalonol, 17β-TbOH or EE2. Of the estrogenic metabolites collected at the four sites, E1 was detected at all of the sites and comprised, on average, 61% of the total mass of the collected estrogens. This is consistent with the results from Soto et al. (2004) in an earlier study on the Elkhorn River. They found E1 at every site they sampled on the Elkhorn River, and it represented up to 46% of the total estrogenic activity at each site (as determined by the E-screen assay).

Progesterone was detected at all of the field sites sampled during May and June, whereas T and 4-A were found at all but one site. Common practices at feedlots help to put these data into perspective. P will be produced endogenously by beef cattle, and is also used in combination implants that would normally be used on some cattle in feedlots (Kolok and Sellin, in press). Consequently, it is at least possible that the P detected at the field sites came from runoff associated with beef cattle feedlots. In contrast, endogenous T is very low in male calves and steers on feedlots, as these animals are castrated (Kolok and Sellin, in press). Furthermore, T is not generally used in implants to promote the growth of beef cattle held on feedlots (Preston, 1997). As such, the cattle feedlots are unlikely sources for the T and 4-A detected in the POCIS extracts.

Trace amounts of MGA, near the detection limit, were detected in almost every POCIS deployed in the field. Given that MGA is typically used to enhance the growth of heifers (Meyer, 2001), it seems unlikely that it would occur in waste from a finishing feedlot typically populated with castrated males. Nevertheless, MGA has been detected in cattle manure several months after use for field fertilization (Schiffer et al., 2001) and there is evidence it can persist in the environment. A method with improved sensitivity for this compound would help to better evaluate its persistence and occurrence in this setting.

Metabolites of the synthetic growth promoters, 17β-trenbolone and α-zearalonol, were not detected in the POCIS extracts. Though zearalanol is not widely used, TBA is the primary ingredient in implants used on both steers and heifers on finishing feedlots (Siemens, 1996; ZoBell et al., 2000; Preston, 1997), and the presence of TB metabolites in the POCIS was expected. The lack of strong evidence for the presence of 17β-TB in the environment is consistent with the recently

published literature (Soto et al., 2004; Durhan et al., 2006), and may reflect a lack of environmental mobility for this metabolite. Soto et al. (2004) detected trace levels of TB metabolites in the water collected from a beef feedlot retention pond; however when compared to water blanks, the validity of the measurements was questionable. Durhan et al. (2006) reported very low levels (5–100 ng/L) of 17α-and 17β-TbOH in discharge from a beef cattle feedlot using HPLC with fluorescence detection; however, variability in method sensitivity prevented GC/MS confirmation of these detections. Furthermore, the occurrence and magnitude of TB metabolites varied considerably from one sampling period to the next, with no clear cut seasonal or spatial pattern. Clearly, improvements in method sensitivity could also help to elucidate the environmental fate and mobility of TB metabolites.

4.2. Laboratory study – POCIS

Individual POCIS ($n = 1$) were deployed in the laboratory aquaria; therefore, it is not possible to quantify the variability associated with these measurements. Nevertheless, the steroidogenic compounds collected by the POCIS in the laboratory sediment study did provide two important pieces of information: EE2 can be released from sediments collected downstream for the Norfolk WWTP and fish in the aquaria secreted steroid metabolites into the water.

Detectable levels of EE2 were found in the POCIS deployed in association with sediments collected downstream from the Norfolk WWTP. This result may not be that surprising considering that EE2 has previously been found in sediments collected downstream from WWTPs (Peck et al., 2004). It is interesting to note, that the POCIS deployed in the field downstream from the Norfolk WWTP (sampling Elkhorn River water) did not detect EE2, whereas the POCIS deployed in conjunction with sediments collected downstream from the Norfolk WWTP did.

Fish in the laboratory were found to excrete steroidal compounds into the water, as has been documented in previous studies (for a review, see Scott and Ellis, 2007). There are two results that act as evidence to suggest that the exogenous compounds detected by the POCIS were excreted from the fish in the aquaria. First, the POCIS in the no-sediment tank collected the same natural steroids, in similar amounts, as did the POCIS in the aquaria containing field sediments. Second, the POCIS detected 11-kT, a major teleost androgen, in each aquarium. While not a focal point of this paper, the detection of steroids produced by 10 male and 10 female fish in a 40-L aquaria receiving continuous water renewal, lends support to our contention that the POCIS represents a sensitive tool for detecting low concentrations of exogenous steroids.

4.3. Fish

Interpretation of the results from the field deployments is limited by the loss of the fish that were deployed at the reference site, Battle Creek. Nevertheless, significant differences in the minnows deployed at the three field sites were apparent.

The Norfolk WWTP adversely impacted the fathead minnows. Fish deployed at the WWTP experienced significantly greater levels of mortality than those at either Sand or Fisher Creeks. Perhaps as a consequence of the mortality of smaller males, the surviving males retrieved from below the Norfolk WWTP were significantly larger than the males retrieved from the other two field sites. In the laboratory, male fish deployed in the no-sediment aquarium had significantly greater levels of HSI than did those fish held in the aquaria containing sediments from the Norfolk WWTP. Sediment organic matter could be an important factor in explaining the differences discussed above. As stated previously, the sediments from the Norfolk WWTP had a 10-fold lower organic content than the sediments from the other three sites. Upon dissection, the intestines of the minnows exposed to the more organic sediments contained visible quantities of sediment, whereas the fish from the Norfolk area did not. The minnows may be grazing on high organic content sediments and these sediments may be nutritionally important. At the WWTP, the option to graze on organically rich sediments was not available.

With respect to hepatic gene expression, four of the genes (AR, ER-α, vg1 and Cyp3A) proved unresponsive to our treatments. This is important, as AR, ER-α and vg1 are known to respond to exogenous steroids (Yadatie et al., 1999; Yamaguchi et al., 2005; Miracle et al., 2006; Hook et al., 2006). In contrast, mRNA expression of StAR and P450scc were almost uniformly responsive in laboratory-treated and field-exposed male and female minnows. While we are unaware of any studies that have focused on expression of these genes in hepatic tissue, our findings suggest that differential hepatic expression of these genes is responsive to environmental cues.

Given the function of StAR and P450scc, it is possible that the differential expression of these genes is related to an up-regulation of pathways involved in the synthesis of stress steroids. The lowest levels of StAR and

P450scc expression were found in fish deployed at Fisher Creek, the site with the lowest mortality (7% or 20% out of 30 deployed fish), and presumably, these fish were the least stressed of the fish deployed in the field. In the laboratory sediment study, StAR and P450scc were consistently found to be lowest in the no-sediment control tank. Considering that the conditions under which the no-sediment fish were maintained were essentially the same as those in their natal tanks, these fish were almost certainly the least stressed. The relationship between hepatic expression of StAR and P450scc and stress clearly deserves more attention.

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