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Endocrine-Disrupting Effects of Cattle Feedlot Effluent on an Aquatic Sentinel Species, the Fathead Minnow

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Over the last decade, research has examined the endocrine-disrupting action of various environmental pollutants, including hormones, pharmaceuticals, and surfactants, in sewage treatment plant effluent. Responding to the growth of concentrated animal feeding operations (CAFOs) and the pollutants present in their wastewater (e.g., nutrients, pharmaceuticals, and hormones), the U.S. Environmental Protection Agency developed a new rule that tightens the regulation of CAFOs. In this study, we collected wild fathead minnows (*Pimephales promelas*) exposed to feedlot effluent (FLE) and observed significant alterations in their reproductive biology. Male fish were demasculinized (having lower testicular testosterone synthesis, altered head morphometrics, and smaller testis size). Defeminization of females, as evidenced by a decreased estrogen:androgen ratio of *in vitro* steroid hormone synthesis, was also documented. We did not observe characteristics in either male or female fish indicative of exposure to environmental estrogens. Using cells transfected with the human androgen receptor, we detected potent androgenic responses from the FLE. Taken together, our morphologic, endocrinologic, and *in vitro* gene activation assay data suggest two hypotheses: *a*) there are potent androgenic substance(s) in the FLE, and/or *b*) there is a complex mixture of androgenic and estrogenic substances that alter the hypothalamic–pituitary–gonadal axis, inhibiting the release of gonadotropin-releasing hormone or gonadotropins. This is the first study demonstrating that the endocrine and reproductive systems of wild fish can be adversely affected by FLE. Future studies are needed to further investigate the effects of agricultural runoff and to identify the biologically active agents, whether natural or pharmaceutical in origin. *Key words:* anabolic steroid hormones, aquatic ecosystem health, concentrated animal feeding operation (CAFO), environmental androgens and estrogens, gene expression, HPG axis, hypothalamic–pituitary–gonadal axis, pharmaceuticals and personal care products (PPCPs), *Pimephales promelas. Environ Health Perspect* 112:353–358 (2004). doi:10.1289/ehp.6591 available via *http://dx.doi.org/* [Online 1 December 2003]

There has been a great deal of research over the last decade examining the endocrine-disrupting action of various environmental pollutants (Ankley et al. 1997; Guillette and Crain 2000). Much of this research has focused on the ability of chemical pollutants to act as estrogen receptor (ER) or androgen receptor (AR) agonists or antagonists (McLachlan 2001). Most of the compounds studied—pesticides and industrial pollutants—exhibit weak receptor affinities compared with endogenous hormones but can produce endocrine responses both *in vitro* and *in vivo* at environmentally relevant doses (Rooney and Guillette 2000; Tyler et al. 1998).

Studies have begun to focus on natural hormones released from animal waste used to fertilize agricultural fields. Significant concentrations of estrogens and androgens have been reported in ponds or streams receiving runoff from fields fertilized with chicken litter (Finlay-Moore et al. 2000; Nichols et al. 1997; Shore et al. 1995). In fact, depending on application rate, concentrations in runoff have been measured as high as 1,280 ng/L (Nichols et al. 1997). Natural hormones, such as estradiol, have also been reported in ponds below cattle holding facilities and have been associated with elevated plasma concentrations of the yolk precursor protein vitellogenin in female turtles (Irwin et al. 2001). Contamination of water systems with endogenous hormones such as 17β-estradiol (E_2) and testosterone (T) is not limited to surface waters because E_2 has been reported in spring water from mantled karst aquifers in agricultural areas (Peterson et al. 2000).

In addition, the presence of endogenous and pharmaceutical estrogens in sewage effluent has been studied as an example of hormonal pollution of the aquatic environment and has been reported as a factor affecting fish development and reproductive activity (Purdom et al. 1994; Tyler et al. 1998). Work performed below sewage treatment plants in Great Britain has documented a significant number of intersex fish compared with rivers with less effluent (Desbrow et al. 1996; Jobling et al. 1998). Furthermore, these studies have reported that many males had elevated levels of estrogen-induced vitellogenin in their blood. This protein does not normally occur in males. Fractionated sewage effluent, derived mostly from domestic sources, exhibited various peaks with estrogenic activity. Those representing ethinyl estradiol and estrone displayed the most potent estrogenic activities (Harries et al. 1996, 1997). In other countries, similar research has supported these observations and extended them by reporting that male fish exposed to sewage effluent not only have detectable plasma vitellogenin concentrations but also display altered plasma concentrations of T and E_2 (Folmar et al. 1996, 2000; Orlando et al. 1999).

These studies have helped focus attention on the possible detrimental roles of pharmaceutical agents released into the environment. A wide array of pharmaceutical agents, including hormonal mimics, have been reported in sewage and open waters in various countries (Daughton and Ternes 1999; Kolpin et al. 2002; Stumpf et al. 1999; Ternes 1998). These agents include drugs commonly prescribed for the treatment of heart disease, stress, inflammation, bacterial infections (antibiotics), and birth control. Further, veterinary drugs, such as growth promoters and antibiotics, are used extensively in agriculture, but few studies have examined their presence in the environment, although some studies have recently reported the presence of these compounds in groundwater near farms (Peterson et al. 2000). Importantly, no studies have examined the possible effects of these compounds on wildlife exposed to runoff from farms using large concentrations of pharmaceutical agents, such as cattle feedlots.

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In the United States, hormone supplements are used in the production of approximately 90% of the beef cattle (Balter 1999). These supplements promote rapid growth and increase the conversion of feed to muscle mass. Currently, marketed hormone implants contain pharmaceutical-grade compounds that have androgenic, estrogenic, or progestogenic activities or a mixture of these activities (Schiffer et al. 2001). Androgenic trenbolone acetate, estrogenic zeranol, and progestogenic melengestrol acetate are commonly used singly or combined with native steroid hormones, including T, E_2, or progesterone (Schiffer et al. 2001).

Recent studies have indicated that there is a basis for concern about the ecologic effects of these pharmaceutical supplements. Trenbolone acetate, a synthetic androgenic anabolic steroid used in cattle production, is metabolized into trenbolone-β, the biologically active molecule, and excreted as trenbolone-α and -β (Schiffer et al. 2001). Trenbolone-β has a half-life in liquid manure of > 260 days, suggesting that it could have ecologic impacts if released into the environment as runoff from feedlots (Schiffer et al. 2001). In another study, estrogenic activity was detected in ponds below feedlots housing a cattle herd in an academic agricultural facility (Irwin et al. 2001).

Responding to a concern over the growth of concentrated animal feeding operations (CAFOs) and the pollutants present in their wastewater (e.g., nutrients, pharmaceuticals, hormones, etc.), the U.S. Environmental Protection Agency (U.S. EPA) recently issued a new agency rule that tightens the regulation of CAFOs (U.S. EPA 2003). The latest rule revises the existing 1976 U.S. EPA requirements on CAFOs in two ways: *a*) more CAFOs will be required to seek discharge permits under the Clean Water Act (1972) (e.g., previously exempt dry litter poultry operations); and *b*) all CAFOs must develop and implement a nutrient management plan.

In our research, we examined whether endocrine activity could be detected in natural stream/river systems below feedlots by studying the reproductive endocrinology and secondary sex characteristics of wild fish populations. We examined adult fathead minnows (FHMs), *Pimephales promelas*, living upstream and downstream of cattle feedlots in Nebraska. The FHM was chosen because it is a well-characterized toxicologic model and native to the study region. FHMs have been proposed as a sentinel species for exposure to environmental androgens and estrogens (Ankley et al. 2001). Untreated male and female FHMs exposed to androgens develop increased head size and nuptial tubercles on the dorsal region of the head. Untreated female and male FHMs exposed to estrogens synthesize the yolk protein vitellogenin (Tyler et al. 1999). We hypothesized that fish populations exposed to effluent from the cattle feedlots would exhibit altered sex steroid hormone titers and altered head morphology compared with FHM populations from the reference site. In addition, we hypothesized that the water would contain hormonally active substances.

Materials and Methods

Research sites. For this initial study, we identified two affected sites: *a*) a stream directly below the effluent outfall of a feedlot with a high density of penned cattle (designated the contaminated site); and *b*) a stream that receives runoff from fields with dispersed cattle and agricultural activity (designated the intermediate exposure site) (Figure 1). Both sites are confluent with the Elkhorn River and have several commercial feedlots that release effluent into retaining ponds, which then drain into the river. In addition to the sites above, we identified a number of reference sites upriver from these feedlots. These streams also flowed into the Elkhorn River but with no apparent feedlot activity in the surrounding area. We were able to capture FHMs in sufficient numbers from only one of these sites (designated the reference site), which is located within the Oak Valley State Wildlife Management Area. At each site, water quality information was obtained that included temperature, pH, dissolved oxygen (DO), and salinity (Table 1).

Fish. During 9 days in June 1999, FHMs (n = 97) were collected at each of the sites using a seine or minnow traps. Immediately upon capture, fish were placed in coolers containing aerated river water. Fish were then transferred to the University of Nebraska in Omaha, where they were anesthetized with tricaine methanesulfonate (MS-222, 150 ppm; A5040, Sigma Chemical Co., St. Louis, MO) and processed. Various morphologic measurements were obtained, including length (0.1 mm), mass (grams), widest head width (HW; 0.1 mm), and interocular (IO) distance (0.1 mm). Hepatic tissue and gonads were removed and mass (grams) obtained; then gonads were immediately transferred to an explant culture. After *in vitro* culturing, the gonads were fixed in neutral buffered formalin and processed for paraffin histology following standard protocol (Humason 1997). To determine the reproductive stage of the gonad, we compared the mean values of four stages of gametogenesis in both sexes between sites (Grier 1981; Selman and Wallace 1989).

Gonad cultures and radioimmunoassays. *In vitro* gonadal synthesis of sex steroid hormones was examined in female and male FHMs following a modification of the protocol described by McMaster et al. (1995). Gonadal tissue culture medium consisted of Media 199 (pH 7.4; no. 21200-027; Gibco, Ontario, Canada),

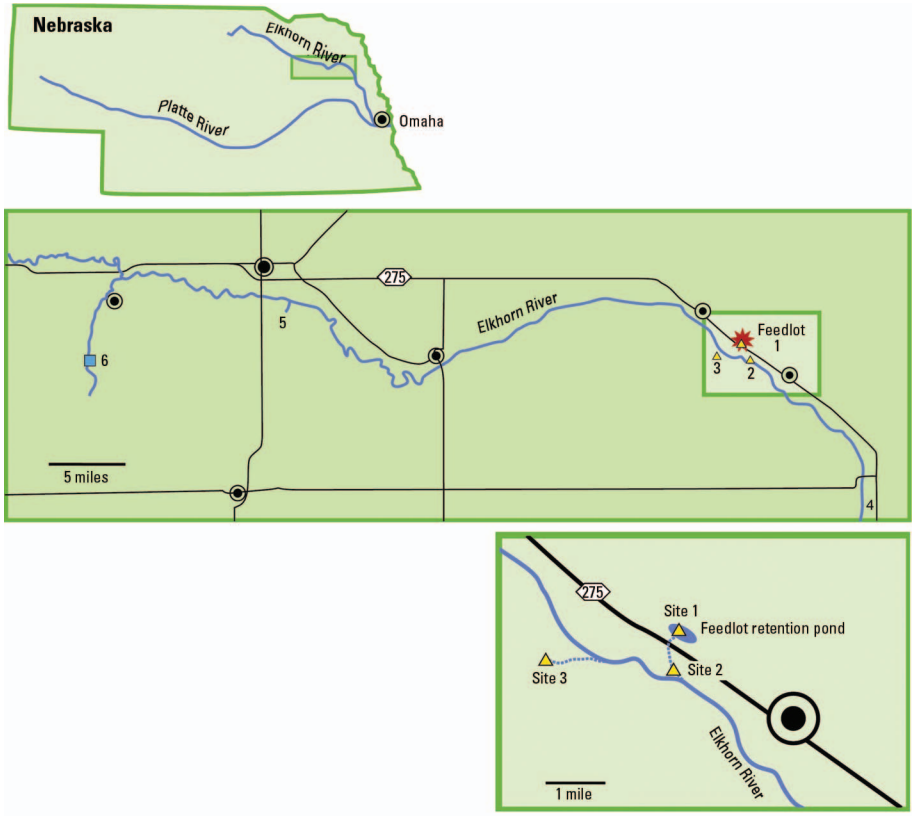

Figure 1. Map of field sites confluent with the Elkhorn River in eastern Nebraska, showing the feedlot retention pond (site 1), contaminated site (site 2), intermediate site (site 3), and reference site (site 6). Figure also presented by Soto et al. (2004).

3-isobutyl-1-methylxanthine (final concentration, 0.1 mM; no. I-7018; Sigma), forskolin (final concentration, 5 µM; no. F-6886; Sigma), and androstenedione (final concentration, 100 ng/mL; no. A-9630, Sigma). Culture medium was sterile-filtered into an autoclaved glass bottle and stored on ice.

After gonads were excised, they were weighed, placed in glass test tubes with 1 mL culture medium, wrapped in Parafilm, and incubated on a rocking plate for 6 hr at 24°C. Parameters of the assay, including the incubation time and quantity of gonadal tissue and culture medium, were determined empirically from a previously conducted pilot study. After incubation, the culture medium was decanted and stored at –80°C until assayed.

In vitro production of E_2 and T in female FHMs and T in male FHMs was measured via radioimmunoassay on extracted culture media as described previously (Guillette et al. 1995). Culture medium samples were extracted twice with ethyl ether, vaporized under a stream of filtered dry air, and resuspended with 100 µL 0.5 M borate buffer (pH 8.0). After resuspension of the steroid hormones, the following assay constituents were added: 200 µL antibody, 100 µL bovine serum albumin borate buffer, and 100 µL ^{3}H-hormone (Amersham Biosciences, Piscataway, NJ). Final sample volume was 500 µL, and all assay tubes were run in duplicate. E_2 and T standards (Sigma Chemical Co.) were also made in duplicate at concentrations of 1.56, 3.12, 6.25, 12.5, 25, 50, 100, 200, 400, and 800 pg/tube.

After incubating all samples and standards overnight at 4°C, we added 500 µL 5% charcoal/0.5% dextran/0.5 M phosphate-buffered saline (PBS) mixture to separate the bound from free hormone. The tubes were vortexed and centrifuged, and the supernatant containing the bound hormone was decanted. Five milliliters of ScintiVerse BD scintillation cocktail (Fisher Scientific, Pittsburgh, PA) was combined with the supernatant, and the tubes were counted on a Beckman scintillation counter (model LS 5801; Beckman, Somerset, NJ). Extraction efficiencies of 95% for E_2 and 99% for T were used to correct raw data to actual medium concentrations. Assays were validated by comparing the slopes of an internal standard curve, a medium dilution curve, and the assay's standard curve. Parallelism between the internal standards, medium dilutions, and assay standard curves was confirmed using homogeneity of slopes for E_2 ($p = 0.24$) and T ($p = 0.11$) (StatView 5.0; SAS Institute, Inc., Cary, NC).

Bioassays for hormonal activity in water samples. Water was sampled in U.S. EPA–approved glass bottles concurrent with collection of the fish at the contaminated, intermediate, and reference sites. In addition, water was obtained from a retaining pond, which is located immediately at the base of the feedlot and whose outfall is the headwaters for the contaminated site. Water was refrigerated upon collection and treated with sodium azide to inhibit bacterial degradation of organic matter in samples. Samples were analyzed for *in vitro* androgenic and estrogenic activity (Soto et al. 2004). Additional water samples were collected 1 year later (June 2000), treated as stated above, and shipped to the U.S. EPA for androgenic activity analysis. Table 1 provides information on sampling conditions and basic water quality parameters.

Preparation of water samples for CV-1 AR-dependent transcriptional activation assay. Dosing medium was made using water that was obtained from the retaining pond immediately below the feedlot, as described above. Powdered Gibco Dulbecco's modified Eagle's medium (Invitrogen Corporation, Carlsbad, CA) with 3.7 g $NaHCO_3$ (ICN Biochemicals, Irvine, CA) was reconstituted with 1 L of retaining pond water and adjusted to pH 7.4. Medium was sterile filtered (0.2 µm, Nalgene bottle-top filters; Fisher Scientific), supplemented with 5% dextran charcoal serum (HyClone, Logan, UT), with added antibiotics, wrapped in aluminum foil, and stored at 4°C until use in the CV-1 transcriptional activation assay.

CV-1 AR-dependent transcriptional activation assay. Several experiments were conducted to determine if feedlot effluent (FLE) induced human AR (hAR)–dependent gene expression in CV-1 cells (monkey kidney line; American Type Culture Collection, Rockville, MD); for a further description of this assay and its use in testing androgenicity of water in other aquatic systems, see Parks et al. (2001). To determine if FLE displayed AR agonist activity, cell medium was made with site water. In this experiment, 200,000 CV-1 cells were plated in a 60-mm dish and then transiently cotransfected with 50 ng pCMVhAR expression vector (from Elizabeth Wilson, University of North Carolina at Chapel Hill) and 5 µg MMTV-luciferase reporter (Boehringer, Mannheim, Germany) using 5 µL Fugene reagent in 95 µL serum-free medium (Boehringer-Mannheim, Basel, Switzerland) (seven replicate studies). Twenty-four hours after transfection, cells were dosed with 4 mL of medium that was made with water from the retention pond site and incubated at 37°C with 5% CO_2. After 24 hr exposure, the medium was removed and the cells were washed once with PBS and then harvested with 500 µL lysis buffer (Promega, Madison, WI). Relative light units of 0.05 mL aliquots of lysate were determined using a Monolight 2010 luminometer (Analytical Luminescence Laboratories, San Diego, CA).

Statistical analyses. We tested for differences between sites for body length and mass, gonad mass, hormones, and head morphometrics in FHMs by one-way analysis of variance (ANOVA) or analysis of covariance and ANOVA on the CV-1 AR-dependent transcriptional activation assays (StatView 5.0). If needed, data were log-transformed to obtain homogeneity of variance. Correlations between various hormones and body parameters were determined using Pearson's correlation or multiple linear regression analyses (StatView). Differences between examined groups were considered significant at $p < 0.05$.

Table 1. Water quality parameters for the three sites confluent with the Elkhorn River in eastern Nebraska from which FHMs were collected.

Site	Temperature (°C)	pH	DO (mg/mL)	Salinity (ppt)
Contaminated	24.8	7.88	2.37	0.8
Intermediate	23.3	NA	2.79	0.2
Reference	21.7	7.64	4.1	0.3

NA, not available (broken pH meter).

Table 2. Morphometric values (mean ± 1 SE) for female FHMs from three sites confluent with the Elkhorn River in eastern Nebraska.

Measurement	Contaminated site ($n = 23$)	Intermediate site ($n = 13$)	Reference site ($n = 19$)
Length (mm)	5.36 ± 0.19	5.68 ± 0.11	5.68 ± 0.12
Soma mass (g)	2.23 ± 0.26	2.48 ± 0.15	2.46 ± 0.14
Gonad mass (g)	0.312 ± 0.05	0.418 ± 0.05	0.405 ± 0.03
Liver mass (g)	0.065 ± 0.008	0.065 ± 0.006	0.076 ± 0.007
IO distance (mm)	4.05 ± 0.18[a]	4.24 ± 0.15[a]	4.72 ± 0.13[b]
HW (mm)	7.07 ± 0.24	7.22 ± 0.19	7.35 ± 0.17

Values with different superscripts within a row of data are significantly different ($p = 0.05$); values in rows with no superscripts are not significantly different.

Results

Morphometrics. No significant difference was noted in length ($p = 0.29$) and mass ($p = 0.70$) among female FHMs from the three sites (Table 2). Further, no significant difference was noted in ovarian ($p = 0.13$) or liver ($p = 0.45$) mass. In contrast, IO distance was significantly different ($F = 5.6$, $p = 0.008$), with females from the contaminated and

intermediate sites having smaller distances than females from the reference site (Table 2). HW, however, was not different (p = 0.47). IO distance was correlated with HW, and the regression lines from each site have similar slopes but significantly different y-intercepts (p = 0.02), with the reference site having a higher y-value than the other two sites.

As with females, no significant difference was noted in length (p = 0.14) or body mass (p = 0.15) among male FHMs collected at the three sites (Table 3). Male fish from all sites were significantly larger than female fish from the three study sites. We found a significant difference in testicular (F = 4.58, p = 0.017) but not hepatic (F = 1.9, p = 0.16) mass in males (Table 3). Males from the contaminated and intermediate sites had significantly smaller testes than did those from the reference site. IO distance was significantly different (F = 4.2, p = 0.02), with males from the contaminated and intermediate sites having reduced distances compared with males from the reference site (Table 3). HW, however, was not different (p = 0.08). IO distance correlated with HW in males, with the regression lines from each site having similar slopes.

Histopathology. No apparent pathology was observed in any of the ovaries or testes using standard histologic techniques. Also, through histologic examination, we confirmed that all FHMs collected were adults and that the reproductive stage of the gonads in males and females did not vary among sites.

Gonadal steroidogenesis. No significant difference in ovarian E_2 synthesis was observed among sites (p = 0.44; Figure 2A). Ovarian mass was not correlated with E_2 synthesis (contaminated: r^2 = 0.074, p = 0.22; intermediate: r^2 = 0.115, p = 0.25; reference: r^2 = 0.169, p = 0.11). Mean ovarian synthesis of T was not different among sites (p = 0.08; Figure 2B). When the data from the females were examined as an estrogen:androgen (E:A) ratio, a significant difference was clearly apparent (F = 5.6, p = 0.02; Figure 2C). Our data indicate that the females from the contaminated and intermediate sites had a defeminized sex hormone ratio, that is, a decreased E:A ratio based on a reduction in E_2 synthesis and an increase in T synthesis (Figure 2A,B).

There was a significant difference in T synthesis *in vitro* from testicular tissue obtained from the fish collected from the three sites (F = 5.6, p = 0.008; Figure 3), and *in vitro* T synthesis was lower in testes obtained from contaminated and intermediate site fish. T synthesis was not correlated with testicular weight at any of the study sites (contaminated: r^2 = 0.14, p = 0.21; intermediate: r^2 = 0.03, p = 0.61; reference: r^2 = 0.11, p = 0.19).

CV-1 AR-dependent transcriptional activation assays. We assessed androgenicity in seven replicate experiments (with duplicates of each replicate). Androgenicity was defined as the ability of FLE or dihydrotestosterone (DHT, Sigma Chemical Co.) to induce AR-dependent luciferase gene expression in a transfected CV-1 cell line. In every sample and in all seven replicates, FLE induced AR-dependent luciferase gene expression. The data presented in Figure 4 are expressed as fold induction over the control medium (without FLE) and compared with the positive control of 1 nM DHT (near maximal concentration in terms of its ability to induce luciferase expression). FLE and DHT each exhibited significantly higher androgen activity than did media (p < 0.0001, for each treatment vs. medium control). DHT- and FLE-induced responses were not significantly different (p = 0.35) from each other.

Discussion

To our knowledge, this is the first study to document endocrine disruption in fish exposed to FLE. Wild fish collected below a feedlot exhibited altered reproductive biology, including decreased T synthesis, altered head morphometrics, and smaller testis size in males and decreased E:A ratio in female fish. We did not observe overt characteristics in either male or female fish suggesting environmental exposure to estrogens. With an *in vitro* assay using cells transfected with hAR, we detected potent androgenic responses from the FLE. Taken together, our morphologic, endocrinologic, and *in vitro* gene activation assay data suggest two hypotheses: *a*) there is an androgenic substance(s) in the FLE and/or *b*) there is a mixture of endocrine-active substances that alter the hypothalamic–pituitary–gonadal axis. Further support for the hypothesis that androgens are present in the FLE comes from observations of androgenic activity (Soto et al. 2004).

Table 3. Morphometric values (mean ± 1 SE) for male FHMs from three sites confluent with the Elkhorn River in eastern Nebraska.

Measurement	Contaminated site (n = 12)	Intermediate site (n = 10)	Reference site (n = 17)
Length (mm)	6.25 ± 0.35	6.68 ± 0.25	6.85 ± 0.07
Soma mass (g)	3.69 ± 0.65	4.06 ± 0.46	4.80 ± 0.18
Gonad mass (g)	0.067 ± 0.01[a]	0.088 ± 0.01[a]	0.111 ± 0.01[b]
Liver mass (g)	0.107 ± 0.02	0.104 ± 0.02	0.143 ± 0.01
IO distance (mm)	5.58 ± 0.48[a]	5.83 ± 0.37[a]	6.77 ± 0.15[b]
HW (mm)	8.3 ± 0.54	8.64 ± 0.39	9.34 ± 0.12

Values with different superscripts within a row of data are significantly different (p = 0.05); values in rows with no superscripts are not significantly different.

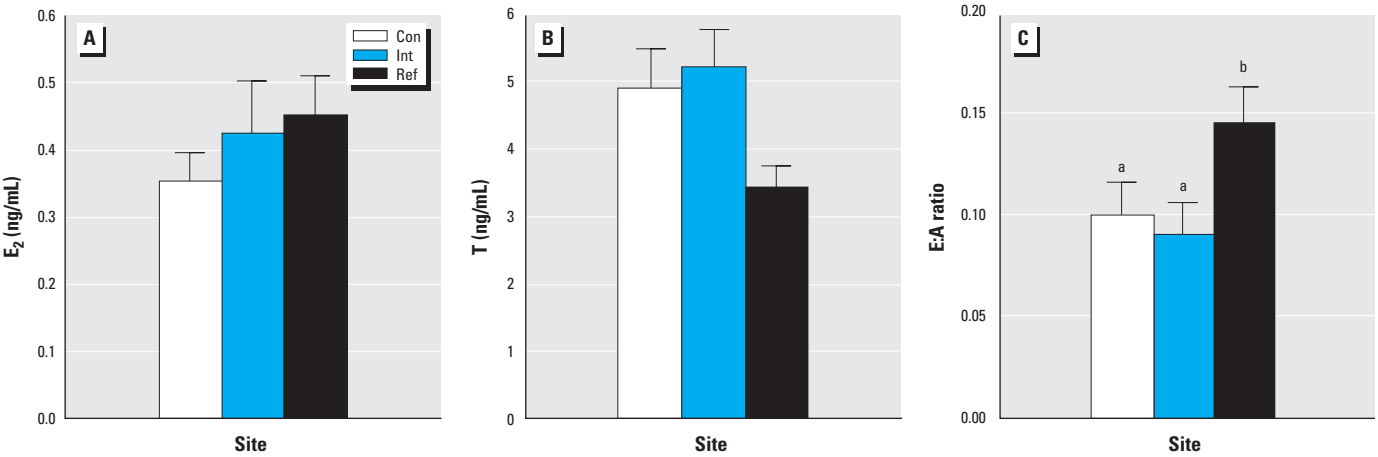

Figure 2. Mean (± 1 SE) *in vitro* synthesis of (*A*) E_2 (p = 0.44) and (*B*) T (p = 0.08) from the ovaries obtained from fish from three Nebraska sites, and (*C*) the E:A ratio. Abbreviations: Con, contaminated; Int, intermediate; Ref, reference. The E:A ratio (*C*) was significantly decreased for ovaries cultured from fish collected from the Con and Int sites (p = 0.02). Values with different superscripts within a row of data are significantly different (p < 0.05); values in rows with no superscripts are not significantly different.

However, Soto et al. (2004) also found estrogenic activity in FLE using the MCF-7 cell *in vitro* E-SCREEN assay, suggesting that there could be a complex mixture of natural and pharmaceutical compounds in the effluent.

Our data clearly demonstrate androgenic activity from water obtained below feedlots. However, it does not identify the causal agents. Androgenic activity could be due to natural androgens found in fecal material or androgenic pharmaceuticals used in growth implants (Meyer 2001). Natural androgens have relatively short half-lives in feces and in the open water of retaining ponds (Meyer 2001). In contrast, recent studies demonstrate that metabolites of synthetic androgens (e.g., trenbolone-β from trenbolone acetate) used in growth implants have longer half-lives. Approximately 27.5% of the initial concentration of trenbolone-β was still present in manure piles 4.5 months after deposition (Schiffer et al. 2001). Natural steroids appear to be rapidly degraded, with half-lives measured on the order of days to hours. No literature could be found regarding the relative persistence of zeranol or melengestrol in feedlot retaining ponds, however.

Trenbolone-β acts as a potent androgen agonist in the CV-1 cell assay used to test FLE in this study (Wilson et al. 2002). In fact, its potency was equal to or greater than that of the positive control, DHT, at similar concentrations. Trenbolone acetate is known to be 8–10-fold more potent than native T in cattle (Schiffer et al. 2001). Furthermore, in an *in utero* screening assay, maternal trenbolone-β increased anogenital distance and attenuated the display of nipples in female rat offspring (Wilson et al. 2002).

In a recent laboratory study, FHMs exposed to trenbolone-β displayed severely altered female and male reproductive biology (Ankley et al. 2003). In females, fecundity decreased, malelike secondary sex characteristics developed (nuptial tubercles), and plasma concentrations of T, E_2, and vitellogenin were all significantly decreased. In males, plasma concentrations of 11-ketotestosterone were decreased and E_2 and vitellogenin were increased. Although difficult to compare directly because of differences in experimental design, data from our field study support the results of this laboratory study.

Trenbolone-β binds the FHM ARs with greater affinity than does T (Ankley et al. 2003). In male FHMs, trenbolone-β could act at the level of the hypothalamus or pituitary to depress gonadotropin–releasing hormone (GnRH) and/or gonadotropic hormone (GtH) synthesis and/or release, leading to decreased T synthesis, testicular mass, and IO distance, as was seen in this study in the males from the contaminated site. Female FHMs exposed to FLE at the intermediate and contaminated sites in this study had decreased E:A ratios caused by a decrease in ovarian E_2 and an increase in T synthesis during *in vitro* culture. That is, if the hormones were examined individually, no significant difference was observed among sites; however, when a ratio was calculated, it was obvious that ovarian steroidogenesis was altered in fish obtained from the intermediate and contaminated sites. This result suggests that some component of the FLE has the potential to inhibit ovarian aromatase, the enzyme that converts T to E_2 (Norris 1997). Interestingly, trenbolone-β at certain concentrations has been shown to weakly bind the FHM estrogen receptor, induce vitellogenesis in male FHMs, and weakly bind the rainbow trout estrogen receptor in an *in vitro* transfected yeast system (Ankley et al. 2003; Le Guevel and Pakdel 2001). Future research should investigate what constituent(s) of the FLE may be inhibiting aromatase synthesis or action.

Other compounds that are strong anabolic agents, such as the mycotoxin zearalanol, are estrogenic in cattle, humans, rainbow trout (*Oncorhynchus mykiss*), and Atlantic salmon (*Salmo salar*) (Arukwe et al. 1999; Le Guevel and Pakdel 2001). Zearalanol is also known to depress concentrations of follicle-stimulating hormone and leutinizing hormone in cattle. Zearalanol, measured as resorcylic acid lactones, was not detected by Soto et al. (2004). Furthermore, we do not know, presently, if zearalanol can interact with GnRH or GtH receptors in fish.

Water quality parameters obtained during this study suggested that the responses observed in fish were unlikely to be complicated by differences in the aquatic environment (Table 1). No fish were found in the retaining pond immediately below the feedlot. This site had very low DO levels (0.7 ppt) and relatively high salinity (1.2 ppt). When the contaminated sites (where fish were obtained) were compared with the reference site, it was apparent that DO was slightly different, as was salinity. The slightly lower observed DO is not surprising given the eutrophic nature of the effluent-laden streams where fish were caught. Salinity was also elevated at the contaminated site versus the other sites, but the levels reported here should have little effect on the fish because the differences were < 1 ppt. Thus, it is unlikely that these variables significantly influenced the end points measured in this study.

We were not able to identify sites (feedlots) where only endogenous fecal steroids would be in the runoff. That is, all the feedlots we identified used growth implants in their cattle. We had hoped to identify sites that had operations raising cattle without hormone supplements and searched extensively for such locations in the same region. All of the operations we identified that did not use hormone implants also did not raise cattle in a feedlot setting. These implant-free cattle are usually free-ranging cattle; that is, they are raised at low density on open rangelands. Future studies are needed to examine fish exposed to slurries of manure from treated and untreated animals. Given the recent publication documenting wide-scale contamination of U.S. water bodies with numerous pharmaceutical agents (Kolpin et al. 2002), future work—such as that presented in this study combined with intensive environmental chemistry—is urgently needed if we are to understand the possible adverse effects of these compounds on aquatic ecosystem health.

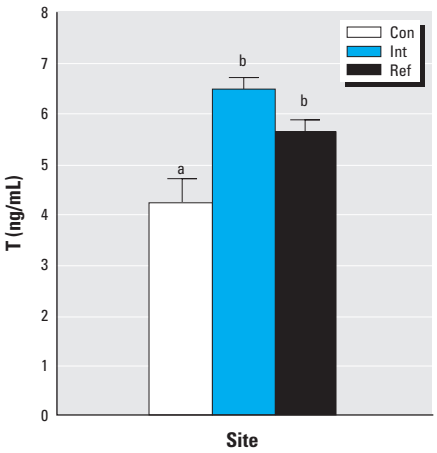

Figure 3. Mean (± 1 SE) *in vitro* T synthesis from testes obtained from fish from three Nebraska sites (p = 0.008). Abbreviations: Con, contaminated; Int, intermediate; Ref, reference. Values with different superscripts within a row of data are significantly different (p = 0.05); values in rows with no superscripts are not significantly different.

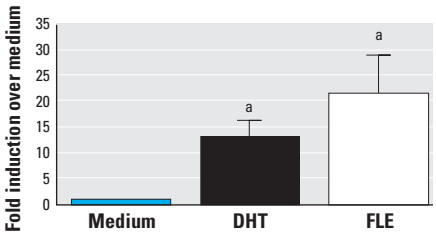

Figure 4. Fold induction of *in vitro* gene expression induced by FLE, DHT (1 nm) (mean ± 1 SE, n = 7), and medium control in CV-1 cells transfected with hAR and the MMTV-luciferase reporter.

[a]FLE and DHT fold induction values were not different from each other (p = 0.35), but FLE and DHT both induced greater gene expression compared with medium alone (p < 0.0001).

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