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

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There has been a great deal of research over the last decade examining 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). De masculinization 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 substances 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, hypotalamic–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]
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 progestogen melengestrol acetate are commonly used singly or combined with native steroid hormones, including T, E<sub>2</sub>, 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 methane-sulfonate (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).

**Gonadal culture 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),...
3-isobutyl-1-methykanthine (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 E2 and T in female FHMs and T in male FHMs was measured via radioimmunoassay on extracted culture media located immediately at the base of the feedlot. Culture medium samples were extracted twice with ethyl ether, vaporized under a stream of nitrogen, and then reconstituted in 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, 0.5 M borate buffer (pH 8.0), and 100 µL 3H-hormone (Amersham). A salting-out procedure with 100 µL of 5% charcoal/0.5% dextran/0.5 M phosphate-buffered saline (PBS) mixture to separate the bound from the 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 E2 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 E2 (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 upon arrival at the laboratory 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 NaHCO3 (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 pg 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 µL of medium that was made with water from the retention pond site and incubated at 37°C with 5% CO2. 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 µL 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.

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

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

<table>
<thead>
<tr>
<th>Site</th>
<th>Temperature (°C)</th>
<th>pH</th>
<th>DO (mg/mL)</th>
<th>Salinity (ppt)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Contaminated</td>
<td>24.8</td>
<td>7.88</td>
<td>2.37</td>
<td>0.8</td>
</tr>
<tr>
<td>Intermediate</td>
<td>23.3</td>
<td>NA</td>
<td>2.79</td>
<td>0.2</td>
</tr>
<tr>
<td>Reference</td>
<td>21.7</td>
<td>7.54</td>
<td>4.1</td>
<td>0.3</td>
</tr>
</tbody>
</table>

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.

<table>
<thead>
<tr>
<th>Measurement</th>
<th>Contaminated site (n = 23)</th>
<th>Intermediate site (n = 13)</th>
<th>Reference site (n = 19)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Length (mm)</td>
<td>5.36 ± 0.19</td>
<td>5.68 ± 0.11</td>
<td>5.68 ± 0.12</td>
</tr>
<tr>
<td>Soma mass (g)</td>
<td>2.23 ± 0.26</td>
<td>2.49 ± 0.15</td>
<td>2.46 ± 0.14</td>
</tr>
<tr>
<td>Gonad mass (g)</td>
<td>0.312 ± 0.005</td>
<td>0.418 ± 0.05</td>
<td>0.405 ± 0.03</td>
</tr>
<tr>
<td>Liver mass (g)</td>
<td>0.006 ± 0.000</td>
<td>0.065 ± 0.006</td>
<td>0.076 ± 0.007</td>
</tr>
<tr>
<td>IO distance (mm)</td>
<td>4.05 ± 0.18</td>
<td>4.24 ± 0.15</td>
<td>4.72 ± 0.13</td>
</tr>
<tr>
<td>HW (mm)</td>
<td>7.07 ± 0.24</td>
<td>7.22 ± 0.19</td>
<td>7.35 ± 0.17</td>
</tr>
</tbody>
</table>

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.
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. 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).

**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).
Approximately 27.5% of the initial concentration of growth implants have longer half-lives. 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 zearanol 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 vitro 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, E₂, and vitellogenin were all significantly decreased. In males, plasma concentrations of 11-ketotestosterone were decreased and E₂ 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 FHMs 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 IQ 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 E₂:Δ ratios caused by a decrease in ovarian E₂ 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 E₂ (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 remaining pond immediately below the feedlot. This site had very low DO levels (0.7 ppm) and relatively high salinity (1.2 ppm). 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.

**REFERENCES**


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