EFFECTS OF THE ANDROGENIC GROWTH PROMOTER 17-β-TRENBOLEONE ON FECUNDITY AND REPRODUCTIVE ENDOCRINOLOGY OF THE FATHEAD MINNOW

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Abstract—Trenbolone acetate is a synthetic steroid that is extensively used in the United States as a growth promoter in beef cattle. The acetate is administered to livestock via slow-release implants; some is converted by the animal to 17-β-trenbolone, a relatively potent androgen receptor agonist in mammalian systems. Recent studies indicate that excreted 17-β-trenbolone is comparatively stable in animal waste, suggesting the potential for exposure to aquatic animals via direct discharge, runoff, or both. However, little is known concerning the toxicity of trenbolone to fish. Our goal was to assess the effects of 17-β-trenbolone on reproductive endocrinology of the fathead minnow (Pimephales promelas). An in vitro competitive binding study with the fathead minnow androgen receptor demonstrated that 17-β-trenbolone had a higher affinity for the receptor than that of the endogenous ligand, testosterone. Male and female fish were exposed for 21 d to nominal (target) concentrations of 17-β-trenbolone ranging from 0.005 to 50 µg/L. Fecundity of the fish was significantly reduced by exposure to measured test concentrations ≥ 0.027 µg/L. The 17-β-trenbolone was clearly androgenic in vivo at these concentrations, as evidenced by the de novo production in females of dorsal (nuptial) tubercles, structures normally present only on the heads of mature males. Plasma steroid (testosterone and β-estradiol) and vitellogenin concentrations in the females all were significantly reduced by exposure to 17-β-trenbolone at concentrations ≥ 0.027 µg/L. The 17-β-trenbolone had a higher affinity for the receptor than that of the endogenous ligand, testosterone. Male and female fish were exposed for 21 d to nominal (target) concentrations of 17-β-trenbolone ranging from 0.005 to 50 µg/L. Fecundity of the fish was significantly reduced by exposure to measured test concentrations ≥ 0.027 µg/L. The 17-β-trenbolone was clearly androgenic in vivo at these concentrations, as evidenced by the de novo production in females of dorsal (nuptial) tubercles, structures normally present only on the heads of mature males. Plasma steroid (testosterone and β-estradiol) and vitellogenin concentrations in the females all were significantly reduced by exposure to 17-β-trenbolone at concentrations ≥ 0.027 µg/L. The 17-β-trenbolone had a higher affinity for the receptor than that of the endogenous ligand, testosterone.

INTRODUCTION

Alterations in reproductive physiology and endocrinology have been clearly documented in fish exposed to certain classes of contaminants and complex mixtures [1]. For example, studies conducted initially in the United Kingdom, and later in other countries throughout Europe and North America, have demonstrated that exposure of fish to some municipal effluents causes estrogenic responses [2–5]. Although many chemicals possibly could contribute to the estrogenicity of municipal effluents, the compounds that appear to be most closely associated with abnormal estrogenic responses in fish are natural and synthetic steroidal estrogens [6–8]. One chemical of particular concern is 17-α-ethyl estradiol (EE₂), a potent and stable estrogen receptor agonist utilized in human birth control formulations.

High-potency synthetic steroids are used intentionally for a variety of applications other than birth control, including as growth promoters for livestock production. One compound extensively utilized in the United States as an anabolic steroid for beef production is trenbolone acetate [9–11]. Trenbolone acetate is administered to livestock via controlled-release implants: upon entry to the blood, the acetate is rapidly hydrolyzed to 17-β-trenbolone, a potent agonist of the mammalian androgen receptor, with a binding affinity approximately equivalent to dihydrotestosterone [12–14]. An important metabolic conversion of 17-β-trenbolone in cattle is epimerization to 17-α-trenbolone, which has a binding affinity for the mammalian androgen receptor about 10-fold lower than 17-β-trenbolone [15,16]. Both stereoisomers are eliminated from cattle dosed with trenbolone acetate, with the 17-α-trenbolone comprising about 95% of the excreted product [17]. Schiffer et al. [17] also demonstrated that the half-lives of 17-α- and 17-β-trenbolone in liquid manure were on the order of 260 d. Further, in soil samples to which the manure had been applied, trenbolone residues were detectable for up to eight weeks. This suggests the potential for exposure of aquatic animals to trenbolone via runoff from feedlots and fields fertilized with manure. Recent studies have indicated that unidentified compo-
of the dorsal pad on the head, and the de novo formation of dorsal (nuptial) tubercles, both characteristics of reproductively active males [23]. To document these types of alterations in the present study, all fish were scored with respect to the occurrence of nuptial tubercles as they were processed. The scoring system considered both the number and relative size of tubercles [25]. In addition, heads from a subsample of female and male fish from the control, 0.5-, 0.5-, and 50-μg/L treatment groups were removed, preserved in the glutaraldehyde:formaldehyde solution, and subjected to histological analysis. A 2-mm-wide piece of dorsal pad (or dorsal skin) and the underlying musculature were dissected from the region above the opercula. Tissues were embedded in methylacrylate, sectioned at 2 to 3 μm, and stained with basic fuchsin and methylene blue-azure A.

Vitellogenin in plasma samples from the fish was measured by using an enzyme-linked immunosorbent assay and steroids (E$_2$, testosterone, and 11-ketotestosterone) were determined by using radioimmunoassay techniques [25,27,28].

A cell-based binding assay was used to determine the ability of 17-β-trenbolone and testosterone to compete with [³H]R1881 (NEN Research Products, Boston, MA, USA) for binding to the fathead minnow androgen receptor [14]. Monkey kidney cells (COS, American Type Culture Collection, Rockville, MD, USA) were plated at 200,000 cells/well and transiently transfected, by using diethylaminoethyl dextran, with 0.5 μg (per well) of a fathead minnow androgen receptor expression vector [29]. Twenty-four hours later, cells were exposed in duplicate to 0.5 nM [³H]R1881 in the presence of unlabeled 17-β-trenbolone (0.01–300 nM) or testosterone (0.3–10 μM), and incubated for 2 h at 37°C. Nonspecific binding was determined by adding a 100-fold molar excess of unlabeled R1881. After incubation, cells were washed with phosphate-buffered saline (pH 7.2) and lysed in 200 μl of ZAP (0.13 M ethyldimethylhexadecylammonium bromide with 3% glacial acetic acid). Radioactivity of the lysate was determined by liquid scintillation counting. Reported data are the mean of two replicate assays with two independent observations (wells) per experiment.

Chemical analyses

Concentrations of 17-β-trenbolone were determined in the stock solution and water samples from each exposure tank twice weekly during the 21-d assay. At the four highest concentrations, water (~1.5 ml) was collected and analyzed on the same day by high-pressure liquid chromatography (HPLC) with fluorescence detection. One-milliliter sample aliquots were directly injected onto an Alltech Adsorbsphere HS column (Deerfield, IL, USA) on an Agilent 1100 high-pressure liquid chromatograph, and chromatographed at 35°C by using a 3-min linear gradient of 50 to 70% methanol in water, followed by an isocratic 70% elution at a flow rate of 1 ml/min. The excitation and emission wavelengths were 359 and 458 nm, respectively. Under these conditions, 17-β-trenbolone eluted at 10 min. The analyte was quantified by using an external standard method. The mean (standard deviation [SD]) recovery of matrix-spiked samples was 91% (6.5%, n = 10). Agreement among duplicate samples was 99% (1.1%, n = 6). The limit of detection using the direct injection technique was approximately 0.02 μg/L.

To quantify 17-β-trenbolone in water from the 0.005-μg/L treatment, it was necessary to first concentrate the samples. Water collected from this treatment was stored in amber glass
vials at 4°C until analyzed. The samples (25–50 ml) were concentrated by using 3-ml C18 columns (J.T. Baker, Phillipsburg, NJ, USA), which were eluted with 2 ml of 70% acetonitrile in water. Aliquots (100 µl) of the eluted samples were injected onto the high-pressure liquid chromatograph and analyzed as described above. The mean (SD) recovery of samples analyzed when using the C18 concentration step was 87% (25.5%, n = 4). The method limit of detection was about 0.0012 µg/L.

Whole-body concentrations of 17-β-trenbolone in the six fish from one replicate tank at each test concentration were measured at conclusion of the 21-d assay. Whole fish (1.1–6.8 g) were extracted by homogenization (8,000 rpm, 1–2 min) in acetonitrile (15 ml). The slurry was centrifuged at 3,010 g for 20 min at −10°C, and the supernatant was concentrated under nitrogen to 5 ml. One hundred-microliter aliquots of the sample were analyzed via HPLC as described above. The mean (SD) recovery of 17-β-trenbolone from spiked tissue samples was 76% (3.3%, n = 4). The limit of detection was 0.7 µg/kg.

A subset of the tissue extracts also was analyzed to determine the possible presence of 17-α-trenbolone, a major trenbolone metabolite in cattle [17]. The 17-α-trenbolone standard (99.65% purity) was a gift from Aventis Pharma S.A. (Antony, France). Concentrations of 17-α-trenbolone were determined by using the same basic conditions as for 17-β-trenbolone, except the retention time for the former was slightly (~30 s) longer than the latter. The mean (SD) recovery of 17-α-trenbolone from spiked tissue samples was 72% (9.4%, n = 2). The detection limit for 17-α-trenbolone was 0.7 µg/kg.

Data analysis

Biological data from the 17-β-trenbolone exposures were transformed (log or square root), when necessary, for normalization and to reduce heterogeneity of variance [24]. Among-treatment differences (based on tank mean values) were determined by using analysis of variance followed by Dunnett’s test (for comparison of exposed animals to controls). When nonmonotonic concentration–response curves were observed, comparisons among all treatments also were assessed with Tukey’s test. Computations were performed with Systat 7.0 (SPSS, Chicago, IL, USA). Differences were considered significant at p ≤ 0.05.

RESULTS

Figure 1 summarizes data from the competitive binding experiments with the fathead minnow androgen receptor that had been transiently transfected into COS cells. The 17-β-trenbolone competed with 0.5 nM [3H]R1881 for binding to the receptor with a concentration inhibiting 50% binding (IC50) determined from a logistic fit of the data, of about 10 nM. To assess comparative affinity of 17-β-trenbolone for the fathead minnow androgen receptor, competitive binding assays with both inert R1881 and testosterone also were conducted. The IC50 values of inert R1881 and testosterone were 1 nM and about 97 nM, respectively, indicating that 17-β-trenbolone has a factor of 10 higher affinity for the fathead minnow androgen receptor than testosterone. The relative affinity of 17-β-trenbolone for the fathead minnow androgen receptor is quite similar to that reported by Wilson et al. [14] for the human androgen receptor transiently transfected into the COS cells (IC50 = 33 nM in competition with 1 nM [3H]R1881).

Fig. 1. 17-β-Trenbolone competitively inhibits 0.5 nM [3H]R1881 binding to the fathead minnow androgen receptor. For comparative purposes, competitive binding of inert R1881 and testosterone for the receptor also are displayed. Data represent the mean of two replicate assays with two independent observations per replicate.

Measured concentrations of 17-β-trenbolone in the fathead minnow test were approximately 80% of the target concentra-

trations in the 5- and 50-µg/L treatments, about 50% of nominal in the 0.05- and 0.5-µg/L treatments, and 30% of the target at the lowest test concentration (Table 1). Although measured values were lower than nominal, within a given treatment group the test concentrations were quite consistent across the six different sampling dates during the 21-d assay, indicating stable performance of the exposure system in terms of delivered dose (Table 1). Measured water concentrations of 17-β-trenbolone across the triplicate exposure tanks within the different treatment groups at any given sampling time also were very consistent, with an average relative percent difference of less than 8% for all treatments for the entire test (data not shown).

The fish (Table 1) accumulated small amounts of 17-β-trenbolone. The androgen was not detectable in animals from the 0.005- and 0.05-µg/L treatment groups. The average bioconcentration factor calculated across fish from the three higher treatment groups was on the order of 13. No 17-α-trenbolone was detected in the fish.

No treatment-related mortality was observed during the assay. Weight of males at the conclusion of the assay was about 10% lower in the 50-µg/L test group than in the control (data not shown). Conversely, the steroid produced a significant concentration-dependent increase in female weight (Fig. 2). This sex-dependent difference in weight gain is similar to what has been observed in rats treated with trenbolone acetate [30].

Fecundity of the fish was decreased by exposure to 17-β-trenbolone (Fig. 3). No spawning occurred in the three highest treatment groups within 1 d of initiation of exposure. Animals from the 0.05-µg/L treatment had a cumulative egg production during the entire test that was less than half of that observed in the control fish; interestingly, organisms in this group seemed to exhibit some recovery over the course of the test with respect to spawning (Fig. 3). No significant difference was found in fecundity between the control animals and those at the lowest 17-β-trenbolone concentration. No significant difference was found in either fertility or hatching success of eggs produced in the control, 0.005-, and 0.05-µg/L treatments (data not shown).
In the ovaries from the two 17-β-trenbolone-exposed females, the majority of corpora lutea were observed in stage 4.5, suggesting hyperproduction of sperm. However, many of the developing and atretic follicles in ovaries from animals in the 0.5- and 0.05-μg/L treatments also were at stage 5, but had a thinned germinal epithelium and greatly expanded, sperm-filled lumens, suggesting hyperproduction of sperm.

Histological evaluation of the control females indicated that the ovaries were at a mean stage of 4.5, spawning-ready [24,25]. The testes of fish from the 0.5-μg/L treatments also were at stage 5, but had a thinned germinal epithelium and greatly expanded, sperm-filled lumens, suggesting hyperproduction of sperm.

No significant differences were found in the gonadosomatic index in males or females across the different treatment groups (data not shown). However, marked histological alterations were found on the gonads of both sexes. Of the three treatments examined, testes of the controls and animals from the 0.05-μg/L groups had a similar appearance; they were at stage 5, spawning-ready [24,25]. The testes of fish from the 0.5-μg/L treatments also were at stage 5, but had a thinned germinal epithelium and greatly expanded, sperm-filled lumens, suggesting hyperproduction of sperm.

Histological evaluation of the control females indicated that the ovaries were at a mean stage of 4.5, spawning-ready and spawning-imminent [24,25]. Corpora lutea indicative of recent spawning were observed in two of the four control females. Ovaries from the two 17-β-trenbolone-exposed groups examined (0.05 and 0.5 μg/L) were at a mean stage of 3.75, and no corpora lutea were observed in either group. Preovulatory atretic follicles were observed in 90% of the ovaries from 17-β-trenbolone-exposed fish. Many of the developing and atretic follicles from the exposed animals, especially those from the 0.5-μg/L treatment, had an unusual appearance in that yolk deposition was reduced for their size (data not shown). The follicles in ovaries from animals in the 0.5-μg/L treatment also tended to undergo atresia at an earlier stage than the control and 0.05-μg/L groups. Overall, histological evidence in ovaries of animals exposed to the 17-β-trenbolone is consistent with decreased vitellogenin concentrations (see below), as well as impaired spawning.

Exposure to 17-β-trenbolone caused an obvious morphological masculinization of the female fathead minnows in terms of tubercle formation (Fig. 4a to c). A significant increase was found in the tubercle score of female fathead minnows from the four highest treatment groups compared to the control (Fig. 5). Many of the exposed females from the 0.05- and 50-μg/L treatment groups also exhibited an incipient dorsal pad that was histologically similar in many respects to that of breeding males. When compared to the dorsal skin of control females, the dorsal pad epithelium of males is thicker, and its loose connective tissue is much thicker (Fig. 6a and b). The dorsal skin epithelium of untreated females can be classified as stratified squamous (squamous epithelial cells), instead of the mucous cells occupying most of the surface epithelial layer present in males (Fig. 7a and b). In addition, the dorsal skin epithelium of untreated females can be classified as stratified squamous (squamous epithelial cells), instead of the mucous cells occupying most of the surface epithelial layer present in males (Fig. 7a and b). The dorsal pad–like structures induced in females at the lower 17-β-trenbolone concentrations did not have an epithelium similar to that of males (Fig. 7c); however, at the 50-μg/L treatment level, dorsal skin epithelium of the females resembled that of males, including the occurrence of abundant mucous cells (Fig. 7d).

![Fig. 2. Effects of 17-β-trenbolone on weight of female fathead minnows at the conclusion of a 21-d exposure. Data are expressed as mean (standard error, n = 3). Values denoted by asterisks differed significantly from the control.](image)

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*Indicated concentrations are the mean from triplicate exposure tanks for each treatment, except the 0.005-μg/L group on days 1, 12, and 15, which are values from one tank.

b Values are for six fish (two males and four females) from each treatment group, except for the 0.5-μg/L treatment (two males and three females).

Nominal (target) concentration (μg/L).

Mean (standard deviation).

ND = not detectable (<0.0012 μg/L for water; 0.7 μg/kg for tissue).

NS = no sample.
Plasma concentrations of 11-ketotestosterone were significantly decreased in males from the 50-µg/L treatment; testosterone concentrations also appeared to be depressed in animals from this group, but not significantly so (Fig. 8a and b). Plasma E₂ concentrations in males from the 50-µg/L group were significantly higher than in control males or animals from the four lower 17-β-trenbolone exposures, all of which were near the method limit of detection (Fig. 8c). In the females, plasma concentrations of testosterone and E₂ exhibited a U-shaped concentration–response relationship (Fig. 9a and b). Concentrations of both steroids were significantly decreased in females exposed to 17-β-trenbolone; however, this reduction was more pronounced in fish from the intermediate than the high treatment groups.

Vitellogenin concentrations in female fathead minnows were significantly reduced by exposure to 17-β-trenbolone; however, as was true for the plasma testosterone and E₂, the concentration–response curve for this was nonmonotonic (Fig. 9c). Vitellogenin concentrations in the 0.5- and 5-µg/L treatments were essentially nondetectable, whereas concentrations of the protein, although depressed, were easily detectable in females from the 50-µg/L group. Vitellogenin was not detectable in male fathead minnows from the control, 0.005-, 0.05-, or 0.5-µg/L treatment groups; however, an indication of a concentration-dependent induction of vitellogenin was found in males from the 5- and 50-µg/L treatments (Fig. 8d). Because of high among-fish variability, vitellogenin induction in the males from the 50-µg/L treatment was significant only at $p \leq 0.1$.

**DISCUSSION**

Development of synthetic chemicals for medicinal purposes, performance enhancement, or pest control focuses on high-potency materials that selectively interact only with species or biological pathways of concern. This is a logical strategy both from the standpoint of economics and minimization of risk to nontarget receptors. However, when biologically conserved pathways are a point of focus, it can be difficult to ensure that adverse effects will not be manifested in nontarget species. This can happen when exposures occur that have not been anticipated as part of existing risk assessment or registration processes. This scenario is exemplified by some chemicals broadly classified as environmental endocrine disruptors. For example, effects on endocrine function are not considered adverse when EE₂ is used in birth control formulations; however, when EE₂ is present in municipal effluents it is potent enough that, even at comparatively small concentrations (low ng/L), alterations in the reproductive endocrinology of fish may occur [2–8]. A similar situation could exist for 17-β-trenbolone, a high-potency androgen that is a metabolite of trenbolone acetate, which has been used extensively since the 1970s as a growth promoter in beef cattle [11,12,31]. The studies we con-
duct with the fathead minnow indicate that 17β-trenbolone binds with high affinity to the androgen receptor in vitro, and reduces fecundity and affects reproductive endocrine function in vivo at concentrations ≥ 27 ng/L. The fact that 17β-trenbolone appears to be a potent androgen in fish, coupled with recent reports that both 17β- and 17α-trenbolone are quite stable in animal wastes [17], indicate a need to assess the occurrence of these chemicals in aqueous environments. Further highlighting this need is a recent report concerning the occurrence of other types of steroidal materials in surface waters of the United States [32].

Although no reports have been published to date of measurements of 17α- or 17β-trenbolone in water (runoff or discharge) from feedlots, evidence exists for the presence of androgenic materials in aqueous wastes from cattle feeding facilities. Guillette et al. [19] (as described by the European Commission [20]) collected female fathead minnows from a site adjacent to a beef feedlot in Nebraska, USA, and reported that E2:testosterone ratios from in vitro incubations of ovarian tissue were indicative of masculinization. Although steroid profiles in female fathead minnows from the present study were significantly altered by 17β-trenbolone, direct comparison of our steroid data to those of Guillette et al. [19] is not possible because of methodological differences (i.e., a short-term analysis of steroid metabolism in vitro vs an integrated measurement of steroid concentrations in vivo). Guillette et al. [19] also found a significant alteration in head morphometrics (interocular distance) of female fathead minnows from their test

Fig. 6. (a) Section of dorsal pad from a spawning-ready control male. Note the thick stratified epithelium and the very thick layer of loose connective tissue overlying the dorsal musculature (M). (b) Dorsal skin, from the same region, of a spawning-ready control female. Note the thin epithelial and connective tissue layers. (c) Dorsal skin of a female from the 0.5-μg/L β-trenbolone treatment. The epithelial and connective tissue layers are thickened, suggesting development of an incipient dorsal pad. Scale bar = 190 μm.
Fig. 7. (a) Dorsal pad epithelium of a control male. The outer layers of the epithelium are almost exclusively mucous cells (arrow). (b) Dorsal skin epithelium of a control female from the same region. The epithelium is stratified squamous cells, with scattered large club (alarm substance) cells (arrowhead) and few mucous cells. (c) Dorsal skin epithelium of a female from the 0.5-μg/L β-trenbolone treatment is similar in structure to that of untreated females, but slightly thicker. (d) Dorsal skin epithelium of a female from the 50-μg/L β-trenbolone treatment. The surface layers of the epithelium contain abundant mucous cells and resemble the control male dorsal pad epithelium. Scale bar = 35 μm.
Toxicity of 17β-trenbolone to fish

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Fig. 9. Effects of 21-d exposure to β-trenbolone on fathead minnow female plasma concentrations of (a) testosterone (T), (b) β-estradiol (E2), and (c) vitellogenin. Data are expressed as mean (standard error, n = 3). Values denoted by asterisks differed significantly from the control. Lettering above the bars indicates similarities and differences across the treatments based on multiple comparisons among all the groups (Tukey’s test).

Fig. 8. Effects of 21-d exposure to β-trenbolone on fathead minnow male plasma concentrations of (a) testosterone (T), (b) 11-ketotestosterone (KT), (c) β-estradiol (E2), and (d) vitellogenin. Data are expressed as mean (standard error, n = 3). Values denoted by asterisks differed significantly from the control.

site; however, they did not report the occurrence of tubercles in the fish, or examine dorsal skin histologically. Significantly, water from the Nebraska site produced a strong androgenic response in an in vitro assay with monkey kidney (CV-1) cells that had been transiently cotransfected with human androgen receptor and luciferase reporter gene constructs [18,33]. Although examination of the data presented by Guillette et al. [19] and Gray et al. [18] suggests that a natural or synthetic androgen could have been present in the feedlot runoff, the possibility was not assessed through analytical measurements, again highlighting the need for this type of information.

In addition to extensive data concerning the overt effects
of trenbolone acetate on performance (weight gain, behavior, and so on) of livestock [11,12,32], a reasonable understanding exists of the basic toxicology of the chemical and some of its metabolites in mammalian models. For example, not only is 17-β-trenbolone an androgen receptor agonist, it is a glucocorticoid receptor antagonist and also binds to the progesterin receptor [14,16,34]. In mammals, 17-β-trenbolone activates androgen-dependent gene expression in vitro with a potency equivalent to or greater than dihydroxytestosterone, a potent natural androgen [14,16,34]. Androgenic effects have been demonstrated in vivo in several mammalian species exposed to trenbolone including rats, mice, rhesus monkeys, humans, pigs, and cattle [World Health Organization; (http://www.inchem.org/documents/jecfa/jecmono/v25je08.htm672)]. Specific in vivo responses vary, but include several typical of exposure to anabolic androgens including growth enhancement, masculinization of females, and alterations in accessory sex organs in males.

Compared to mammalian studies, there has only been limited work concerning the effects of trenbolone acetate or its metabolites on fish. Galvez et al. [21], Davis et al. [22], and Galvez and Morrison [35] assessed the effects of trenbolone acetate on channel catfish and tilapia from an aquacultural perspective. They found that administration of the acetate through the diet to swim-up channel catfish fry for 60 d produced populations of 100% phenotypic males at feed concentrations of ≥50 mg/kg [21]. Qualitatively similar results were obtained when blue tilapia fry received trenbolone acetate at ≥25 mg/kg diet for 28 d [35]. In further studies with the channel catfish, Davis et al. [22] found that the trenbolone acetate treatment regime used in their earlier study [21] did not produce functional males, in that the treated fish had decreased plasma testosterone concentrations at maturity and were infertile. We are aware of one study that attempted to assess the effects of 17-β-trenbolone on fish. Peterson et al. [36] exposed mature Japanese medaka to 17-β-trenbolone for 14 d at nominal water concentrations ranging from 0.002 to 2 μg/L. They found that concentrations ≥ 0.2 μg/L inhibited fecundity of the fish (C. Foran, West Virginia University, Morgantown, WV, USA, personal communication). Examination of concentration–response data from the present study suggests that the fathead minnow may be an order of magnitude more sensitive than medaka to the effects of 17-β-trenbolone on fecundity; however, this conclusion must be tempered by the fact that Peterson et al. [36] used a static-renewal (as opposed to a flow-through) test system, and did not measure concentrations of 17-β-trenbolone in their test system. Peterson et al. [36] also reported an inhibition of vitellogenin production in female medaka exposed to the higher 17-β-trenbolone concentrations, which is similar to one of the responses we observed in the fathead minnow. However, again, because of differences between the studies with respect to exposure systems and characterization, it is difficult to make quantitative across-study comparisons.

An interesting observation from this study was the occurrence of U-shaped concentration–response relationships for three parameters in the female fathead minnow: plasma concentrations of testosterone, E2, and vitellogenin. Although one result of this type might be considered spurious, observation of three very similar relationships within the reproductive endocrine system of the fish would not seem to be a chance occurrence. This is especially true when considering that the three measures can be plausibly linked to one another from a functional perspective. Specifically, testosterone is the metabolic precursor to E2 via conversion by CYP19 aromatase [37], and E2 is the primary signaling molecule for production of vitellogenin in female oviparous vertebrates, including fish [38]. A number of reports have been made that androgenic and antiandrogenic chemicals can display U-shaped dose responses in mammalian test systems (reviewed by Gray et al. [39]). In many cases these nonmonotonic responses result from separate mechanisms of action at different dosage levels. For example, two separate mechanisms of action appear to be involved in producing a U-shaped dose–response curve for the effects of testosterone on rat testis in vivo. At low doses, testosterone treatment initiates a cascade of effects including inhibition of pituitary secretion of luteinizing hormone, reduced testosterone production by Leydig cells, reduced intratesticular testosterone, and inhibition of sperm production and fertility. However, as the dose of administered testosterone is increased, serum concentrations of testosterone rise, followed by an increase in intratesticular testosterone and restoration of spermatogenesis and fertility [40,41]. The U-shaped dose responses observed in the female fathead minnow treated with 17-β-trenbolone possibly also result from different mechanisms of action at different test concentrations. As in the rat exposed to testosterone, female fathead minnow pituitary gonadotropin secretion may be reduced by 17-β-trenbolone exposure, resulting in ovarian dysgenesis in the low treatment groups, whereas at higher concentrations, the androgen acts directly on the ovary to restore steroidogenic activity. Alternatively, high concentrations of 17-β-trenbolone might downregulate androgen receptor in the pituitary, resulting in a loss of feedback inhibition of gonadotropin secretion and restoration of ovarian endocrine activity. Further investigation, including determination of the status of other hormonal signals in the hypothalamic–pituitary–gonadal axis, is required to determine the biological basis of the nonmonotonic concentration–response relationships observed in female fathead minnows exposed to 17-β-trenbolone.

An additional observation of interest from this study was the marked difference between males and females with respect to test concentrations that resulted in biological responses. Significant effects in males (changes in plasma concentrations of 11-ketotestosterone, E2, and vitellogenin) were consistently observed only in the 50-μg/L treatment group, whereas in the females, alterations in a number of parameters (tubercle production and plasma testosterone, E2, and vitellogenin) were seen at much lower 17-β-trenbolone concentrations. This marked sensitivity to 17-β-trenbolone may, in part, reflect the critical role that androgens play in the normal reproductive endocrinology of female fish [42].

A final point that should be noted in terms of concentration–response relationships generated for the females concerns identification of a no-effect concentration for 17-β-trenbolone. Neither reductions in fecundity nor masculinization of the females were observed in fish from the lowest treatment group (0.0015 μg/L, measured). However, consideration of the concentration–response curves for several of the endpoints in the females (body weight, testosterone, E2, and vitellogenin) suggests that subtle (albeit not significant) responses could have occurred even at the lowest 17-β-trenbolone concentration tested (Figs. 2 and 8a to c). If these changes were indeed real, this study did not effectively document a no-effect concentration for all the endpoints examined; this suggests that further
studies with fish and 17-ß-trenbolone may need to include test concentrations below 1 ng/L.

The fathead minnow reproduction assay utilized in this study originally was developed in response to a legislated mandate to the U.S. Environmental Protection Agency to develop a screening and testing program to identify chemicals with the potential to cause adverse reproductive effects through alterations in the hypothalamic–pituitary–gonad axis [23,26,43,44]. Much of the attention associated with this program has been on estrogen mimics; however, concern is increasing for environmental contaminants that have other endocrine-based modes of action, including chemicals that act as androgen mimics [33,45–47]. Hence, it is critical that methods developed to support the screening and testing program effectively identify androgenic chemicals. In initial studies, the synthetic steroid methyltestosterone was used as a model androgen to assess performance of the fathead minnow assay [23]. Methyltestosterone was androgenic in the fish (e.g., females were masculinized); however, the chemical also caused at least one response (vitellogenin induction in males) indicative of an estrogen receptor agonist. This appears to be due to the fact that the methyltestosterone was aromatized to methylestradiol by the fish [48], thus giving the appearance of a chemical with a mixed mode of action. Because 17-ß-trenbolone is not an aromatizable androgen [49], we did not anticipate this type of paradoxical response. But, vitellogenin induction was observed in males exposed to comparatively high concentrations of the chemical. At least two explanations are possible for this response: Plasma E2 in the males was elevated sufficiently to induce vitellogenin or 17-ß-trenbolone directly activated the estrogen receptor of the fish thereby resulting in vitellogenin production. Precedent exists for the latter explanation; Le Guevel and Pakdel [50] reported that 17-ß-trenbolone had weak activity in a recombinant yeast reporter gene system expressing rainbow trout estrogen receptor. In addition, recent studies from our laboratory have shown that 17-ß-trenbolone exhibits specific, low-affinity binding to the fathead minnow estrogen receptor (unpublished data).

In summary, 17-ß-trenbolone is a potent androgen in fish, producing masculinization of females and reducing fecundity at a measured concentration of 0.027 µg/L in a 21-d exposure with the fathead minnow. Given the extensive use of trenbolone acetate in certain types of livestock feeding operations, as well as the relative persistence of its androgenic metabolites, additional studies are warranted to ascertain potential ecological risk.

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