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Characterization of responses to the antiandrogen flutamide in a short-term reproduction assay with the fathead minnow☆

Kathleen M. Jensen[a,*], Michael D. Kahl[a], Elizabeth A. Makynen[a], Joseph J. Korte[a], Richard L. Leino[b], Brian C. Butterworth[a], Gerald T. Ankley[a]

[a] *U.S. Environmental Protection Agency, Office of Research and Development, National Health and Environmental Effects Research Laboratory, Mid-Continent Ecology Division, 6201 Congdon Boulevard, Duluth, MN 55804 USA*

[b] *Department of Anatomy and Cell Biology, School of Medicine, University of Minnesota Duluth, 10 University Drive, Duluth, MN 55812 USA*

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Abstract

A short-term reproduction assay with the fathead minnow (*Pimephales promelas*) has been developed to detect chemicals with the potential to disrupt reproductive endocrine function controlled by estrogen- and androgen-mediated pathways. The objective of this study was to use the assay to characterize responses of fathead minnow reproductive endocrinology and physiology to the mammalian antiandrogen, flutamide. Male and female fish were exposed to nominal (target) concentrations of 50 and 500 μg flutamide/l for 21-days, following which plasma steroid and vitellogenin concentrations were determined and gonadal morphology assessed. Fecundity of the fish was significantly reduced by exposure to a measured test concentration of 651 μg flutamide/l. In addition, embryo hatch was significantly reduced at this concentration. Qualitative histological assessment of ovaries from females exposed to flutamide indicated a decrease in mature oocytes and an increase in atretic follicles. Testes of males exposed to flutamide exhibited spermatocyte degeneration and necrosis. Concentration-dependent increases in plasma testosterone and vitellogenin concentrations were observed in the females. Flutamide also altered reproductive endocrinology of male fathead minnows. Males exposed to 651 μg flutamide/l exhibited elevated concentrations of β-estradiol and vitellogenin. In summary, the results of this study with the fathead minnow demonstrate that flutamide affects reproductive endocrine function in fish and that the type of hormonal pattern and histopathology effects observed are consistent with an antiandrogenic mode-of-action. Consequently, our findings suggest that the 21-day reproduction assay utilizing fathead minnows is a sensitive short-term screening method for the detection of endocrine-disrupting chemicals, including antiandrogens.
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Keywords: Flutamide; Fathead minnow; Antiandrogen; Endocrine disruption; Reproduction

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* Corresponding author. Tel.: +1 218 529 5177; fax: +1 218 529 5003.
E-mail address: jensen.kathleen@epa.gov (K.M. Jensen)

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1. Introduction

Regulatory organizations throughout the world are implementing screening and testing programs for chemicals that may adversely affect endocrine function in humans and wildlife. Emphasis of the programs to date has been on reproductive endocrine function, in particular, pathways and endpoints controlled by estrogens and androgens (U.S. EPA, 1998; OECD, 1999, 2000). A number of in vitro and in vivo tests have been developed to identify these classes of endocrine-disrupting chemicals (EDCs); although differences exist among different regulatory organizations, all incorporate assays with fish as part of the EDC testing suite. In the U.S., a 21-day reproduction assay with the fathead minnow (*Pimephales promelas*) has been developed for Tier 1 screening (U.S. EPA, 1998, 2002; Ankley et al., 2001). This test has been proposed as a part of a harmonized EDC screening and testing program for member countries of the Office of Economic Cooperation and Development (U.S. EPA, 2002; OECD, 1999, 2000; Huet, 2000).

The short-term fathead minnow reproduction assay is initiated with sexually-mature fish with a documented history of successful spawning (Ankley et al., 2001; U.S. EPA, 2002). Over the course of a 21-day chemical exposure, fecundity and embryo viability (fertility, hatching success) are monitored. At conclusion of the assay, evaluations of secondary sexual characteristics are made, and gonadal status is assessed via determination of the gonadosomatic index (GSI) and by histological analyses. In addition, concentrations of vitellogenin and plasma sex steroids (β-estradiol; testosterone; 11-ketotestosterone) are determined. Based on tests with known EDCs, the assay appears to be sensitive, as well as discriminatory for identifying chemicals that affect pathways involving estrogens and androgens (U.S. EPA, 2002). For example, the test described by Ankley et al. (2001), as well as relatively similar variants of the assay, respond in a reliable and predictable manner to both weak and strong agonists of the estrogen receptor (Kramer et al., 1998; Panter et al., 1998; Miles-Richardson et al., 1999a, b; Harries et al., 2000; Korte et al., 2000). Similarly, the test effectively identifies androgen receptor agonists (Ankley et al., 2001, 2003), as well as chemicals that affect steroid metabolism (Ankley et al., 2002).

Antiandrogens (generally androgen receptor antagonists) represent a group of potentially important environmental EDCs (Kelce and Wilson, 1997; Monosson et al., 1997; U.S. EPA, 1998). In mammals, antiandrogens affect aspects of sexual differentiation and development, causing feminization and demasculinization of male offspring dosed in utero during sensitive developmental stages (Gray et al., 1994, 1999; Kelce et al., 1994). There are indications that antiandrogenic chemicals also can affect sexual differentiation in some species of fish exposed during early life stages (Koger et al., 1999; Bayley et al., 2002). However, effects of exposure to antiandrogens are less well characterized in adult animals, including fish (Monosson et al., 1997). Baatrup and Junge (2001) found that three mammalian antiandrogens, vinclozolin, *p,p'*-DDE and flutamide, altered the sexual characteristics of the adult male guppy (*Poecilia reticulata*). Makynen et al. (2000) exposed sexually-mature fathead minnows to vinclozolin and found that the chemical (or its antiandrogenic metabolites; Kelce et al., 1994) affected gonadal condition (GSI, histology) of the females. However, Makynen et al. (2000) did not assess the full suite of endpoints subsequently recommended by Ankley et al. (2001) for EDC testing in the fathead minnow. A further uncertainty relative to characterizing vinclozolin as an antiandrogen in the fathead minnow reproduction assay was that Makynen et al. (2000) found little, if any, competitive binding affinity of vinclozolin or its metabolites to the fathead minnow androgen receptor at the concentrations tested.

The objective of this study was to evaluate the effects of another antiandrogen, flutamide, on reproductive endocrinology of the adult fathead minnow using the complete protocol described by Ankley et al. (2001). Both flutamide and its 2-hydroxyflutamide metabolite are antagonists of the mammalian androgen receptor, and commonly used as 'model' antiandrogens for EDC testing in mammalian systems (U.S. EPA, 1998). Our previous work has shown that, as opposed to vinclozolin and associated metabolites, flutamide does bind to the androgen receptor of the fathead minnow (Makynen et al., 2000; Ankley et al., 2004).

2. Materials and Methods

2.1. Experimental conditions

The flutamide used for these studies was reported as 99% pure (Sigma, St. Louis, MO, USA). A chemical 'saturator' was prepared by dissolving 3.5 g of flutamide in 40 ml of acetone, which was evenly dried on the interior of an 18 l carboy. Stock concentrations of flutamide were generated at about 21 mg/l with an 8 ml/min flow of filtered (0.5 μm) Lake Superior water through the carboy. Three coated vessels supplied a usable stock for the 21 days of chemical exposure. Target flutamide concentrations in the test system were generated by combining solution from the saturator with clean (Lake Superior) dilution water. The test tanks (40 cm × 20 cm × 25 cm; L × W × H) contained approximately 10 l of test solution, which was renewed at a flow rate of about 45 ml/min. The fish were maintained at 25 ± 1 °C under a 16:8 h L:D photoperiod and fed adult brine shrimp (San Francisco Bay Brand, Newark, CA, USA) twice daily. The mean (range) water quality characteristics during the test were pH, 7.48 (7.20–7.68); hardness, 41.7 (41.0–42.0) mg/l as $CaCO_3$; alkalinity, 37.7 (37.0–38.0) mg/l as $CaCO_3$; and dissolved oxygen, 6.6 (5.0–7.8) mg/l.

2.2. Experimental design

Exposures were conducted using the general experimental design and test methods described in detail by Ankley et al. (2001) and the U.S. EPA (2002). Briefly, each tank contained four female and two male adult fathead minnows from an on-site culture at the Duluth EPA laboratory. Animals (six months old) exhibiting sexual dimorphism but not yet actively spawning were placed in the test tanks to begin spawning. The fish were monitored for two weeks prior to initiation of chemical exposure to document reproductive success. Based on the results of an initial range-finding study (data not shown), the 21-day reproduction test was conducted at target flutamide concentrations of 50 and 500 μg/l. There were three replicate tanks at each flutamide treatment level, plus three clean-water control tanks.

Fish in the test tanks were visually examined daily to assess survival, general appearance (including secondary sex characteristics), reproductive behavior, and spawning activity. Eggs were removed from the spawning substrate, counted, and examined microscopically to determine fertility. Embryos (n = 50 when possible) were selected from a subset of randomly chosen spawns (n = 10–16/treatment) and maintained in clean water for five days to determine hatching success.

After 21 days of flutamide exposure, fish were removed from the test tanks, anesthetized with MS-222 (100 mg/l buffered with 200 mg $NaHCO_3$/l), and blood was collected from the caudal artery/vein with a heparinized microhematocrit tube. Plasma was isolated by centrifugation and stored with aprotonin (0.13 units) at −80 °C until determination of vitellogenin and sex steroids. Fish and gonads were weighed for determination of GSI, and one gonad from each fish was preserved in 1% glutaraldehyde/4% formaldehyde in 0.1 M phosphate buffer for histological analysis. Heads from the male fish were removed and preserved in 10% neutral buffered formalin for assessment of nuptial tubercles, which were scored with respect to the number and relative size of the tubercles (Jensen et al., 2001).

For histological examination of the gonads, tissues from randomly selected fish (three males and five females/treatment) were embedded in methacrylate, sectioned at 2–3 μm in a step-wise fashion, and stained with hematoxylin and eosin. For each ovary, three slides were made with one section from 500 μm deep into the organ and two sections from 1000 μm deep. Testes were sectioned in a similar manner, except that the sections were taken at 250 and 500 μm depths. The gonads were evaluated with no knowledge of the exposure group and conducted as described elsewhere (U.S. EPA, 2002). Briefly, ovarian maturity was evaluated with respect to oocytes in the following stages: (1) primary growth, (2) cortical alveolus, (3) early vitellogenic, (4) late vitellogenic, and (5) mature spawning oocyte. In addition to assessing overall stage of the ovary, the numbers of early-stage (1, 2) follicles per 10× field and atretic follicles per section were determined. Testicular staging was based on the degree of germ cell differentiation and relative size and sperm content of the seminiferous tubules: (1) resting germ cells, (2) spermatogonia, (3) spermatocytes, (4) spermatids and some spermatozoa in lumen of seminiferous tubules with small tubule lumen, and (5) abundant sperm in lumen of seminiferous tubules with expanded lumina (U.S. EPA, 2002).

Plasma vitellogenin concentrations were determined using an enzyme-linked immunosorbent assay

(ELISA) with a polyclonal antibody to fathead minnow vitellogenin, and purified fathead minnow vitellogenin as a standard (Parks et al., 1999; Korte et al., 2000; Ankley et al., 2001; U.S. EPA, 2002). Plasma β-estradiol (E2) and testosterone (T) in both sexes and 11-ketotestosterone (KT) in males were measured using radioimmunoassay (RIA) techniques (Ankley et al., 2001; Jensen et al., 2001; U.S. EPA, 2002).

2.3. Analytical methods

Concentrations of flutamide were determined in the stock solution and water samples from each exposure tank twice weekly during the 21-day assay. Water samples were analyzed by direct injection onto a Hewlett-Packard 1050 high-pressure liquid chromatograph (HPLC) equipped with a diode-array detector (at 310 nm). A gradient program with a mobile phase starting at 40% acetonitrile, 20% methanol, and 40% water; and increasing to 80% acetonitrile, and 10% methanol and water (each) at a flow-rate of 1.0 ml per min was used, with an Alltech Nucleosil C18 AB 4.6 mm × 250 mm column (Deerfield, IL, USA). The external standard method of quantitation was used.

After removing tissue (plasma, gonad) samples, the remaining carcass of the fish was wrapped in foil, put into plastic zip-lock bags and frozen at −20 °C until extraction. Acetonitrile (15 ml) was used for extraction using an Ultra-Turrax T25 tissue homogenizer (Janke and Kunkel, Germany) at 8000 rpm. Approximately 3 g of each homogenate was removed and used for lipid analysis. The remaining mixture was transferred to a conical polypropylene centrifuge tube and centrifuged at 3010 × *g* for 20 min at −5 °C. Supernatant was concentrated under nitrogen to a final volume of 10 ml and stored at −20 °C until analysis. Acetonitrile tissue extracts were diluted with equal portions of HPLC grade water before injection. Analysis was by HPLC using the same method as described for water samples.

A subset of the tissue extracts, one male and one female fish from each treatment, was analyzed for 2-hydroxyflutamide using a Finnigan-MAT TSQ700 mass spectrometer and Varian 3400 gas chromatograph (GC/MS). Analyses were done by multiple-ion detection, monitoring mass to charge ratios of 216.0 and 292.1 for hydroxyflutamide and 311.9 for the internal standard dibromobiphenyl. The presence of hydroxyflutamide was confirmed using ion ratios with the normally accepted criteria of ±20% of the mean of the standards.

Total lipid content was determined microgravimetrically using a slight modification of the method described by Radin (1981). A 3:2 hexane/isopropanol mixture was added to the homogenized acetonitrile-fish slurry and mixed using a wrist action shaker followed by centrifugation and washing of the supernatant with warm sodium sulfate solution.

2.4. Data analyses

An unexplained shift in the E2 standard curve occurred while analyzing one set of female plasma samples by RIA. Since it was impossible to reanalyze the samples (due to an insufficient plasma volume), the E2 values from these fish were omitted from subsequent statistical analyses. Consequently, the reported E2 results are based on plasma samples from only a subset of fish (4–5/treatment) used in the experiment.

Differences between treatments at conclusion of the test were assessed with ANOVA followed by Dunnett's procedure (U.S. EPA, 2002). When necessary, data were transformed for normalization and/or to reduce variance heterogeneity. Analyses were performed using SYSTAT 9 (SPSS, Chicago, IL). Results were considered significant at $P \leq 0.05$. All data are presented as mean (±S.E.) of the three replicate exposure tanks unless otherwise noted.

3. Results

3.1. Water and tissue analyses

Water concentrations of flutamide were stable and relatively close to the target values of 50 and 500 μg/l; the respective mean (±S.E., $n = 6$) measured concentrations were 62.7 ± 5.9 and 651 ± 45.0 μg/l over the course of the 21-day test (Table 1). Mean water concentrations in the triplicate tanks at each flutamide treatment were similar, with average coefficients of variation (averaged across the duration of the test) of 5.5 and 3.4%, respectively, for the low and high treatments. There was no detectable flutamide in the control water.

There was a slight accumulation of flutamide by the fish (Table 1), with bio-concentration factors (based on

Table 1
Summary of water and tissue concentrations from a 21-day fathead minnow exposure to flutamide

Treatment[a]	Flutamide water concentration (μg/l)[b]							Flutamide tissue concentration[c]			
	Day of exposure							Wet weight (ng/g)		Lipid normalized (ng/mg lipid)[d]	
	1	5	8	13	15	20	Mean (S.E.)	Male	Female	Male	Female
Control	ND[e]	ND	ND	ND	ND	ND	–	ND	ND	ND	ND
50	61.7	48.9	43.2	82.2	70.3	69.8	62.7 (5.9)	804.5 (127.8)	1373 (240.4)	59.1 (8.5)	55.4 (16.2)
500	822	623	505	730	616	608	651 (45.0)	11,050 (1540)	15,362 (1863)	623 (30.0)	450 (72.6)

[a] Nominal (target) concentration (μg/l).
[b] Water concentrations are the mean from triplicate exposure tanks for each treatment.
[c] Values represent the mean (S.E.) of 4 fish from each treatment group.
[d] Mean (S.E.) lipid concentrations in males and females (across treatments) were 1.48 (0.14) and 3.14 (0.27)%, respectively.
[e] ND; not detectable (35 μg/l for water; 14–24 ng/mg lipid for tissue depending on fish weight).

wet weight) ranging from about 13 (in males from the low treatment) to 24 (females from the high treatment). On a wet weight basis, males accumulated concentrations of flutamide approximately 60–70% of that in females; however, this difference between the sexes was reduced through lipid normalization, especially in the low flutamide treatment (Table 1). It was possible to confirm the presence of 2-hydroxyflutamide in two of the tissue extracts; a male from the low concentration and a female from the high concentration contained 79.2 and 893 ng hydroxyflutamide/g fish, respectively (4–18% the concentration of flutamide).

3.2. Biological endpoints

No treatment-related mortality was observed during the assay. There were no significant treatment-related differences in male or female weight, nor were there any discernable alterations in external morphology, including secondary sexual characteristics of fish exposed to flutamide. Mean tubercle scores ranged from 35.8 ± 2.5 to 39.8 ± 2.0 in the control and high treatments, respectively.

There were significant effects of flutamide on fecundity of the fathead minnows (Fig. 1). Mean fecundity of

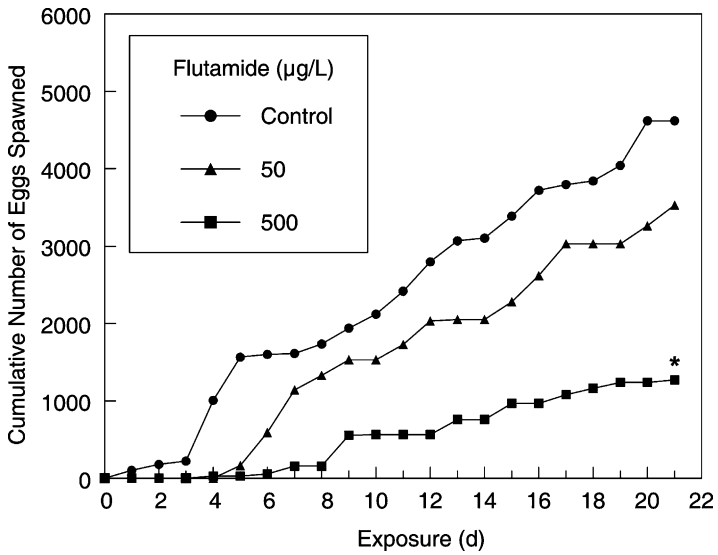

Fig. 1. Cumulative fecundity of fathead minnows exposed to flutamide for 21 days. Each line represents the cumulative fecundity of three replicates at each of two flutamide concentrations and the control. Asterisk (*) indicates value significantly different from control ($P \leq 0.05$).

the fish over the 21-day test was 18.3 ± 4.6, 14.0 ± 3.8, and 5.0 ± 1.4 eggs/female/day in the control, low, and high treatments, respectively. The decrease in fecundity was related both to a significant reduction in the mean number of spawns/female in the high treatment: 3.2 ± 0.2, 2.2 ± 0.3, and 1.4 ± 0.3, respectively, in the control, low, and high treatments, and to a decrease in mean number of eggs per spawn (120.3 ± 27.1, 148.2 ± 50.3, and 73.1 ± 13.8 in the control, low, and high treatments, respectively). Fertility ranged from 94 to 100% and was not affected by treatment; however, there was a significant decrease in mean embryo hatch observed in the high treatment (60.6 ± 1.3%) compared to the control (94.0 ± 3.0%). Hatching success in the low flutamide treatment (88.6 ± 5.8%) was not significantly affected.

No significant effects of flutamide on either the male or female GSI were observed (male GSI was 1.70 ± 0.05, 1.72 ± 0.08, and 1.81 ± 0.32 in the control, low, and high treatments, respectively and female GSI was 12.0 ± 1.06, 12.4 ± 0.63, and 11.1 ± 0.83 in the control, low, and high treatments, respectively). There were, however, histological alterations in the gonads of both sexes. There were no differences in testicular stage between the control and low treatment; all were at stage 5 with abundant spermatozoa in an expanded lumen. One testis of a fish from the high treatment was classified as less mature, stage 4, with some spermatozoa in a small lumen. The testes of two males from both flutamide treatment levels had small numbers of cells with pycnotic nuclei located in the seminiferous tubule epithelium. No pycnotic cells were seen in the control group. The exact identity of these cells was not determined, but they appeared to be aborted spermatogonia or sperm precursor cells (Fig. 2). Histological evaluation of the ovaries indicated that there were

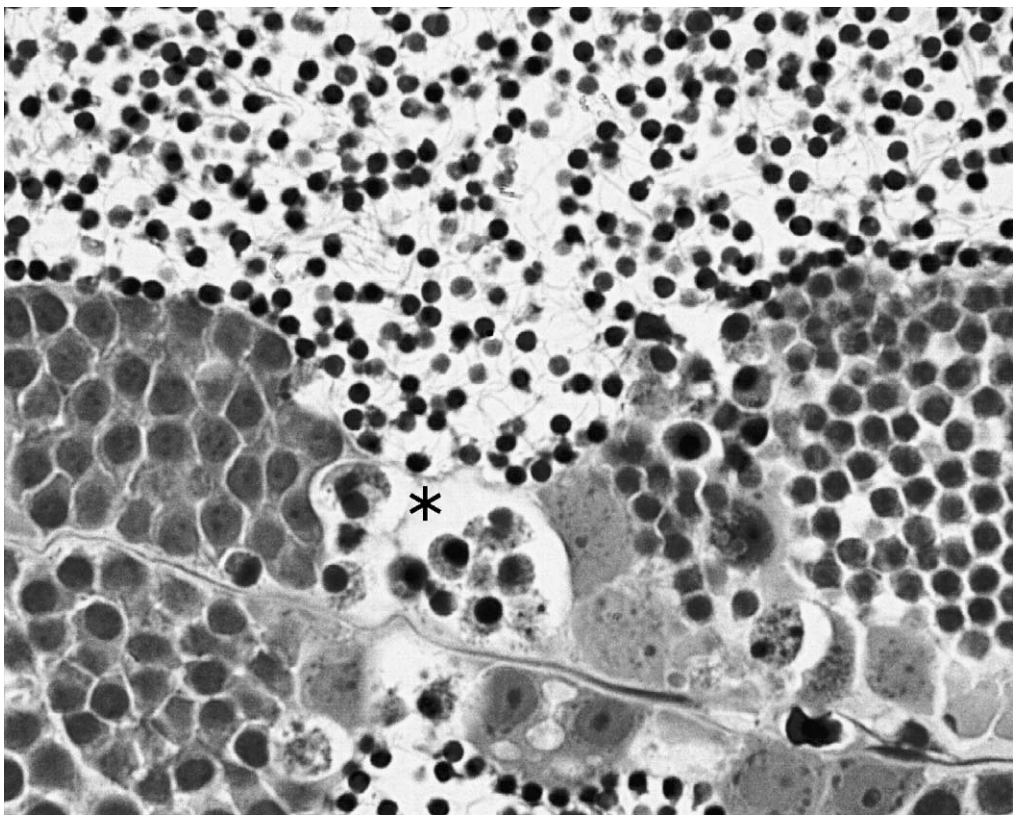

Fig. 2. Section of seminiferous tubule from a male exposed to flutamide (500 μg/l) showing clusters of pycnotic and degenerating cells (e.g., near asterisk) among healthy-appearing cysts (1580×).

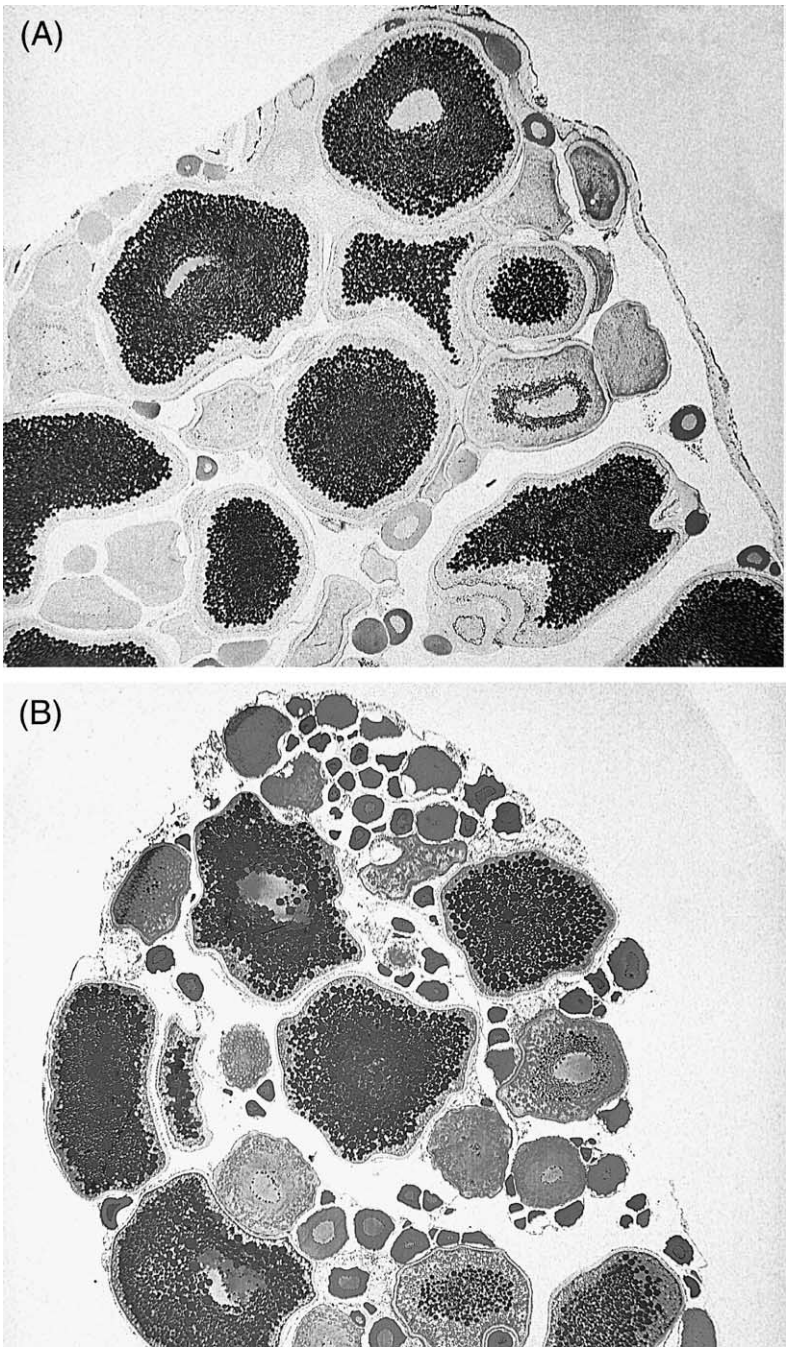

Fig. 3. (A) Section of control ovary showing mature follicles typical of an actively spawning female. (B) Section of ovary from female exposed to flutamide (500 μg/l) showing oocyte atresia and relatively greater numbers of early-stage follicles compared to the control (50×).

no differences in ovarian stage among the control and flutamide-exposed fish. Most ovaries were classified as either stage 4 or 5, spawning ready. There was, however, an increase in the number of early-stage follicles observed in females from the high flutamide treatment group compared to the control (Fig. 3); the mean number of these follicles in sections from the control, low, and high treatments was 29 ± 3.8, 28 ± 5.2, and 36 ± 7.2, respectively. In addition, an increase in oocyte atresia was observed in females from the high treatment compared to the control (Fig. 3); the mean number of atretic follicles in sections from the control, low, and high treatments was 40 ± 10.8, 35 ± 15.8, and 66 ± 22.7, respectively.

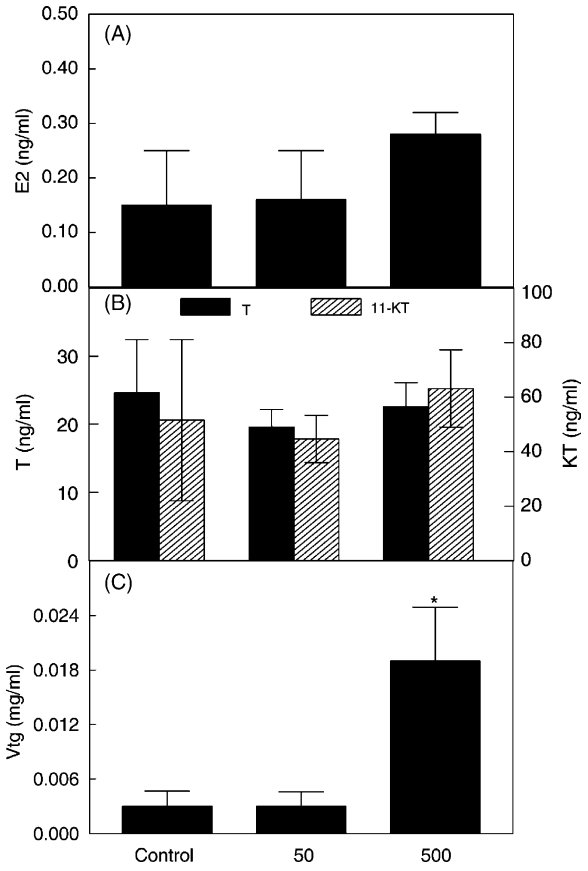

Fig. 4. Plasma concentrations of (A) β-estradiol (E2), (B) testosterone (T) and 11-ketotestosterone (KT), and (C) vitellogenin (Vtg) in male fathead minnows exposed to flutamide for 21 days. Data represent the mean (S.E.) of three replicate tanks at each flutamide concentration (μg/l) and the control. Asterisks (*) indicate values that are significantly different from control ($P \leq 0.05$).

A slight, but not significant, increase in plasma E2 was observed in males exposed to the high flutamide concentration (Fig. 4A). Male plasma concentrations of T and KT were not significantly affected by exposure to flutamide (Fig. 4B). There was, however, a significant increase in the ratio of KT to T in fish exposed to the high treatment. Vitellogenin concentrations were very low or non-detectable in male fathead minnows from the control and low treatments; however, there was a significant increase in males from the high treatment (Fig. 4C). In the females, plasma concentrations of E2

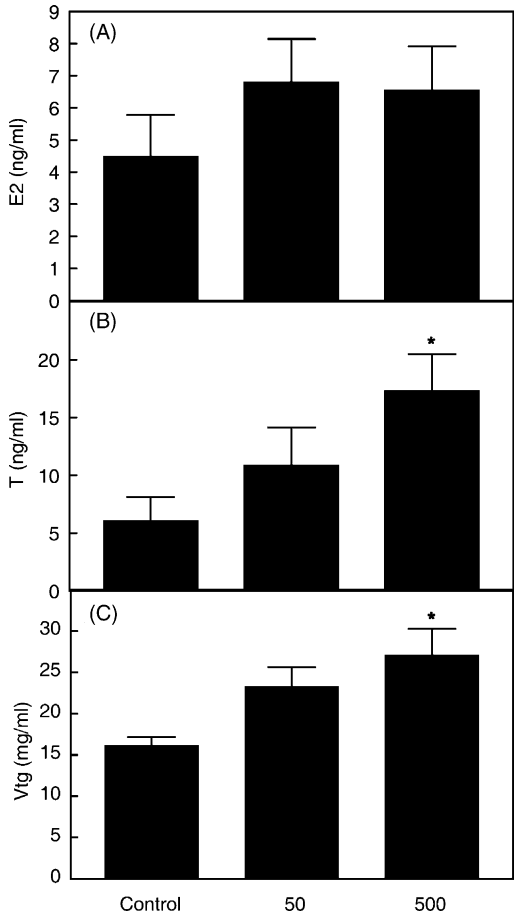

Fig. 5. Plasma concentrations of (A) β-estradiol (E2), (B) testosterone (T), and (C) vitellogenin (Vtg) in female fathead minnows exposed to flutamide for 21 days. Data represent the mean (S.E.) of three replicate tanks at each flutamide concentration (μg/l) and the control (except for E2 where the data represent the mean of 4–5 fish at each treatment). Asterisks (*) indicate values that are significantly different from control ($P \leq 0.05$).

appeared to increase with exposure to flutamide, but the small sample size and variability precluded statistical significance in this endpoint (Fig. 5A). Concentration-dependent increases in plasma T and vitellogenin concentrations were observed in the females (Fig. 5B and C).

4. Discussion

The objective of this study was to evaluate effects of a model antiandrogen, flutamide, on reproductive biology of the fathead minnow using a short-term assay specifically designed to detect chemicals which affect pathways controlled by estrogens and/or androgens (Ankley et al., 2001; U.S. EPA, 2002). Exposure to flutamide for 21 days significantly reduced fecundity of the fish and subtly affected different aspects of reproductive endocrine function in both males and females. For example, plasma steroid and vitellogenin concentrations were affected in both sexes. At the tissue level, there were effects of flutamide exposure on gonadal histology, characterized by spermatocyte degeneration and necrosis in the males and retarded oocyte maturation accompanied by oocyte atresia in the females. Based on these results, we conclude that reproductive endocrine function in the fathead minnow is affected by chemicals identified as antiandrogens in mammalian models. This lends further support to the utility of the short-term fathead minnow reproduction test as a method for effectively identifying EDCs.

In previous work, both in our lab and elsewhere, a model system based on reproductively-active fathead minnows has proven to be very useful in terms of identifying certain groups of EDCs. For example, both estrogen and androgen receptor agonists adversely affect fecundity of the fathead minnow, and the two different groups of chemicals are easily differentiated in that the former strongly induces vitellogenin in males (Kramer et al., 1998; Harries et al., 2000; Korte et al., 2000) and the latter masculinizes females (Ankley et al., 2001, 2003). These types of diagnostic responses are comparatively easy to interpret (or, even, anticipate) relative to current knowledge concerning the reproductive endocrinology of fish. One of the challenges in interpreting results of the current study, however, was the difficulty in speculating what an antiandrogen should look like in terms of effects on fish. The complete role of androgens (and the androgen receptor) in reproductively-active fish (particularly females) is not well defined (Borg, 1994), so possible effects associated with antagonist(s) of the system are uncertain. There have been limited studies with adult fish that are suggestive as to the effects of antiandrogens on spawning behavior and reproductive endocrinology (Makynen et al., 2000; Baatrup and Junge, 2001). Some also have assessed the impact of antiandrogens, including flutamide, on sexual differentiation of fish exposed during early development (Koger et al., 1999; Bayley et al., 2002). Results of these studies are qualitatively quite similar to responses observed in rats exposed in utero to antiandrogens, where effects include feminization and demasculinization of male offspring (Gray et al., 1994, 1999). This suggests a common mechanism of action for flutamide (and probably other antiandrogens) in developing mammals and fish. Also, flutamide, administered with methyltestosterone, appeared to inhibit the effects of the androgen by decreasing epidermal thickness in juvenile rainbow trout (Sower et al., 1983). In more recent work, Ankley et al. (2004) found that flutamide effectively blocked masculinization of females exposed to β-trenbolone, a potent androgen receptor agonist in fish (Ankley et al., 2003), thus providing direct in vivo evidence that flutamide is an antiandrogen in the fathead minnow. Based on this evidence, we feel that at least some of the effects observed in the flutamide-exposed fish in our study were likely mediated through antagonism of the androgen receptor.

The 21-day fathead minnow reproduction study with flutamide considered the full range of endpoints recommended by Ankley et al. (2001) as suitable for testing (anti-) estrogenic/androgenic EDCs. Our previous work with the mammalian antiandrogen vinclozolin also included a 21-day exposure of reproductively-mature fathead minnows (Makynen et al., 2000). Because the vinclozolin experiment did not assess some of the endpoints measured in the flutamide study (e.g., plasma vitellogenin), a comprehensive comparison of the two experiments is not possible. But there are some notable similarities in their results. Although not statistically significant in the flutamide study, an increase in plasma E2 concentrations of about the same magnitude (ca. 80–100%) was observed in males from both experiments. A similar increase in female plasma E2 concentrations was observed, though lack of statistical significance may have been the result

of small sample size in both studies. Plasma T concentrations in the females were also affected; a three-fold increase was observed in females exposed to 500 μg flutamide/l and a slight, but not significant, increase of 25% was observed in females exposed to 700 μg vinclozolin/l. Both antiandrogens affected ovarian condition; there was a significant decrease in GSI of females exposed to 700 μg vinclozolin/l (Makynen et al., 2000), and observed histopathological alterations in fish from that treatment group were similar to those observed in females from the high (500 μg/l) flutamide treatment.

The hormonal patterns observed in this study with fathead minnows are similar to those described in studies with male rats exposed to flutamide (Yamada et al., 2000; O'Connor et al., 2002). For example, O'Connor et al. (2002) observed significant increases in serum T and E2 concentrations (concomitant with increases in follicle stimulating hormone [FSH], lutenizing hormone [LH], and dihydrotestosterone [DHT]) at dosages between 5 and 100 mg flutamide/kg/day. In the intact-male rat assay, an androgen receptor antagonist such as flutamide typically increases LH secretion to stimulate the Leydig cells to produce more T (and subsequently the metabolic products DHT and E2) as a mechanism to compensate for the decreased androgenic stimulus at the hypothalamic-pituitary level (O'Connor et al., 2002). Although gonadotropin was not measured in this study with fathead minnows, it is likely that the increases in steroid concentrations were the result of a similar feedback mechanism. Androgens, specifically KT, control the stimulation of male secondary sex characteristics (Borg, 1994). The maintenance of peripheral androgen levels by the males via a feedback system offers an explanation as to why a reduction in tubercle expression (i.e., tubercle score) was not observed in this study. The increased plasma T concentrations could be further attributed to the capacity of flutamide and 2-hydroxyflutamide to competitively inhibit binding to the fathead minnow androgen receptor (Ankley et al., 2004).

Retarded oocyte maturation and an increase in oocyte atresia were observed in females exposed to flutamide, consistent with the previous exposure of fathead minnows to vinclozolin (Makynen et al., 2000). In addition, testicular histopathology was observed in males exposed to 500 μg flutamide/l. Similar gonadal histopathology has been reported for medaka (*Oryzias latipes*) exposed to the antiandrogens cyproterone acetate and vinclozolin (Kiparissis et al., 2003). In addition, disruption and/or inhibition of spermatogenesis have been observed in adult male guppies (*Poecilia reticulata*) exposed to flutamide (Kinnberg and Toft, 2003) and mature male sticklebacks (*Gasterosteus aculeatus*) exposed to cyproterone acetate (Rouse et al., 1977). It is also interesting to note that the testicular histopathology (i.e., spermatocyte degeneration/necrosis) observed in fathead minnows is similar to that observed in flutamide-exposed male rats (O'Connor et al., 2002).

Somewhat unexpectedly, plasma vitellogenin concentrations were modestly increased by flutamide in both males and females. Hepatic vitellogenin production is mediated via activation of the estrogen receptor (Specker and Sullivan, 1994); in males concentrations of the protein are normally close to non-detectable, unless there has been exposure to exogenous estrogen receptor agonists (Sumpter and Jobling, 1995). We could find no evidence that flutamide effectively binds to estrogen receptor(s) of any species, so it seems unlikely that this would explain elevated concentrations of vitellogenin in the males. It is possible, therefore, that the observed vitellogenin induction was due to the slight increase in endogenous E2 in the males. Flutamide also increased vitellogenin in the female fathead minnows; in this case we speculate that elevations of the protein in the plasma may have been due to the increase in E2 and/or decreased deposition in the oocytes. Specifically, flutamide reduced fecundity of the fish, largely by reducing frequency of spawning. This indicates that flutamide caused a delay in egg maturation (and/or release), which is consistent with the histological observation of a greater number of immature primary oocytes in the ovaries of some exposed fish. The inability of the females to essentially clear vitellogenin via deposition to the eggs could explain increased concentrations observed in the plasma.

A significant reduction in embryo hatch was observed in animals from the high treatment group. Information concerning early lifestage fathead minnow toxicity of flutamide could not be found, but our high concentration (500 μg/l, nominal) is well below the 96-h LC50 value of 3.6 mg/l reported by Hagino et al. (2001) for newly hatched medaka. We have not observed significant effects on hatching success of other EDCs, including estrogens, androgens, and chemicals which affect steroid metabolism (Ankley et al., 2001,

2002, 2003). Further studies are needed to ascertain whether decreased hatching success is an indication of exposure to antiandrogenic chemicals, or whether it is more specific to flutamide.

Work in mammals indicates that 2-hydroxyflutamide is a much more potent antiandrogen than the parent flutamide (Moguilewsky et al., 1986). In the present study, we detected 2-hydroxyflutamide in the whole body extracts of exposed fish at a comparatively small concentration (<20%) relative to the parent material, indicating that fish possess the enzymatic systems necessary for this biotransformation. It is difficult to ascertain, however, whether responses observed in the fathead minnows were due to the flutamide or the hydroxylated metabolite(s). Competitive binding studies with the mammalian (rat) androgen receptor suggest that 2-hydroxyflutamide has an order of magnitude, or greater, affinity for the receptor than flutamide (Kelce et al., 1994). Recent data from our laboratory indicates that binding of 2-hydroxyflutamide to the cloned fathead minnow androgen receptor also is an order of magnitude greater than relative binding affinity of the parent chemical (Ankley et al., 2004). Hence, it is quite possible that the effects we observed were due to the metabolite, despite the fact that its concentrations in the fish were more than an order of magnitude and lower than those of flutamide.

In summary, the results of this short-term reproduction study with the fathead minnow demonstrates that the mammalian antiandrogen, flutamide, affects reproductive endocrine function in fish, and that the observed effects are consistent with an antiandrogenic mode-of-action. The type of hormonal pattern and histopathology effects described in this study, along with Makynen et al. (2000), should be useful in identifying chemicals with antiandrogenic activity. Consequently, our findings suggest that the 21-day reproduction assay utilizing fathead minnows is a sensitive short-term screening method for the detection of endocrine-disrupting chemicals, including antiandrogens.

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