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Effects of a 3β-Hydroxysteroid Dehydrogenase Inhibitor, Trilostane, on the Fathead Minnow Reproductive Axis

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A number of environmental contaminants and plant flavonoid compounds have been shown to inhibit the activity of 3β-hydroxysteroid dehydrogenase/Δ^5-Δ^4 isomerase (3β-HSD). Because 3β-HSD plays a critical role in steroid hormone synthesis, inhibition of 3β-HSD represents a potentially important mode of endocrine disruption that may cause reproductive dysfunction in fish or other vertebrates. The objective of this study was to test the hypothesis that exposure to the model 3β-HSD inhibitor, trilostane, would adversely affect reproductive success of the fathead minnow (*Pimephales promelas*). Results of *in vitro* experiments with fathead minnow ovary tissue demonstrated that trilostane inhibited 17β-estradiol (E2) production in a concentration- and time-dependent manner, and that the effect was eliminated by providing a substrate (progesterone) that does not require 3β-HSD activity for conversion to E2. Exposure of fish to trilostane caused a significant reduction in spawning frequency and reduced cumulative egg production over the course of the 21-day test. In females, exposure to 1500 μg trilostane/l reduced plasma vitellogenin concentrations, but did not cause significant histological alterations. In males, average trilostane concentrations as low as 50 μg/l significantly increased testis mass and gonadal somatic index. Trilostane exposure did not influence the abundance of mRNA transcripts coding for 3β-HSD or other steroidogenesis-regulating proteins in males or females. As a whole, results of this study support the hypothesis that 3β-HSD inhibition can cause reproductive dysfunction in fish, but did not yield a clear profile of responses at multiple levels of biological organization that could be used to diagnose this mode of action.

Key Words: **fish; steroidogenesis; reproduction; gene expression; endocrine disruption.**

Fish reproduction is regulated via the coordinated interaction of the hypothalamic-pituitary-gonadal (HPG) axis and liver. In theory, disruption of signaling, biosynthesis, and/or metabolism at any point along this multiorgan axis has the potential to adversely affect reproductive success. Consequently, a conceptual model of the teleost HPG and liver axis was developed (Villeneuve *et al*., 2007a) and used to identify specific biochemical targets potentially vulnerable to modulation by small, bioavailable, organic xenobiotics. Targets identified include steroid hormone receptors (e.g., estrogen receptors [ER] and androgen receptors [AR]), any of a number of enzymes involved in steroid biosynthesis, cholesterol transport proteins (e.g., steroidogenic acute regulatory protein [StAR], peripheral benzodiazepine receptor), and neurotransmitter receptors involved in regulating the production and release of peptide hormones (e.g., gonadotropin releasing hormones and gonadotropins; Trudeau, 1997; Trudeau *et al*., 2000). This study is part of an overall program of research in which chemicals known or hypothesized to interact with these specific biochemical targets are being systematically evaluated in a fathead minnow (*Pimephales promelas*) 21-day reproduction test. The goal of the research program is to improve our understanding of the diversity of molecular initiating events and chemical modes of action within the HPG axis that can lead to reproductive dysfunction in fishes and, where possible, identify profiles of responses that may be useful for diagnosing chemical mode of action.

A number of modes of action within the HPG axis have been previously examined using model chemicals. For example, previous studies with fathead minnow have demonstrated that exposure to ER and AR agonists and antagonists reduce fecundity, alter secondary sex characteristics, and impact gonad morphology and/or histology (Ankley *et al*., 2003, 2004; Jensen *et al*., 2004; Makynen *et al*., 2000; Miles-Richardson *et al*., 1999; Parrott and Blunt, 2005). Similarly, exposure to various inhibitors of steroidogenic cytochrome P450s (CYPs), including fadrozole, prochloraz, and ketoconazole, has been shown to reduce fathead minnow fecundity over the course of a 21-day reproduction test (Ankley *et al*., 2002, 2005a, 2007). The reproductive impacts of various inhibitors of steroidogenic CYPs are consistent with the important roles they play. For example, CYP11A (cholesterol side chain cleavage) and aromatase (CYP19a, CYP19b) are thought to be rate limiting for steroid and 17β-estradiol (E2) production, respectively. CYP17 α-hydroxylase/17, 20-lyase (CYP17) regulates the shift between androgen/estrogen production needed to support early

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stages of germ cell development versus progestin production to support final germ cell maturation (Miller, 1988, 2005; Pandey and Miller, 2005; Patiño *et al*., 2001). The potential relevance of inhibition of other steroidogenic enzymes is less clear.

To date, there have been few, if any, studies that have investigated whether inhibition of hydroxysteroid dehydrogenases, which also play critical roles in steroid biosynthesis (Miller, 1988, 2005), would result in adverse reproductive impacts. As an example, 3β-hydroxysteroid dehydrogenase/Δ^5-Δ^4 isomerase (3β-HSD; E.C. 1.1.1.145; E.C. 5.3.3.1) catalyzes the conversion of Δ^5-3β-hydroxysteroids (e.g., pregnenolone, 17α-hydroxypregnenolone, dehydroepiandrosterone) to Δ^4-3-ketosteroids (e.g., progesterone, 17α-hydroxyprogesterone, androstenedione [AD]; Norris, 2007). A variety of xenobiotic contaminants including mercury, polychlorinated biphenyls, and tributyltin have been reported to modulate 3β-HSD activity (Andric *et al*., 2000; McVey and Cooke, 2003; Mondal *et al*., 1997). Additionally, plant flavonoids including genistein, daidzein, biochanin A, and formononetin have been shown to inhibit 3β-HSD activity *in vitro* (Ohno *et al*., 2002, 2004; Wong and Keung, 1999). While genistein and other plant flavonoids are derived from "natural" sources (i.e., plant material), loading into aquatic environments has been associated with human activities including discharges from pulp mills, ethanol plants, and wastewater treatment plants, runoff from pasture lands, and may also be associated with wastewater from food production facilities, particularly those that process soybean products (Erbs *et al*., 2007; Fukutake *et al*., 1996; Kawanishi *et al*., 2004; Kiparissis *et al*., 2001; Novak personal communication). Thus, there is reason to believe that inhibition of 3β-HSD is an environmentally relevant mode of action. However, we are not aware of any studies that have explicitly examined the impact of 3β-HSD inhibition on fish reproduction.

In this study, we examine the effect of trilostane on fathead minnow reproduction and reproductive parameters after 21 days of waterborne exposure. Trilostane is a pharmaceutical inhibitor of 3β-HSD activity originally developed as a treatment for Cushing's syndrome (Komanicky *et al*., 1978). While it is unlikely to be environmentally relevant as a contaminant, trilostane is well suited as a model chemical since it is known to be a competitive, and fairly specific, inhibitor of 3β-HSD (Komanicky *et al*., 1978; Potts *et al*., 1978; Touitou *et al*., 1984). In contrast, plant flavonoids and xenobiotics reported to modulate 3β-HSD activity may cause endocrine modulation and/or toxicity through additional modes of action. Given the specificity of the model chemical trilostane, this study represents the first robust test of the hypothesis that inhibition of 3β-HSD is a relevant mode of endocrine disruption that could cause reproductive dysfunction in fish.

METHODS

Chemical and test organisms. Trilostane (CAS 13647-35-3; purity 98.0%; Lot#345-13B) used for this study was provided by Sanofi-Synthelabo Research (Malvern, PA). All fish used in the study were reproductively mature adult fathead minnows (5–6 months old) obtained from an on-site culture facility at the US EPA Mid-Continent Ecology Division (Duluth, MN). All laboratory procedures involving animals were reviewed and approved by the Animal Care and Use Committee in accordance with Animal Welfare Act and Interagency Research Animal Committee guidelines.

Range-finding experiment. There were no previous reports regarding the toxicity of trilostane to fathead minnows or other aquatic organisms. Consequently, a range-finding experiment was conducted to aid in identification of appropriate concentrations for the 21-day reproduction test. For the range-finding experiment, nominal (target) concentrations of 0, 80, 400, or 2000 μg trilostane/l were delivered in UV-filtered Lake Superior water, without the use of carrier solvents, to 20 l aquaria at a continuous flow rate of 32 ml/min. There was one test tank per concentration and each tank contained three male and three female fathead minnows (6 months old) and three breeding substrates. The fish were exposed for 9 days during which survival and spawning activity were monitored daily. At the termination of the experiment, surviving fish were euthanized in a buffered solution of tricaine methanesulfonate (MS-222; Finquel; Argent, Redmond, WA) and plasma samples collected for determination of plasma testosterone (T) and E2 concentrations.

In vitro *steroid production assays.* Several *in vitro* steroid production experiments were conducted to evaluate whether trilostane was able to effectively inhibit fathead minnow 3β-HSD. The methods used for these assays were adapted from those previously described by McMaster *et al*. (1995) as modified by Villeneuve *et al*. (2007b). The first experiment was a concentration-response characterization. Medium 199 (M2520; Sigma, St Louis, MO) supplemented with 0.1mM IBMX (3-isobutyl-1-methylxanthene, Sigma I7018) and 1 μg 25-hydroxycholesterol (Sigma H1015)/ml (hereafter referred to as SSM-standard–supplemented medium) was spiked with 0, 12, 60, 300, or 1000 μg trilostane/l (0–3.0μM) delivered in ethanol, with ethanol concentrations held constant at 0.99% in all treatments. Previous experiments have shown that ethanol concentrations up to 1.0% do not adversely affect steroid production over a 12-h period (Villeneuve *et al*., 2007b). Ovary tissue was removed from control adult female fathead minnows and ovaries from each fish were mechanically separated into 25 explants of approximately equal size (≈5–15 mg) while immersed in ice-cold, nonsupplemented, Medium 199. Individual explants were transferred to glass test tubes containing 500 μl of trilostane- or ethanol-spiked SSM and incubated in a shaking water bath at 25°C for 12 h. A total of five replicate explants, per fish, were tested at each concentration and the experiment was replicated three times using tissue from different fish. Samples of trilostane-spiked and control medium were incubated without tissue to serve as assay blanks. Following incubation, the medium from each tube was transferred to a microcentrifuge tube and stored at −20°C until extracted, and then the tissue from each tube was removed and weighed. Medium samples were extracted twice by liquid:liquid extraction with diethyl ether (Sigma-Aldrich 309958) as described previously (Villeneuve *et al*., 2007b). Concentrations of E2 in the extracts were determined by radioimmunoassay (RIA) using an adaptation of methods developed for quantifying steroids in fathead minnow plasma samples (US EPA, 2002; Villeneuve *et al*., 2007b). To normalize for variations in basal steroid production by ovary tissue from different fish, E2 production was expressed relative to the mean control production (set equal to 1.0) on a per-fish basis.

The second *in vitro* experiment was a time-course characterization. SSM was spiked with either 50 μg trilostane/l (0.15μM; delivered in methanol) or methanol only, with 0.535% methanol in both treatments. The 50 μg/l dose was chosen as a moderate concentration that appeared effective but would still allow for some measurable level of steroid production. Ovary tissue from nonexposed adult female fathead minnows was removed and mechanically separated into explants as described above. Ten explants, per fish, were transferred to test tubes containing trilostane-spiked SSM, while another 10 explants were transferred to control tubes. Tissue from six different fish was tested for a total of 60 replicate tubes per treatment. Explants were incubated in a shaker water bath at 25°C. After 1, 2, 3, 4, 6, 8, and 10 h of incubation, six replicate tubes per treatment (one per fish per treatment) were removed from the water bath

and placed on ice, and the medium was transferred to a microcentrifuge tube and stored at −20°C until extracted. Explants from each tube were transferred to a preweighed tube containing RNAlater (Sigma, R0901) and tubes were re-weighed to determine tissue weight by subtraction. After 12 h, the remaining three replicate tubes, per fish, per treatment, were placed on ice and medium samples were collected and stored in the same manner as above. The three replicate explants, per fish, per treatment, were transferred sequentially to a preweighed tube of RNAlater, weighing the tube after each transfer, such that the weight of each explant was determined but the tissue was pooled into a single tube. Media samples were extracted and analyzed by RIA as described above. The additional replication at the 12-h time point (i.e., three replicates, per fish, per treatment) was used to facilitate comparison of fish-to-fish variability versus explant-to-explant variability.

The final *in vitro* steroid production experiment involved six different treatments: (1) SSM, (2) SSM + 50 μg trilostane/l (0.15μM), (3) SSM + 500 μg trilostane/l (1.5μM), (4) medium 199 supplemented with 0.1mM IBMX and 1.0 μg progesterone (Sigma)/ml (hereafter referred to as PSM-progesterone–supplemented medium), (5) PSM + 50 μg trilostane/l, and (6) PSM + 500 μg trilostane/l. All treatment solutions contained 0.5% ethanol. Four replicate explants per female fathead minnow were transferred to glass test tubes containing 500 μl of the appropriate treatment solution and incubated in a shaker water bath at 25°C for 8 h, and the experiment was replicated three times using tissue from different fish. Eight hours was selected to help assure that steroids would be measured during the linear phase of production for both substrates (based on previous, unpublished experiments). Three 500-μl samples of each medium treatment were incubated without tissue to serve as assay blanks. Following incubation, medium samples were collected, each explant was weighed, and media was extracted and analyzed by RIA as described above.

In vitro *aromatase assay.* Aromatase catalyzes the conversion of C19 androgens (e.g., T, AD) to C18 estrogens (e.g., E2, estrone), reactions that occur downstream of those catalyzed by 3β-HSD (Miller, 1988). An *in vitro* aromatase assay was conducted to evaluate whether trilostane treatment could directly inhibit or enhance fathead minnow aromatase activity. Ovaries were removed from six adult female fathead minnows, weighed, and homogenized in 4-μl phosphate buffer (10mM K_2HPO_4, 100mM KCl, 1mM EDTA, 1mM dithiothreitol, pH 7.4) per mg tissue. Postmitochondrial supernatants (PMS) were prepared by centrifugation as described previously (Villeneuve *et al*., 2006a), and PMS from all fish were pooled together. A set of nine 50 μl aliquots of ovary PMS was placed in a 85°C water bath for 10 min for use as heat-inactivated blanks. Three replicate 50-μl aliquots of PMS and one heat-inactivated blank were then treated with 0, 0.46, 1.37, 4.11, 12.3, 37.0, 111, 333, or 1000 μg trilostane/l (0–3.0μM) delivered in phosphate buffer. Reactions were initiated by adding 160nM 1β-^{3}H-androstenedione (Perkin Elmer NET-926, Boston, MA, specific activity 25.3 mCi/mmol) and 1mM β-NADPH (Sigma N1630), bringing the total reaction volume to 150 μl, and incubated for 4 h at 25°C. Following incubation, the samples were extracted and release of tritiated water was quantified as a measure of aromatase activity, using methods described elsewhere (Ankley *et al*., 2005b).

Fathead minnow reproduction assay. Solvent-free stock solutions of trilostane were prepared by dissolving the chemical in UV-filtered Lake Superior water. Stock solution was diluted in Lake Superior water and delivered to 20-l tanks, containing 10 l of water, at a continuous flow of 44 ± 5 ml/min (mean ± SD) to achieve nominal test concentrations of 0, 60, 300, or 1500 μg trilostane/l over the course of the 21-day exposure period. General water quality characteristics measured in the test system over the course of the study (mean ± SD) were hardness 46.2 ± 0.8 mg/l as $CaCO_3$, alkalinity 41.4 ± 1.5 mg/l as $CaCO_3$, pH 7.4 ± 0.1, dissolved oxygen 6.3 ± 0.5 mg/l (79 ± 3% saturation), and temperature 25.6 ± 0.3°C.

The basic experimental design was described by Ankley *et al.* (2001) except that a paired rather than group spawning approach was used (Ankley *et al*., 2005a; Villeneuve *et al*., 2006b). Prior to starting chemical exposure, reproductively mature fish were paired (one male and one female) and loaded into tanks at a density of two pairs per tank. Each pair was separated by a water permeable mesh divider and each pair had their own breeding substrate. The fish were held in the test system, receiving Lake Superior water only, for a 14-day acclimation period during which the fecundity of each pair was assessed daily. The animals were kept under a 16:8 h light:dark cycle and were fed adult brine shrimp twice daily. After 14 days, exposures were initiated using pairs that had spawned successfully during acclimation. In total, 10 pairs of fish were exposed at each trilostane treatment level. In the case of controls, 14 pairs of fish were included in the test.

Over the course of the exposure, the total number of eggs spawned and the number of fertile eggs produced by each pair was recorded daily. Water samples were collected from each tank every 2–3 days (9/21 days in total) and trilostane concentrations in the water were measured by high-performance liquid chromatography (HPLC) with diode array detection (254 nm; Model 1100; Agilent Techologies, Santa Clara, CA). The HPLC was equipped with a Zorbax SB-C18 column (75 × 2.1 mm). Two hundred microliters of sample was directly injected and an isocratic elution with 60% methanol/25mM formic acid at a flow rate of 0.2 ml/min (oven temperature 30°C) was used for the analysis. The method detection limit was 20 μg/l. At the conclusion of the assay, the fish were anesthetized in buffered MS-222. Whole-body wet weight was measured and then urine was expelled from males by applying gentle pressure on the fish's abdomen and collected using nonheparinized micro-capillary tubes. The presence or absence of sperm in the urine sample was noted and samples were stored at −80°C for subsequent metabolomic analyses (pending; see also Ekman *et al*., 2007). Blood was collected from males and females using heparinized microhematocrit tubes, and plasma was separated by centrifugation. Plasma samples were stored at −80°C until extracted and analyzed. Following biofluid sampling, secondary sex characteristics (e.g., nuptial tubercles) were evaluated, and then liver, gonads, dorsal fat pad, brain, and pituitary were removed. Tubercles were scored based on their relative number and size as described previously (Jensen *et al*., 2001; US EPA, 2002). Liver samples were transferred to preweighed microcentrifuge tubes and snap frozen in liquid nitrogen immediately after weighing each tube (allowing determination of liver weight by subtraction). Whole gonads were weighed and subdivided into several pieces. Approximately one-third of the total gonad tissue was preserved in RNAlater, one-third snap frozen in liquid nitrogen, and one-third was preserved in Davidson's fixative for histological analysis. Dorsal fatpads (if present, generally males only) and brains were removed and transferred to preweighed microcentrifuge tubes containing RNAlater. After all samples were collected, the tubes containing samples were reweighed to determine sample weight by subtraction, and then stored at −20°C until extracted. Pituitary samples were collected with a fine forceps, preserved in RNAlater and stored at −20°C. All dissection tools were washed with RNaseZap (Ambion, Austin, TX) between each sample to prevent sample cross-contamination or degradation by RNAses.

Histological and biochemical analyses. Gonads preserved for histological analysis were embedded in paraffin. Sectioning and analyses were conducted by Experimental Pathology Laboratories (Herndon, VA). Briefly, gonads were sectioned longitudinally at 4–5 μm and stained with hematoxylin and eosin. Three sections were assessed from each gonad, beginning at the midline and then at 50-μm intervals following the first section. Severity scores (1 = minimal, 2 = mild, 3 = moderate, and 4 = severe) were qualitatively assigned to pathological findings for each specimen based on the relative amount of affected gonad section compared to that of the controls. Relative maturational stage of the gonads was also evaluated using criteria and a scoring system described previously (Villeneuve *et al*., 2007d).

Plasma vitellogenin (VTG) concentrations were determined using an enzyme-linked immunosorbent assay with a fathead minnow polyclonal antibody and a purified fathead minnow vitellogenin standard (Korte *et al*., 2000; US EPA, 2002). Plasma E2 and T (equivalents, see"Results") concentrations were quantified by RIA following liquid:liquid extraction with diethylether (Jensen *et al*., 2001; US EPA, 2002).

Gene expression analyses. Relative abundance of selected mRNA transcripts was quantified using real-time quantitative PCR (QPCR). Gonad or brain samples preserved in RNAlater were transferred to TriReagent (Sigma) and total RNA was extracted from the tissue according to the manufacturer's protocol. Total RNA concentrations were measured using a Nanodrop ND 1000

spectrophotometer (Nanodrop Technologies, Wilmington, DE). Purity of the samples was evaluated based on optical density at 260/280 nm. The ratio for all samples was between 1.7 and 2.2 with the exception of one ovary and three testis samples which had ratios ranging from 1.63 to 1.69. Total RNA samples were diluted to 10 ng/μl for use in QPCR assays. Relative abundance of 11β-hydroxysteroid dehydrogenase (11β-HSD) mRNA transcripts was determined using the primers, probes, and protocol described by Martinović *et al*. (2008). Relative abundance of StAR, 3β-HSD, aromatase (A isoform, predominantly expressed in ovary; CYP19A), CYP11A, CYP17, and follicle-stimulating hormone receptor (FSHR) transcripts were determined using primers, probes, and protocols described previously (Villeneuve *et al*., 2006a, 2007c). Relative transcript abundance was normalized to the total RNA used in each one-step QPCR reaction and a gene-specific mRNA standard curve and data were expressed as approximate number of copies per ng total RNA (see Villeneuve *et al*., 2007c for details).

Data analysis. Data from all experiments were tested for normality using a Kolmogorov-Smirnov test, and homogeneity of variance using Levene's test. QPCR results for testis 3β-HSD and CYP17 and for ovary aromatase A and FSHR were log-transformed to meet the assumption of parametric statistics. Aromatase assay results, QPCR results for ovary 3β-HSD and testis StAR, and female morphological data (i.e., whole body mass, gonadal somatic index [GSI]) were not normally distributed, even after transformation. In all other cases, nontransformed data met the assumptions of parametric statistics.

Data from the *in vitro* steroid production assays were analyzed using general linear models (GLM) ANOVA. Explant weight, fish, and either trilostane concentration or exposure duration (time) were used as independent variables in the analyses. In the case of the third *in vitro* experiment, data for each substrate were analyzed separately. In all experiments, neither explant weight nor any interactions between explant weight and other independent variables were significant. Consequently, explant weight was removed from the final statistical model and only the effects of fish, concentration, or time, and their interactions were considered. Duncan's multiple range test was used to assess *post hoc* differences between treatments or time points. For the concentration-response experiment, the EC_{50} for reduced E2 production, and associated 95% confidence interval, was estimated using GraphPad Prism 4.03 (GraphPad software, San Diego, CA).

Repeated measures ANOVA was used to test for effects of trilostane treatment on cumulative egg production over time, using pair as the unit of replication ($n = 10$ for trilostane treatments, $n = 14$ for controls). All other parametric data from the 21-day reproduction assay were analyzed using GLM ANOVA with treatment and replicate as independent variables. In cases where there was no replicate effect or significant interaction between treatment and replicate, replicate was removed from the model and a one-way ANOVA was used to test for differences across treatment groups. Duncan's multiple range test was used for all parametric *post hoc* testing to determine differences between all treatment groups. Data that did not conform to the assumptions of parametric statistics were analyzed using a nonparametric Kruskall-Wallis test, and Dunn's *post hoc* test.

All statistical analyses were conducted using SAS 9.1 (SAS Institute, Cary, NC), with the exception of Dunn's test which was performed using GraphPad Instat v. 3.01 (GraphPad Software). All tests were two tailed and differences were considered significant at $p < 0.05$, unless otherwise noted.

RESULTS

Range-Finding Experiment

One female from the 400 μg trilostane/l treatment died on d 7 of the 9-day range-finding experiment. There were no other mortalities, even at the greatest concentration tested (e.g., 2000 μg trilostane/l nominal) nor were there any alterations in fish behavior, which suggested that the animals were significantly stressed. Consequently, the mortality observed was assumed to be nontreatment related and concentrations up to 2000 μg/l appeared nonlethal. No effects on plasma steroid concentrations in males or females were obvious after 9 days of exposure. However, over the exposure period, fish from the control tanks produced 213 eggs, while those from the 80, 400, and 2000 μg trilostane/l nominal tanks produced 47, 0, and 0 eggs, respectively. While not statistically robust, the results suggested that concentrations in a similar range would be appropriate for the 21-day reproduction test. Consequently, 1500, 300, 60, and 0 μg trilostane/l were identified as target concentrations for the reproduction assay.

In Vitro *Experiments*

Trilostane significantly impacted E2 production by fathead minnow ovary tissue exposed *in vitro*. In the first experiment, trilostane caused a concentration-dependent decrease in the production of E2 by fathead minnow ovary explants (Fig. 1A). The estimated EC_{50} for reduction in E2 production was 37.8 μg trilostane/l, although the precision of the estimate was limited by high fish-to-fish variability in basal E2 production (95% confidence interval 8.35–171 μg/l). *In vitro* exposure to 50 μg trilostane/l inhibited time-dependent production of E2 relative to control ovary explants (Fig. 1B). There was a significant time-dependent increase in E2 production by control explants, but not by trilostane-treated explants (Fig. 1B), and a significant interaction between treatment and time was detected. In a third experiment, *in vitro* exposure to either 50 or 500 μg trilostane/l significantly inhibited E2 production by ovary explants provided with a substrate dependent on 3β-HSD activity for conversion to E2 (i.e., 25-hydroxycholesterol; Fig. 1C). However, trilostane exposure did not significantly inhibit E2 production by explants provided with a substrate that can be readily converted to E2 without the involvement of 3β-HSD (i.e., progesterone; Fig. 1C). Finally, treatment of fathead minnow ovary PMS with trilostane did not cause significant inhibition of aromatase activity *in vitro* (Supplementary Fig. SI-1). While not direct measures of 3β-HSD inhibition, results of all four *in vitro* experiments were entirely consistent with trilostane's anticipated mode of action.

Effects of trilostane on *in vitro* androgen production by fathead minnow gonad tissue could not be reliably determined. Preliminary *in vitro* steroid production experiments (data not shown) suggested that trilostane may cause a concentration-dependent increase in T production. However, further investigation, including analysis of trilostane-spiked media blanks and trilostane standards alone using our standard T RIA method (US EPA, 2002; Villeneuve *et al*., 2007b) demonstrated that trilostane cross-reacted with our T antibody, yielding a concentration-dependent false-positive response (Supplementary Fig. SI-2). After identifying the cross-reactivity in the T RIA, we attempted to assess trilostane's effects on androgen production by fathead minnow gonad tissue by

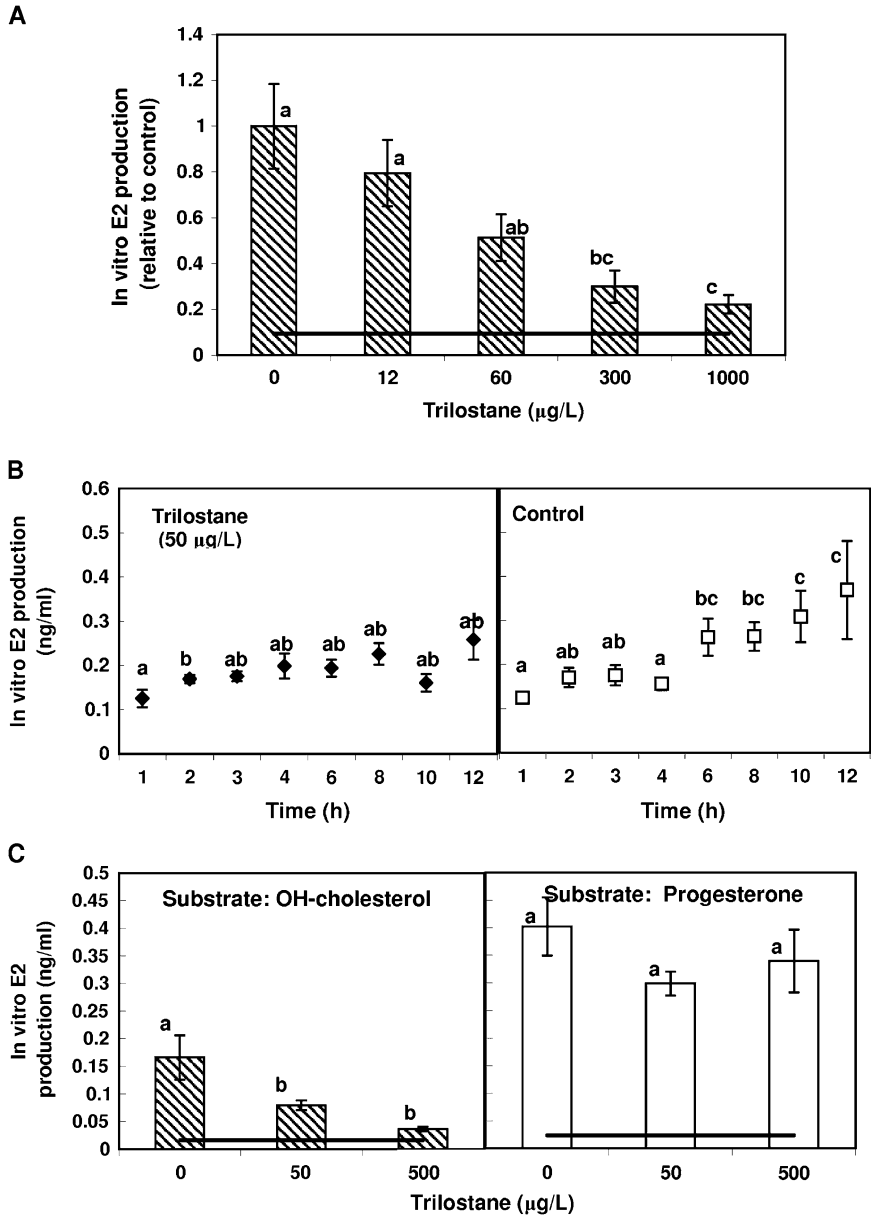

FIG. 1. Results of *in vitro* steroid production experiments with trilostane. Means ± SE. Different letters indicate statistically significant differences among treatments within a given experiment ($p < 0.05$). (A) *In vitro* E2 production by fathead minnow ovary tissue exposed to varying concentrations of trilostane for 12 h; 25-hydroxycholesterol (OH-cholesterol) was provided as a substrate. E2 production expressed relative to control production normalized on a fish-to-fish basis; $n = 4$ replicate explants per treatment per fish, $n = 3$ replicate fish. Horizontal line indicates method detection limit of the radioimmunoassay. (B) *In vitro* E2 production by fathead minnow ovary tissue exposed to 50 µg trilostane/l medium or control medium for variable durations. OH-cholesterol was provided as a substrate; $n = 6$ replicates per treatment and time point. (C) *In vitro* E2 production by ovary tissue incubated in trilostane-spiked or control medium for 8 h and provided either OH-cholesterol or progesterone as a substrate. Horizontal line indicates the method detection limit of the radioimmunoassay.

examining *in vitro* androstenedione (AD) and 11-ketotestosterone (11-KT) production. However, trilostane alone and trilostane spiked into SSM yielded false-positive responses in our AD RIA and in a commercial 11-KT ELISA (Cayman Chemical, Ann Arbor, MI; data not shown) as well. Within the range of T concentrations typically produced by fathead minnow gonad tissue in culture and the range of trilostane concentrations effective in reducing E2 production, the T measurements could not be adequately corrected for the cross-reactivity. Therefore, effects of trilostane on androgen production *in vitro* could not be established.

Fathead Minnow Reproduction Assay

Averaged over the course of the 21-day reproduction assay, measured trilostane concentrations were close to nominal, but

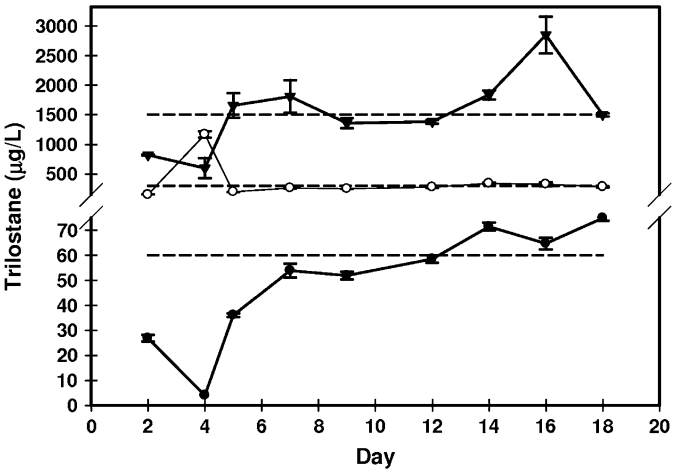

FIG. 2. Mean (± SD) concentration of trilostane detected in water sampled from the exposure tanks ($n = 5$ replicate tanks per trilostane treatment) over the course of the exposure. No trilostane was detected in control tanks ($n = 7$ replicate tanks). Concentrations less than 20 µg/l (method detection limit) are approximate only. Horizontal dashed lines indicate the nominal target concentrations; ▼, 1500 µg/l nominal; ○, 300 µg/l nominal; ●, 60 µg/l nominal.

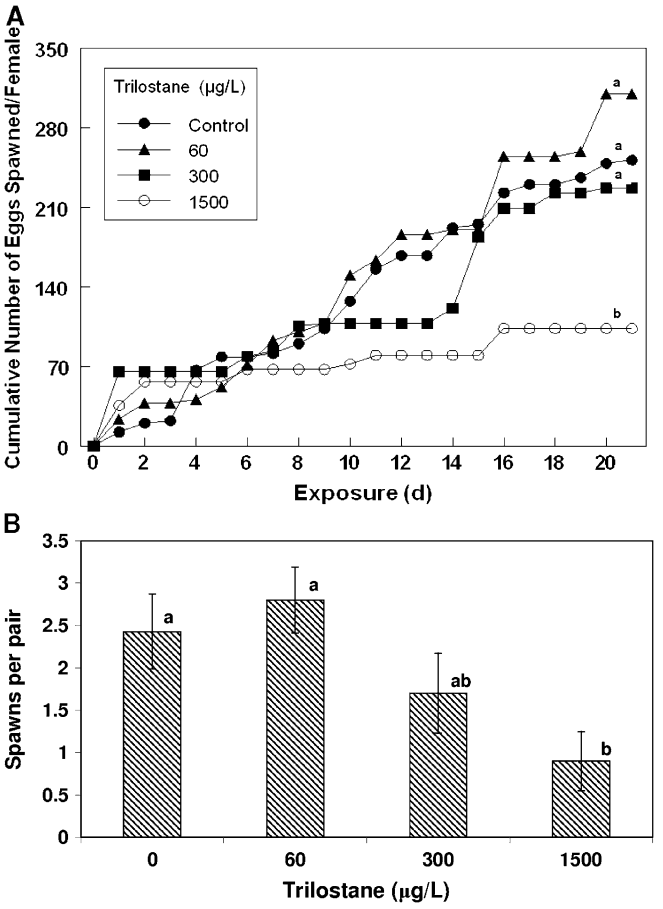

Fig. 3. Effects of trilostane on (A) cumulative egg production and (B) spawning frequency. Spawning frequency represented as mean (± SE) number of spawns per pair ($n = 10$–14 pairs) over the duration of the 21-day test; different letters indicate statistically significant differences between treatments ($p < 0.05$).

there were some fluctuations over time (Fig. 2). Initial concentrations measured on the second day of exposure were approximately 50% of nominal (Fig. 2). A new stock solution was put online the following day; however, a power outage between days 3 and 4 resulted in excess trilostane delivery to the 300 µg/l treatment group and deficient chemical delivery to tanks in the 60 and 1500 µg/l groups (Fig. 2). Concentrations were corrected and remained relatively steady for the remainder of the test, with the exception of concentrations in the 1500 µg/l treatment reaching approximately 2800 µg/l on d 16 (Fig. 2). While exposure concentrations fluctuated somewhat over time, there was relatively little variability among replicate tanks within a treatment. Mean (± SD, $n = 9$ days sampled) coefficients of variation among replicate tanks within each treatment were 3.1 ± 1.4, 3.7 ± 2.7, and 6.7 ± 5.5%, in the 60, 300, and 1500 µg/l treatment groups, respectively. Overall mean (± SD) concentrations over time were 50 ± 22, 348 ± 285, and 1555 ± 638 in the 60, 300, and 1500 µg/l treatment groups, respectively. No trilostane was detected in the control tanks.

Two fish died over the course of the exposure. One female from the 1500 µg/l treatment group died on d 10 of the exposure. Additionally, one female from the control group died on d 11. The overall mortality across all treatment groups was 2.3%, which was well within the criteria (≥ 90% adult survival in control treatments) established for an acceptable 21-day reproduction assay (Ankley *et al.*, 2001).

Exposure of the fish to trilostane over a 21-day period altered a number of reproductive parameters. Most notably, there was a significant reduction in cumulative egg production by pairs of fathead minnows from the 1500 µg/l treatment group (Fig. 3A). Correspondingly, the mean number of spawns per pair, over the 21-day period, was reduced in a concentration-dependent manner (Fig. 3B). The number of eggs produced per spawning event was not affected by treatment, and there was no reduction in the proportion of eggs fertilized. Thus, as a whole, it appeared that spawning frequency rather than the productivity associated with each spawning event was impacted by trilostane exposure.

VTG concentrations were the primary reproductive parameter affected in females. Females from the 1500 µg/l treatment had VTG concentrations that were significantly less than those in control fish (Fig. 4A). Plasma E2 concentrations also tended to be lower in females from the 1500 µg/l treatment, but the difference was not statistically significant (Fig. 4B). Histological analysis of ovaries from trilostane-exposed fish did not reveal any notable pathologies or significant changes in median ovary maturational stage as evaluated based on the proportions of follicles of different sizes and their characteristics (e.g., presence and location of cortical alveoli, size and shape of yolk granules and germinal vesicles; Villeneuve *et al.*, 2007d).

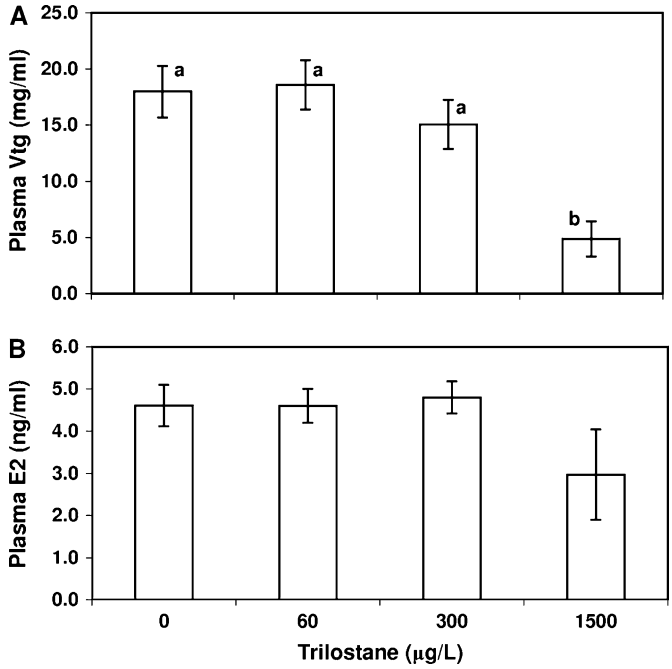

Fig. 4. Effects of trilostane on mean (± SE; $n = 8$–13) (A) VTG and (B) plasma estradiol (E2) concentrations in females. Different letters indicate statistically significant differences between treatments ($p < 0.05$).

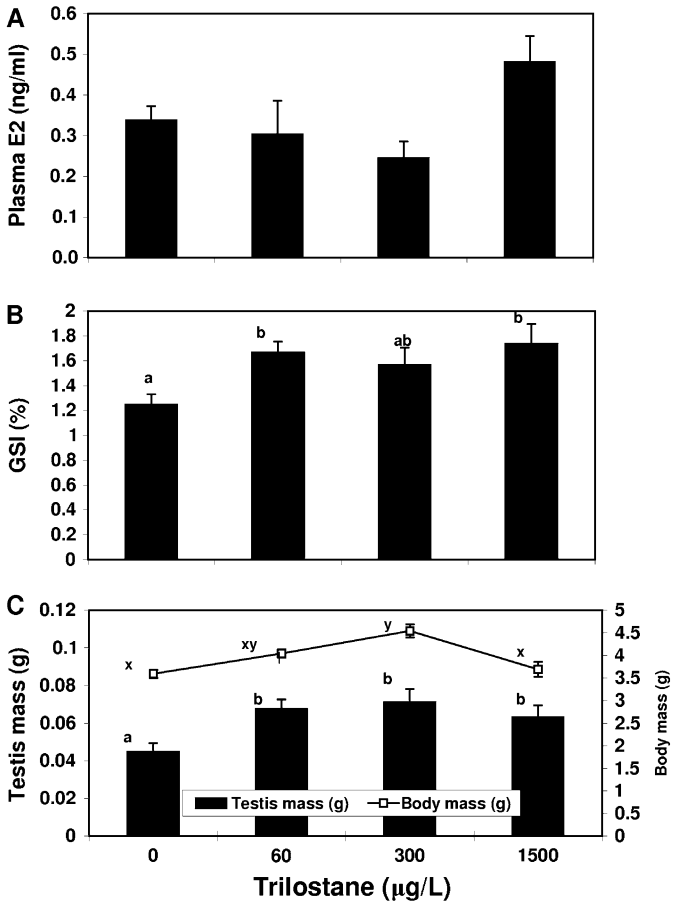

Fig. 5. Effects of trilostane on mean (± SE; $n = 10$–14) (A) plasma estradiol (E2), (B) GSI, and (C) testis and whole body mass of males. Different letters indicate statistically significant differences between treatments ($p < 0.05$).

Similarly, there were no significant effects on female body mass or GSI. At the molecular level, no significant effects on the relative abundance of StAR, 3β-HSD, CYP19A, or FSHR transcripts in ovary tissue were detected (Supplementary Fig. SI-3).

Trilostane exposure had no effect on VTG concentrations in males (data not shown), although a potential decrease would not be easily detected since basal expression of the lipoprotein in males is generally below our method detection limit. Plasma E2 concentrations tended to be greater in males from the 1500 µg/l treatment group, but, as with females, the difference among treatments was not statistically significant (Fig. 5A; $p = 0.0635$; df = 3). Histological examination of testis revealed minimal to mild asynchronous development and mineralization in trilostane-treated fish, but not controls. However, the incidence and severity of these conditions did not increase in a concentration-dependent manner and both observations tend to be common background–type findings even in control animals. Furthermore, there were no significant effects on testes maturational stage as evaluated based on sperm maturation, relative abundance, and characteristics of the germinal epithelium and tubule lumina (Villeneuve *et al.*, 2007d). Despite the lack of clear histological impacts, trilostane did cause significant morphological alterations in males. GSI was significantly increased in male fathead minnows exposed to 60 and 1500 µg trilostane/l (Fig. 5B). Trilostane exposure was associated with significant increases in testis mass at all concentrations tested; however, the increase in testis mass in males from the 300 µg/l treatment group was offset somewhat by a significant increase in whole body mass (Fig. 5C). Neither dorsal pad index (i.e., the ratio of dorsal pad mass to whole body mass; data not shown) nor tubercle scores ($p = 0.0548$; Supplementary Fig. SI-4) were significantly impacted by trilostane exposure. Finally, at the molecular level, the abundance of 3β-HSD, 11β-HSD, CYP11A, CYP17, and StAR transcripts in testis and the abundance of 3β-HSD transcripts in brain were not significantly impacted by trilostane exposure (Supplementary Fig. SI-5). Overall, increased testis mass relative to body mass appeared to be trilostane's primary effect on males.

The ability to determine trilostane's *in vivo* effects on circulating testosterone concentrations was confounded with its cross-reactivity in the T RIA. Without an ability to measure trilostane concentrations directly in the plasma samples (due to limited volume), there was no reliable way to correct for the artifact. Consequently, the effect of trilostane exposure on circulating androgen concentrations could not be determined.

DISCUSSION

Verification of Trilostane's Mode of Action

The primary objective of the *in vitro* experiments was to verify that trilostane could act as an inhibitor of steroidogenesis, in general, and 3β-HSD activity, in particular, in the fathead minnow gonad. Trilostane has been used previously to inhibit steroidogenesis and/or germinal vesicle breakdown in *in vitro* experiments with several other fish species including freshwater perch (*Anabas testudineus*), striped bass (*Morone saxitalis*), mummichog (*Fundulus heteroclitus*), and common carp (*Cyprinus carpio*) (Bhattacharyya *et al*., 2000; King *et al*., 1994; Mukherjee *et al*., 2006; Petrino *et al*., 1989; Weber and Sullivan, 2000). Effective concentrations tested in those experiments ranged from approximately 0.01 to 10 μg/ml. The ability of trilostane to inhibit E2 production by fathead minnow ovary tissue exposed *in vitro*, in a concentration- and time-dependent manner, at concentrations similar to those used in other *in vitro* experiments with fish, was consistent with previous reports that trilostane inhibits steroidogenesis in fish gonad tissue. Efficient conversion of 25-hydroxycholesterol to E2 requires the activity of CYP11A, CYP17, 3β-HSD, 17β-HSD, and aromatase. The fact that trilostane did not directly inhibit aromatase activity implies that the effect was upstream of aromatase in the E2 synthesis pathway. Conversion of progesterone to E2 requires the activity of CYP17, 17β-HSD, and aromatase, but not CYP11A or 3β-HSD. Therefore, the observation that trilostane's effect on E2 production could be effectively eliminated by providing progesterone as a substrate suggests that trilostane was inhibiting 3β-HSD and/or CYP11A. Had it been feasible to reliably measure T production *in vitro*, it would have been possible to discriminate effects on 3β-HSD activity from a potential effect on CYP11A activity. Nonetheless, the *in vitro* data strongly support the assumption that trilostane functions as an inhibitor of 3β-HSD activity in fathead minnow ovary, just as it was designed to act in mammalian adrenal tissue as a treatment for Cushing's syndrome (Komanicky *et al*., 1978).

Reproductive Impacts of Trilostane Exposure

Exposure to trilostane reduced the spawning frequency, and consequently cumulative fecundity, of fathead minnows exposed *in vivo* over a 21-day period (Fig. 3). Results of previous *in vitro* experiments with fish ovarian follicles suggest that trilostane can block final oocyte maturation and germinal vesicle breakdown (Bhattacharyya *et al*., 2000; King *et al*., 1994; Mukherjee *et al*., 2006; Petrino *et al*., 1989; Weber and Sullivan, 2000). In fish, final oocyte maturation is thought to be regulated by 17α-hydroxylated progesterone derivatives including 17α,20β-dihydroxy-4-prenen-3-one (17,20β-P) and 17α,20β,21-trihydroxy-4-pregnen-3-one (20β-S) (Nagahama, 1997; Thomas *et al*., 2002). Endogenous synthesis of these compounds is dependent on reactions catalyzed by 3β-HSD (Nagahama, 1997). Therefore, inhibition or delay of oocyte maturation, and consequently ovulation, due to reduced production of maturation-inducing steroids is a plausible mechanistic explanation for the reduced spawning frequency observed in trilostane-treated fathead minnows. Measurements of 17,20β-P concentrations in ovarian tissue and/or plasma, coupled with quantitative characterization of germinal vesicle breakdown via microscopic examination of intact oocytes (as opposed to histological sections), would provide for a more definitive assessment of this hypothesis in future experiments.

In addition to a hypothesized effect on oocyte maturation, data from this study provided direct evidence that VTG concentrations were reduced in trilostane-exposed females (Fig. 4A). Uptake of vitellogenin from the plasma into the oocytes is a critical part of oocyte development in fish (Arukwe and Goksøyr, 2003; Tyler and Sumpter, 1996). Previous research with the fathead minnow has demonstrated a strong correlation between VTG concentrations in females and fecundity (Miller *et al*., 2007). Thus, it is plausible that oocyte development was retarded by decreased availability of vitellogenin in the plasma resulting in slower oocyte growth and less frequent ovulation. Ultimately, both impaired vitellogenesis and final ooctye maturation may have played a role in mediating trilostane's adverse effect on reproduction.

Surprisingly, however, no notable histological or morphological changes in ovary were detected. Previous experiments with chemicals that disrupt vitellogenesis (e.g., fadrozole, trenbolone, prochloraz) have caused obvious reductions in yolk deposition and oocyte size, increases in preovulatory atretic follicles, and/or changes in median ovarian stage (Ankley *et al*., 2002, 2003, 2005a). No such alterations were observed in this study. Whether this points toward other mechanisms as the dominant cause of the decrease in spawning frequency or simply suggests a less severe effect on vitellogenesis than observed in previous studies remains to be determined.

The major impact of trilostane in male fathead minnows was a significant increase in the mass of the testes (absolute and relative to body mass; Figs. 5B and 5C). In another recent fathead minnow study, ketoconazole, an inhibitor of steroidogenic CYPs, caused a significant increase in GSI (Ankley *et al*., 2007). In the ketoconazole study, the increased testis size was associated with a proliferation of the interstitial cells involved in steroid production (Ankley *et al*., 2007). Thus, it was hypothesized that proliferation of steroid-producing cells, and a related increase in testis mass relative to body mass, was part of a compensatory response aimed at offsetting the effect of the stressor (Ankley *et al*., 2007). While the increase in GSI and testes mass caused by trilostane could be viewed as part of a compensatory response to presumed inhibition of 3β-HSD–mediated androgen production, there was little other corroborating evidence. Neither plasma nor *in vitro* concentrations of androgens could be effectively measured due to trilostane's cross-reactivity with our T, AD, and 11-KT antibodies. Furthermore, in this study, there was no histological evidence

of either interstitial cell proliferation or increased sperm accumulation (due to less frequent spawning) that would explain the increased GSI, nor was the effect on testes mass noticeably concentration dependent. Thus, an explanation for trilostane's effect on testis size remains elusive.

At the mRNA transcript level, trilostane did not elicit effects indicative of either inhibition of gene expression or compensatory upregulation of gene expression to offset the direct effects of presumed 3β-HSD inhibition on steroid production. There was no significant upregulation of 3β-HSD transcripts in ovary or testes (Supplementary Figs. SI-3 and SI-5), where 3β-HSD plays a role in the synthesis of reproductive steroids. Similarly, there was no upregulation of 3β-HSD transcripts in male brain (Supplementary Fig. SI-5), despite evidence that 3β-HSD is expressed in the brain and central nervous system of fish, frogs, and birds and is thought to play a role in synthesis of neurosteroids (Mathieu *et al*., 2001; Mensah-Nyagan *et al*., 1994; Sakamoto *et al*., 2001; Ukena *et al*., 1999). Compensatory upregulation of various mRNA transcripts coding for steroidogenesis-regulating proteins has been observed in previous studies with the fathead minnow. Exposure to fadrozole, a competitive inhibitor of aromatase, has been shown to upregulate expression of aromatase A in fathead minnow ovary tissue (Villeneuve *et al*., 2006a). Similarly, exposure to ketoconazole resulted in significant upregulation of CYP11A and CYP17 (Ankley *et al*., 2007). Neither of these responses to steroidogenesis inhibition was observed in this study (Supplementary Fig. SI-5). Given the role of StAR in the cholesterol transport process thought to be rate limiting for acute steroidogenesis (Miller, 1988; Stocco, 2001; Stocco and Clark, 1996), we hypothesized that StAR transcripts might be more abundant in the gonads of trilostane-exposed fish. Additionally, we hypothesized that expression of mRNAs coding for FSHR, which is involved in mediating signals from the pituitary that support vitellogenesis and oocyte growth (Kumar *et al*., 2001; Tyler and Sumpter, 1996), and 11β-HSD which is involved in the synthesis of 11-KT, one of the primary androgens in male fish (Borg, 1994), might also be upregulated in ovary and testis, respectively, as part of a compensatory response to 3β-HSD inhibition. However, none of these hypothesized transcript-level responses were observed. Consequently, in contrast to previous studies with steroidogenesis inhibitors, there was no evidence of a compensatory response at the transcript level, at least at the time point examined (21 days).

Implications for Ecological Risk Assessment

There is evidence in the literature to suggest that inhibition of 3β-HSD is an environmentally relevant mode of action. For those environmental contaminants previously reported to modulate 3β-HSD activity (e.g., plant flavonoids, mercury, PCBs, tributyltin) (Andric *et al*., 2000; McVey and Cooke, 2003; Mondal *et al*., 1997; Ohno *et al*., 2002, 2004; Wong and Keung, 1999), 3β-HSD inhibition is just one of the multiple modes of potential toxic action. However, there may be other environmental contaminants for which 3β-HSD inhibition is a dominant mode of action. Both empirical evidence and the known role of steroids in regulating reproduction suggest that inhibition of 3β-HSD could cause reproductive dysfunction in fish. Results of this study support that assumption, showing clear reductions in spawning frequency and cumulative fecundity. However, the study did not yield a distinctive pattern of responses spanning multiple levels of biological organization (e.g., molecular/biochemical to histological/morphological, to whole organism) that would be considered readily diagnostic of this mode of action. Thus, for the time being, additional diagnostic experiments employing approaches similar to the *in vitro* assays performed as part of this study, or more direct measures of 3β-HSD activity, would be needed to assign this mode of action to an "unknown" chemical. Nonetheless, confirmation of 3β-HSD inhibition as a relevant cause of reproductive dysfunction in fish helps to substantiate the use of conceptual systems models as relevant tools for identifying potential modes of toxic action, as an aid to the risk assessment process.

SUPPLEMENTARY DATA

Supplementary data showing aromatase assay results (Figure SI-1), trilostane's cross-reactivity in the T RIA (Figures SI-2), QPCR results (Figures SI-3 and SI-5), and tubercle score results (Figure SI-4), are available online at http://toxsci.oxfordjournals.org/.

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