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TEMPORAL EVALUATION OF EFFECTS OF A MODEL 3β-HYDROXYSTEROID DEHYDROGENASE INHIBITOR ON ENDOCRINE FUNCTION IN THE FATHEAD MINNOW

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Abstract—Inhibition of enzymes involved in the synthesis of sex steroids can substantially impact developmental and reproductive processes controlled by the hypothalmic-pituitary-gonadal (HPG) axis. A key steroidogenic enzyme that has received little attention from a toxicological perspective is 3β-hydroxysteroid dehydrogenase (3β-HSD). In these studies, we exposed reproductively-active fathead minnows (*Pimephales promelas*) to the model 3β-HSD inhibitor trilostane at two test concentrations (300 and 1,500 μg/L) over a 16-d period that included both 8-d exposure and 8-d recovery phases. Plasma concentrations of 17β-estradiol (E2) in females were depressed within hours of exposure to the drug and remained decreased at the highest trilostane concentration throughout the 8-d exposure. Reductions in E2 were accompanied by decreases in plasma concentrations of the estrogen-responsive protein vitellogenin (VTG). During the recovery phase of the test, plasma E2 and VTG concentrations returned to levels comparable to those of controls, in the case of E2 within 1 d. Up-regulation of ovarian expression of gene products for follicle-stimulating hormone receptor (*fshr*) and aromatase (*cyp19a1a*) suggested active compensation in trilostane-exposed animals. Effects of trilostane on HPG-related endpoints in exposed males were less pronounced, although, as in females, up-regulation of gonadal *fshr* was seen. Data from these time-course studies provide insights as to direct impacts, compensatory responses, and recovery from effects associated with perturbation of a comparatively poorly characterized enzyme/pathway critical to sex steroid synthesis. This information is important to the design and interpretation of approaches for assessing the occurrence and effects of HPG-active chemicals in both the laboratory and the field. Environ. Toxicol. Chem. 2011;30:2094–2102. © 2011 SETAC

Keywords—Fathead minnow Reproduction Endocrine function Toxicity mechanisms Steroidogenesis

INTRODUCTION

Research concerning chemical disruption of the vertebrate hypothalamic-pituitary-gonadal (HPG) axis has focused mostly on substances that interact with estrogen or androgen receptors and inhibitors of cytochrome P450 (CYP) isozymes involved in sex steroid production. Comparatively less is known concerning the effects of chemicals on steroidogenic hydroxysteroid dehydrogenases (HSDs). One of the key HSDs involved in steroid synthesis is 3β-HSD, which converts Δ^5-3β-hydroxysteroids such as pregnenolone and dehydroepiandrosterone to Δ^4-3-ketosteroids such as progesterone and androstenedione [1]. In vivo or in vitro evidence indicates that chemicals such as mercury, polychlorinated biphenyls, tributyltin and plant flavonoids can modulate (generally depress) 3β-HSD activity [2–6], indicating that this may be an environmentally relevant pathway for disruption of HPG axis function. To investigate the possible biological significance of 3β-HSD inhibition in terms of adverse effects in fish, we previously conducted a short-term reproduction assay with fathead minnows (*Pimephales promelas*) exposed to the pharmaceutical trilostane [7]. Trilostane was chosen as a model chemical for the work of Villeneuve et al. because it is a specific competitive inhibitor of 3β-HSD [8–10]. Exposure of the fish to waterborne trilostane (1,500 μg/L) for 21 d caused a significant decrease in egg production, coincident with a depression in plasma concentrations of the estrogen-responsive protein vitellogenin (VTG; egg yolk precursor) in females. Decreased vitellogenesis in vivo was consistent with inhibition of 17β-estradiol (E2) production by fathead minnow ovary tissue exposed to trilostane in vitro [7].

Our interest in trilostane and its inhibition of 3β-HSD stems from a larger effort aimed at defining a comprehensive suite of toxicity/adverse outcome pathways associated with perturbation of the HPG axis in fish [11]. A primary goal of the overall project is the identification and development of molecular and biochemical indicators that are diagnostic of chemical perturbation of specific targets in the axis or are predictive of adverse outcomes (e.g., decreased egg production). Because of the dynamic nature of the HPG axis, an important aspect of the work has involved evaluation of changes in the system over time, both during exposure to test chemicals and during recovery after cessation of exposure [12–15]. These temporal studies are critical to defining linkages of endpoints across biological levels of organization, identifying sensitive and stable indicators of exposure and effects, and understanding the complex interplay between direct and compensatory responses to chemicals.

In this article, our initial work with trilostane [7] is expanded on relative to describing temporal effects of the 3β-HSD inhibitor on aspects of reproductive endocrinology of the adult fathead minnow, using two different time courses: a long-term study in which males and females were sampled periodically over the course of an 8-d chemical exposure, and a subsequent 8-d recovery phase in clean water, and a short-term experiment in which samples were collected from females over the course of a 12-h exposure. Endpoints

All Supplemental Data may be found in the online version of this article.

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included plasma sex steroid (E2, testosterone [T]) and VTG concentrations, ex vivo steroid production by excised gonads, and expression of several ovarian and testicular genes coding for proteins involved in regulation of HPG function, including steroid production.

MATERIALS AND METHODS

Chemical exposure and analytical verification

Trilostane (98% pure) was obtained from Sanofi-Synthelabo Research.

Solvent-free stock solutions, designed to achieve target concentrations of 300 and 1,500 μg/L in the test tanks, were prepared in sand filtered, ultraviolet-treated, Lake Superior water (USA). These target concentrations were selected based on data from Villeneuve et al. [7], who found a significant decrease in egg production and female VTG concentrations in a 21-d fathead minnow test at 1,500, but not 300, μg trilostane/L. To confirm exposure conditions, water samples were collected periodically during tests and analyzed on an Agilent Technologies model 1100 high-pressure liquid chromatograph. Samples (100 μl) were directly injected onto a Zorbax SB-C18 column (2.1 × 75 mm; Hewlett-Packard) and eluted isocratically with 65% methanol/formic acid (25 mM) at a flow rate of 0.2 ml/min and column temperature of 30°C. Concentrations were determined using diode array absorbance at 254 nm and an external standard method of quantification. Quality control samples such as procedural blanks, spiked recoveries, and duplicate analyses constituted 10% of the sample load. The mean ± standard deviation (SD) agreement among duplicate analyses was 99.7 ± 0.4% ($n = 27$), and the average trilostane recovery was 93 ± 3.6% ($n = 20$). No trilostane was detected in any of the procedural controls (detection limit, 50 μg/L).

Experimental design

The experimental design and basic test conditions were similar to those described elsewhere for other types of HPG-active chemicals [13–15]. Fish were reproductively mature adult (4–6 months old) fathead minnows from an on-site culture facility. All procedures involving the fish conformed with institutional guidelines concerning the care and use of animals for experimental work. Exposures were conducted in glass aquaria containing 10 L water that was continuously renewed at a rate of approximately 45 ml/min with clean Lake Superior water (control) or trilostane dissolved in Lake Superior water. In the long-term study, 16 replicate tanks were used for each of three treatment groups (control, 300, and 1,500 μg trilostane/L). All tanks contained four male and four female fish. Fish from two replicate tanks per treatment were sampled 1, 2, 4, and 8 d after starting the trilostane exposure, and 1, 2, 4, and 8 d after stopping chemical delivery (i.e., depuration/recovery in clean Lake Superior water). A short-term study was conducted in conjunction with the long-term study by placing unexposed females from the culture into control or 1,500-μg/L treatment tanks after fish had been sampled from the tanks for the long-term test. Loading of the fish for the 12-h study was staggered so that two replicate tanks containing four females from each of the two groups were sampled after 1, 2, 4, 8, or 12 h of trilostane exposure. All exposures were conducted at 25 ± 0.5°C under a 16:8 light:dark photoperiod. In the long-term study, fish were fed twice daily to satiation with adult brine shrimp. Fish were not fed during the short-term study.

Sample collection and biological measurements

Fish were anesthetized with a buffered solution of tricaine methane sulfonate (MS-222; Argent). After weighing, blood was collected from a caudal incision using a heparinized microhematocrit tube. The blood was centrifuged to obtain plasma, which was stored at −80°C. Gonads were removed and weighed to calculate the gonadal-somatic index. Two subsamples of gonad tissue were snap frozen in liquid nitrogen for subsequent gene expression or metabolomic analyses. A third gonad subsample was used immediately for an ex vivo steroidogenesis assay. Additional tissues (e.g., liver, brain, pituitary) were collected and preserved but were not analyzed as part of the present work.

Plasma concentrations of VTG were measured using an enzyme-linked immunosorbent assay with a fathead minnow polyclonal antibody and fathead minnow VTG as a standard [16]. Plasma E2 and T concentrations were determined using a small-volume radioimmunoassay method [17]. Ex vivo steroid production in ovary and testis samples was determined with the basic method of McMaster et al. [18] as modified by Villeneuve et al. [15], using radioimmunoassay to measure T and E2 in the culture media.

Real-time quantitative polymerase chain reaction (QPCR) was used to measure expression of select genes in the ovaries and testes. Target genes included several whose expression had been observed to be altered in past studies in our laboratory with HPG-active chemicals (e.g., [13–15]), as well as gene products that plausibly could be linked to inhibition of 3β-HSD through either direct effects or compensation [19]. In testes, we evaluated expression of genes for 3β-HSD (*3βhsd*), 11β-HSD (*11βhsd*), lutenizing hormone receptor (*lhr*), follicle-stimulating hormone receptor (*fshr*), and CYP c17α-hydroxylase/17, 20-lyase (*cyp17*). Transcripts measured in ovaries included *3βhsd*, *11βhsd, 20βhsd*, *fshr*, *cyp17*, CYP side-chain cleavage (*cyp11a*), and aromatase (*cyp19a1a*). Primer and probe sequences for all of these genes have been described in other studies from our laboratory [7,20–22].

Samples for QPCR analyses were extracted and DNAse treated (DNA free; Applied Biosystems/Ambion), and 250 ng total RNA was reverse transcribed to complementary DNA (cDNA), using methods described by Biales et al. [23]. In most cases, transcripts were quantified using Taqman EZ reverse transcription polymerase chain reaction (RT-PCR) kits (Applied Biosystems). Reactions were 12 or 20 μl in volume and contained 2.0 μl template cDNA, 150 nM gene-specific Taqman probe, and 200 nM forward and reverse primers. Forty cycles of amplification (melt 94°C for 20 s, anneal and extend, 58°C for 60 s) were performed using a 7500 RT-PCR system (Applied Biosystems). Relative transcript abundance was estimated based on a standard curve consisting of multiple dilutions of a gene-specific messenger RNA standard. Data were not corrected for amplification efficiency, but all slopes of the standard curves were between −3.2 and −3.8, indicating no major variations in efficiency. Standards were prepared by ultra-high 7-yield in vitro transcription using Megascript T7 kits (Ambion/Applied Biosystems) as described previously [21].

For ovary samples from days 1, 2, and 8 of the exposure period, abundance of transcripts coding for *cyp17, cyp11a, 20βhsd, fshr,* and *3βhsd* were determined using Power SYBR Green PCR Master Mix (Applied Biosystems). SYBR Green reactions included 2.0 μl cDNA template, 200 nM forward and reverse primers, and 2X Master Mix in a 12.5-μl reaction. Forty cycles of amplification (melt 95°C, 15 s; anneal and extend

60°C, 60 s) were conducted on a 7500 RT-PCR system (Applied Biosystems). After amplification, a dissociation curve was generated for all samples (dissociation 95°C–60°C) to verify product specificity. Relative transcript abundance was estimated based on a standard curve generated by analyzing multiple dilutions of a gene-specific amplicon, initially amplified from fathead minnow cDNA using the same gene-specific primers used for QPCR. Dilution of the amplicons was optimized to yield a standard curve over threshold cycles ranging from approximately 15 to 35.

Data analysis

Approaches for data reduction and analysis were the same as described in Ankley et al. [14] and Villeneuve et al. [15]. Briefly, statistical evaluations focused on comparison of treatment groups within rather than across time points. To facilitate presentation, data (and their significance) are depicted as fold-change (log 2) relative to controls at each sampling time. Data normality and homogeneity of variance were determined with Kolmogorov-Smirnoff and Levene's tests, and parametric data were analyzed by one-way analysis of variance (three treatments) or *t* tests (two treatments). When analysis of variance indicated significance, treatment groups were compared using Duncan's multiple range or Dunnett's tests. Nonparametric data either were log10 transformed and compared using analysis of variance or analyzed using the Kruskal-Wallis test followed by Dunn's test. Results were considered significant at $p \leq 0.05$.

RESULTS

Exposure verification

Water samples were collected from all tanks containing fish on five occasions during the 8-d exposure phase of the long-term test and analyzed for trilostane. Overall, test concentrations were relatively stable and close to target values. The mean ± SD concentration of trilostane in the low treatment was 276 ± 49 μg/L ($n = 88$), and the average concentration in the high treatment was 1,553 ± 240 μg/L ($n = 96$). Within 1 d of ceasing delivery of trilostane to the test tanks (i.e., for the 8-d recovery phase of the experiment), the concentration of trilostane was below the detection limit (50 μg/L) in all treatment tanks. The mean concentration of trilostane in tanks used for the short-term exposures was 1,654 ± 60 μg/L ($n = 28$). No trilostane was detected in any of the control tanks over the course of the experiments.

Biological responses: Females

No treatment-related mortality of females was seen during the short- or long-term tests. The gonadal-somatic index did not vary in a significant or consistent manner as a function of treatment (data not shown).

Exposure to 1,500 μg trilostane/L significantly reduced plasma VTG concentrations in female fathead minnows (Fig. 1a). Vitellogenin levels were decreased to some extent within as little as 2 d of exposure and were significantly reduced by days 4 and 8 of the trilostane treatment. Concentrations of the

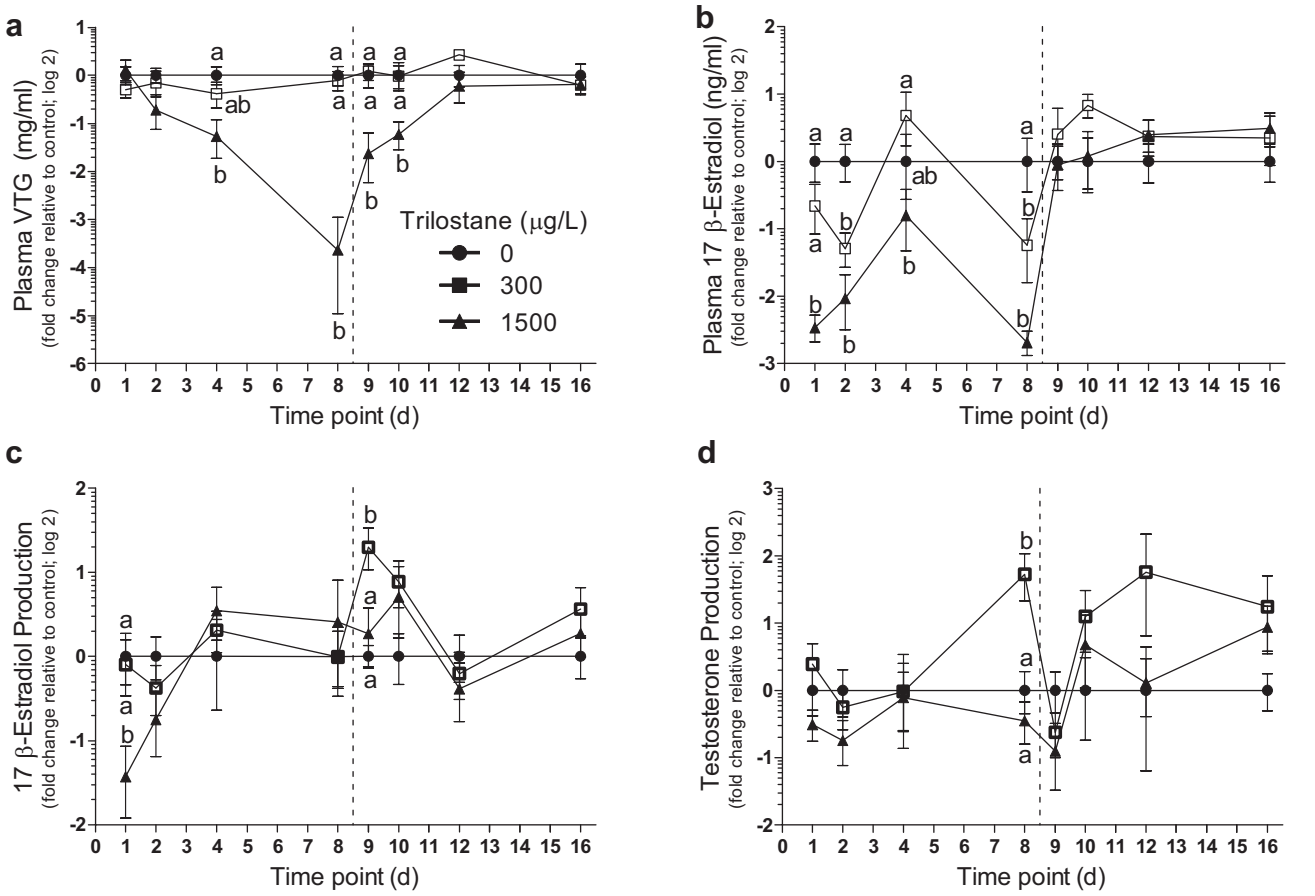

Fig. 1. Effects of an 8-d trilostane exposure/recovery on female fathead minnow: (**a**) plasma vitellogenein concentration, (**b**) plasma 17β-estradiol (E2) concentration, (**c**) ex vivo ovarian E2 production, and (**d**) ex vivo ovarian testosterone production. Vertical dashed line indicates cessation of chemical delivery. Data points indicate the mean ± standard error of the mean fold-change, relative to controls from the same time point, expressed as log 2 transformed units. Different lowercase letters indicate statistically significant differences between treatments, within a given day.

lipoprotein remained decreased on days 1 and 2 of the recovery phase of the test and returned to levels comparable to those of controls by day 8 of depuration. Effects of 300 μg trilostane/L on VTG in females were less pronounced, with a slight depression observed only on day 4 of the exposure phase of the test (Fig. 1a).

Trilostane also decreased plasma E2 concentrations in the females (Fig. 1b). In the 1,500 μg/L group, E2 concentrations were depressed within 1 d and remained significantly lower than controls throughout the 8-d exposure. Within 1 d of cessation of trilostane exposure, plasma concentrations of E2 in the 1,500-μg/L treatment group had returned to control levels. The 300 μg/L treatment also decreased plasma concentrations of E2 in the females, although to a much lesser extent than the higher treatment (Fig. 1b). In the lower treatment, E2 concentrations were significantly reduced by 2 d, returned to levels comparable to controls at 4 d, were again significantly depressed at 8 d and, finally, returned to concentrations comparable to or higher than controls during the recovery phase of the experiment.

Because plasma E2 concentrations were reduced within 1 d in the longer study, this endpoint was examined further in samples from the shorter-term time course. In that experiment, we found that 1,500 μg trilostane/L significantly reduced plasma E2 concentrations within 2 h of exposure, and that E2 remained depressed at the 4-, 8-, and 12-h sampling times (Fig. 2a).

Given the marked reductions in plasma E2 concentrations associated with the trilostane exposure, we anticipated that ex vivo steroid production also would be depressed. However, this was not the case. Only one occurrence was seen, E2 on day 1 in the 1,500-μg/L treatment, where ex vivo steroid production was lower than in controls in either the short- or long-term study (Figs. 1c, d; 2b, c). In two instances, ex vivo steroid production in trilostane-treated females was higher than the controls, E2 in the 300-μg/L treatment on day 1 of the recovery phase of the longer study, and T in fish from the same treatment group on day 8 of the exposure period (Figs. 1c, d).

Initial analysis of QPCR data suggested consistent transcriptional up-regulation in the trilostane-treated females on day 4 of the exposure, regardless of the gene examined (Supplemental Data, Fig. S1). However, 11 samples (one from 300 μg/L, day 1 of exposure; five from 300 μg/L, day 4 of exposure; four from 1,500 μg/L, day 4 of exposure; and one from 1500 μg/L, day 1 of recovery) were identified as extremes based on principal components analysis of the fold-change data for the seven genes analyzed across all time points (Supplemental Data, Fig. S2). These samples typically had fold change values far in excess of those observed in the same treatment group at any other time point. For example, these samples had fold changes of 8 to17 for *11βhsd*, compared with a mean (± SD) fold-change of 1.58 ± 2.1 for the 300-μg/L group and 1.57 ± 2.7 for the 1,500-μg/L group, respectively. This consistent, extreme response among different transcripts was not explained by either differences in cDNA template concentration, as determined spectrophotometrically (Nanodrop ND-1000 spectrophotometer; Nanodrop Technologies) or differences in 18S ribosomal RNA abundance (data not shown). Nonetheless, the unusual consistency of responses among different genes and the pronounced nature of the observed fold-change values suggested that data from these 11 samples were likely technical artifacts. Consequently, we conducted subsequent analyses without the 11 suspect samples identified by principal component analysis. Figures in the *Results* section are based on the censored data; however, for transparency, figures depicting initial analysis of the complete dataset also are provided (Supplemental Data, Fig. S2).

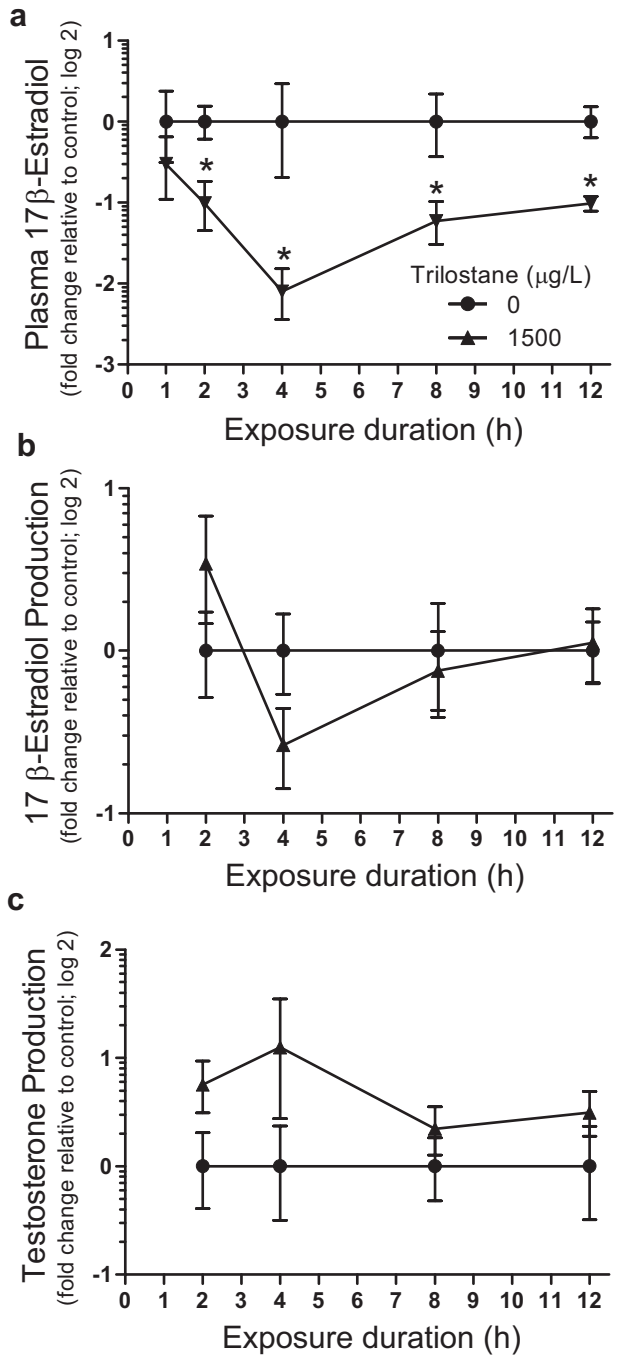

Fig. 2. Effects of a 12-h trilostane exposure on female fathead minnow: (**a**) plasma 17β-estradiol (E2) concentration, (**b**) ex vivo ovarian E2 production, and (**c**) ex vivo ovarian testosterone (T) production. The 1-h ex vivo data have been omitted because of a sampling error. Data points indicate the mean ± standard error of the mean fold-change, relative to controls from the same time point, expressed as log 2 transformed units. Asterisks indicate statistically significant differences between treatments at a given time point.

Ovarian expression of seven genes was monitored over the entire time course of the long-term study. No consistent changes were seen in expression of *3βhsd*, *11βhsd,* or *20βhsd*, nor were significant changes found in *cyp11a* or *cyp17* expression (Fig. 3a–e). Ovarian transcripts of *cyp19a1a* were most abundant in fish exposed to 1,500 μg trilostane/L, with significant

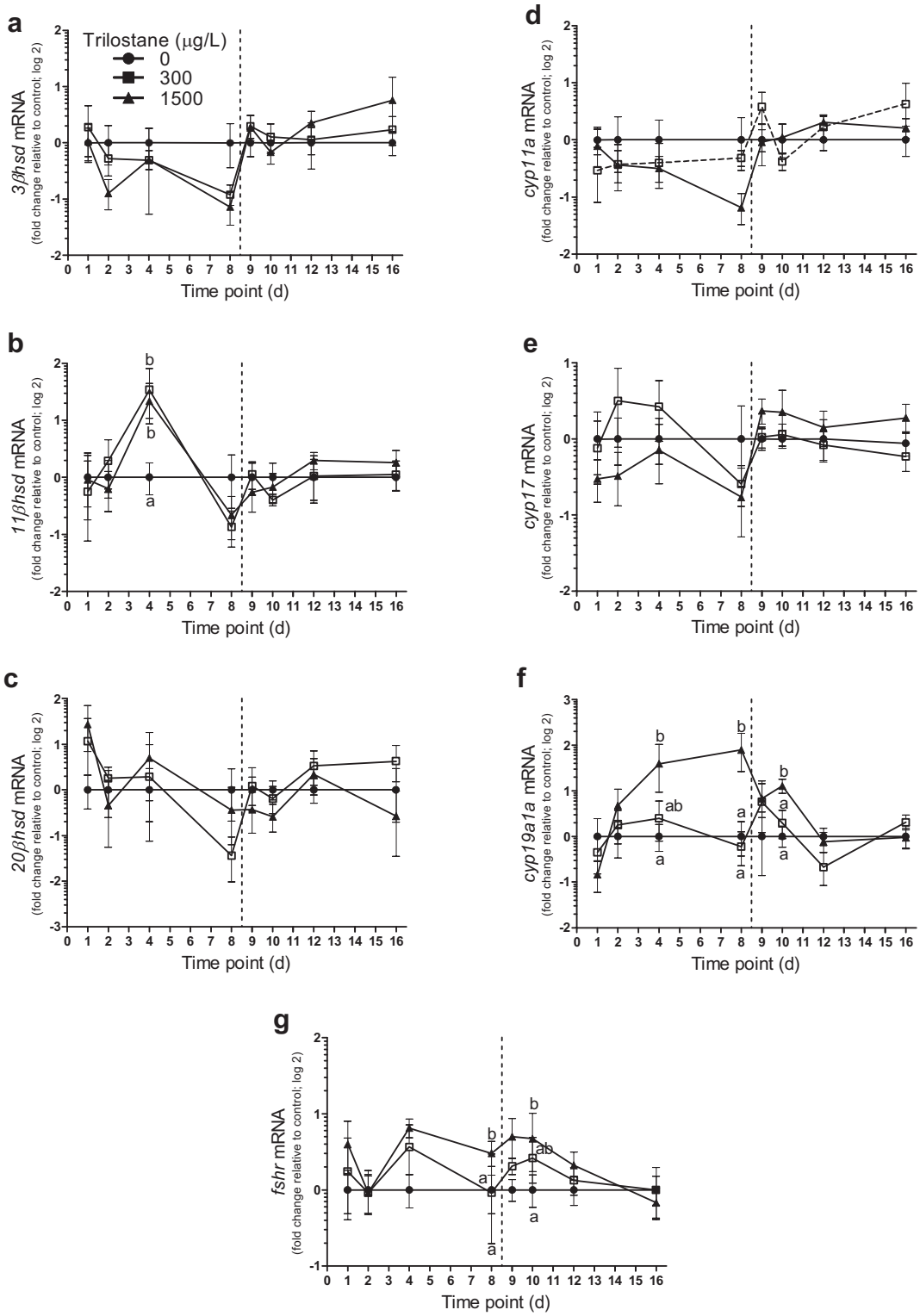

Fig. 3. Effects of an 8-d trilostane exposure/recovery on ovarian expression in fathead minnows of: (**a**) *3βhsd*, (**b**) *11βhsd*, (**c**) *20βhsd*, (**d**) *cyp11a*, (**e**) *cyp17*, (**f**) *cyp19a1a*, and (**g**) *fshr*. Vertical dashed line indicates cessation of chemical delivery. Data points indicate the mean ± standard error of the mean fold-change, relative to controls from the same time point, expressed as log 2 transformed units. Different lowercase letters indicate statistically significant differences between treatments, within a given day. Data from 11 suspect samples were excluded (see text for details).

differences occurring on days 4 and 8 of the exposure and day 2 of the recovery phase of the test (Fig. 3f). A slight elevation of *cyp191a1* expression was apparent in the 300-μg/L treatment on day 4 of the exposure (Fig. 3f). Expression of ovarian *fshr* also was affected by trilostane; transcript levels were significantly increased in the 1,500-μg/L treatment group on day 8 of the exposure (Fig. 3g).

Biological responses: Males

No male fish died in the study, and gonadal-somatic index values in trilostane-exposed males did not differ significantly from controls (data not shown). Plasma concentrations of VTG in males were not affected by trilostane, remaining low to undetectable during the 8-d exposure (data not shown).

Trilostane had no significant effect on plasma T concentrations in males over the course of the 8-d exposure followed by 8 d of recovery (Fig. 4a). Ex vivo production of T by testis was significantly affected on one occasion; a slight increase over controls occurred on day 1 of the recovery phase in the 300-μg/L treatment group (Fig. 4b).

Initial QPCR analyses were conducted on testis samples from fish exposed to trilostane for 1, 4, or 8 d (Fig. 5a–e). No changes in expression of *cyp17* and *lhr* were observed on these days, so no additional time points were examined. A complete time course dataset was generated for *fshr, 11βhsd*, and *3βhsd* regulation. Expression of *fshr* was significantly elevated by trilostane on exposure day 1 in the 1,500-μg/L treatment and on exposure day 4 in the 300-μg/L (and, perhaps, 1,500-μg/L) group (Fig. 5a). Both *3βhsd* and *11βhsd* transcripts were significantly elevated by trilostane only on exposure day 4; this response was neither concentration dependent nor consistent over time (Fig. 5c, e).

DISCUSSION

3β-Hydroxysteroid dehydrogenases catalyze the formation of key intermediates in the production of both corticosteroids and sex steroids [1]. The properties of trilostane relative to inhibition of 3β-HSDs were first described in the open literature more than 30 years ago [9]. Originally developed to decrease synthesis of corticosteroids, trilostane has been used in both humans and pets (e.g., dogs), as a therapeutic agent for various conditions arising from hypercortisolism [8,24,25]. Although possibly less effective for inhibiting sex steroid synthesis [9], trilostane also has been used in treatment of estrogen-dependent breast cancer [26,27]. Interestingly, trilostane seems to act as an anti-estrogen not only through inhibition of sterod synthesis but via direct antagonism of the estrogen receptor [28].

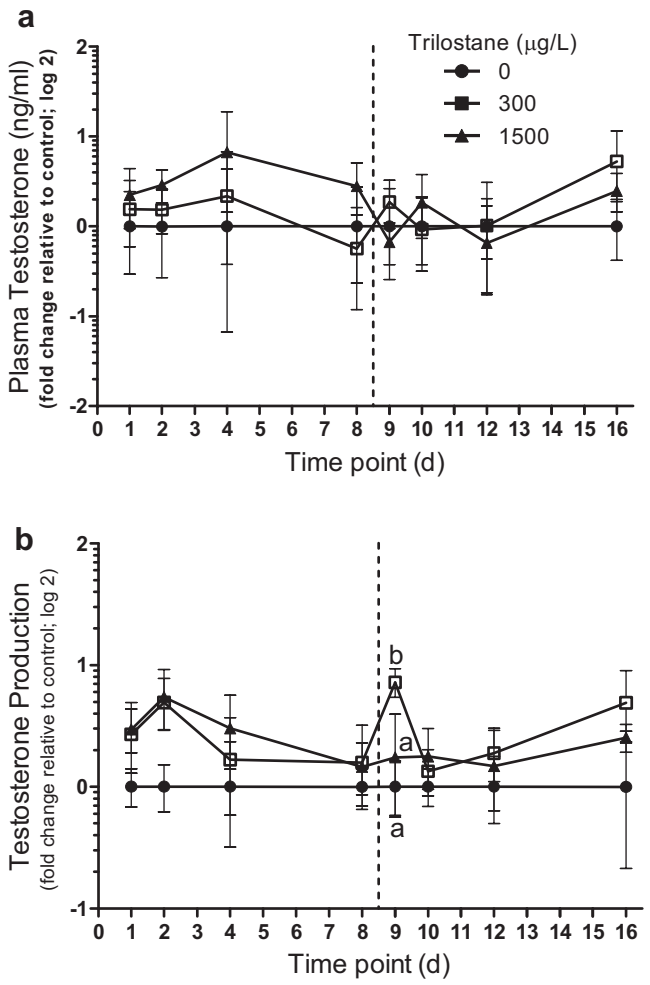

Fig. 4. Effects of an 8-d trilostane exposure/recovery on male fathead minnow: (**a**) plasma testosterone (T) concentration, and (**b**) ex vivo testicular T production. Vertical dashed line indicates cessation of chemical delivery. Data points indicate the mean ± standard error of the mean fold-change, relative to controls from the same time point, expressed as log 2 transformed units. Different lowercase letters indicate statistically significant differences between treatments, within a given day.

Most research with trilostane has been done in mammals; however, the drug also can inhibit sex steroid synthesis in fish. For example, Ohta et al. [29] used trilostane to inhibit androgen production in studies with Japanese eel testis cultures. In vitro studies in our laboratory with fathead minnow ovaries showed that trilostane inhibits E2 synthesis when hydroxy-cholesterol is used as a precursor, but not progesterone, a substrate that does not require 3β-HSD to produce E2 [7]. Complementary in vivo studies showed that exposure of spawning fathead minnows to waterborne trilostane (1,500 μg/L) for 21 d reduced egg production, concomitant with reduced plasma E2 and VTG concentrations in females. In males, trilostane concentrations as low as 50 μg/L increased size of the testis, which we hypothesized could be a compensatory response of the fish to decreased androgen signaling [7].

The present work expands on the findings of Villeneuve et al. [7] by examining the effects of trilostane on HPG function in fathead minnows over time, during both exposure and recovery. Consistent with our original work, a test concentration of 1,500 μg trilostane/L significantly reduced plasma concentrations of E2 and VTG in females, whereas 300 μg/L of the drug caused little or no effect on either endpoint. In this study, we found that exposure to trilostane affects plasma E2 concentrations in female fathead minnows very rapidly—within 2 h—and that recovery to control E2 levels after cessation of exposure also occurs quickly (≤1 d). Consistent with the role of E2 in stimulating vitellogenesis, effects of trilostane on concentrations of VTG in the females lagged behind changes in circulating E2 levels, with respect to both initial impact and recovery. Nonetheless, VTG concentrations that had been depressed to less than 10% of control levels after 8 d exposure to 1,500 μg trilostane/L fully rebounded by the end of the 8-d recovery phase of the test. The return of E2 and VTG to control levels in fish removed from the trilostane exposure demonstrates the highly dynamic/adaptive nature of the HPG axis in response to contaminant stress.

Studies documenting the temporal effects of chemicals on HPG function are rare. However, these types of studies are critical to developing approaches for assessing the risk of endocrine-active materials. For example, identification of optimal experimental designs for detecting alterations in HPG function for laboratory-based chemical screening programs requires knowledge of how quickly responses occur, as well as how long changes persist both during and after exposure. Knowledge of chemically induced alterations in HPG function over time also is critical in field monitoring work, especially because most environmental exposures are not constant, but fluctuating. To address the need for time-course data, we have conducted studies with the fathead minnow using an experimental design similar to that described here for trilostane with a series of chemicals that perturb other pathways within the HPG axis [11]. Results to date indicate that the rapidity of responses of the HPG axis with respect to initial effects and recovery are

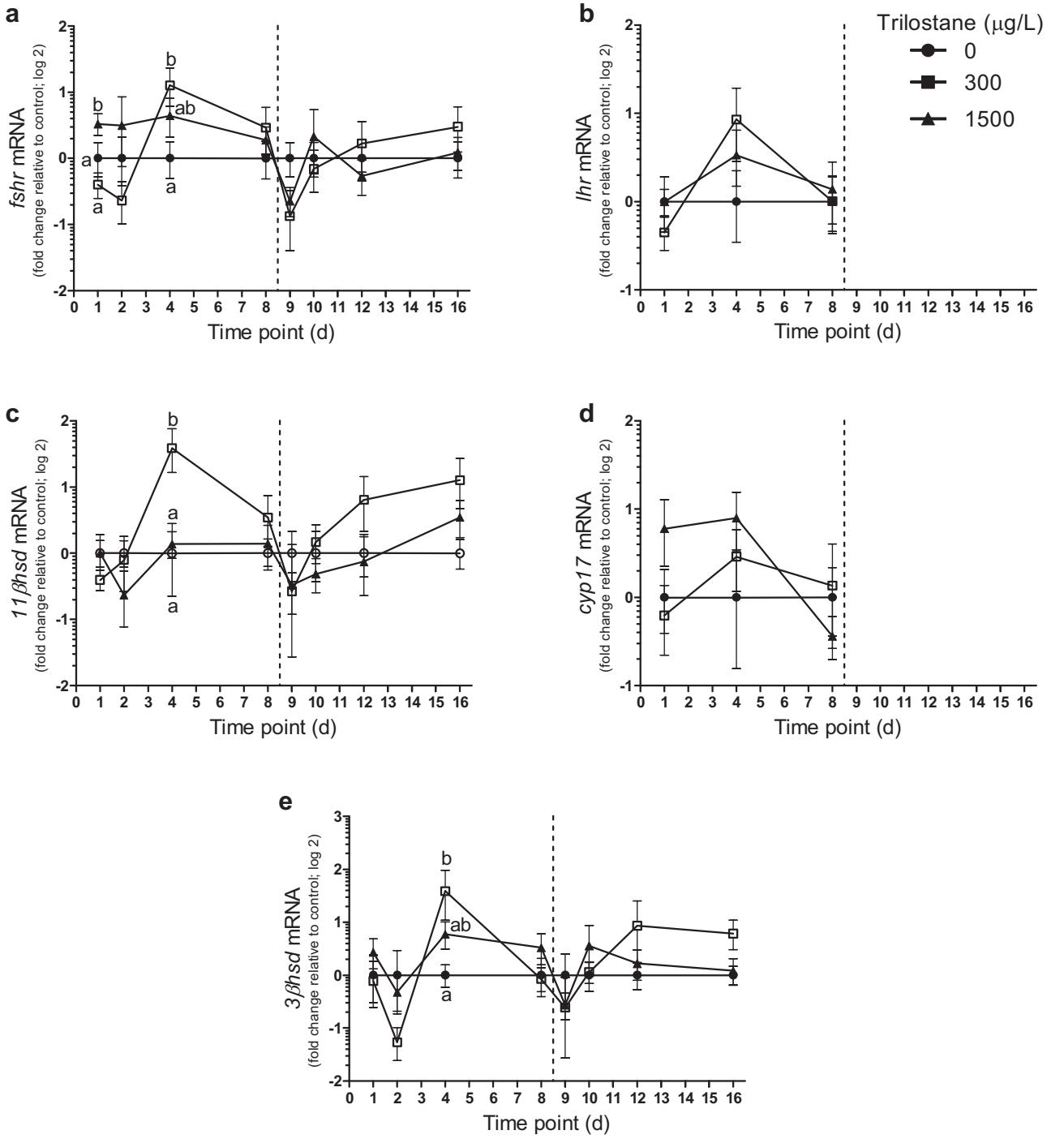

Fig. 5. Effects of an 8-d trilostane exposure/recovery on testicular expression in fathead minnow of: (**a**) *fshr*, (**b**) *lhr*, (**c**) *11βhsd*, (**d**) *cyp17*, and (**e**) *3βhsd*. Vertical dashed line indicates cessation of chemical delivery. Data points indicate the mean ± standard error of the mean fold-change relative to controls from the same time point, expressed as log 2 transformed units. Different lowercase letters indicate statistically significant differences between treatments, within a given day.

not unique to trilostane. For example the fungicide prochloraz, which decreases steroidogenesis through inhibition of CYP17 and CYP19, can depress circulating E2 concentrations in female fathead minnows after as little as 6 h treatment, with recovery to control levels within 1 d of cessation of an 8-d exposure [14,30]. We have observed comparable behavior in plasma E2 concentrations in female fathead minnows exposed to fadrozole, a relatively specific inhibitor of CYP19 [15].

An important component of the rapid recovery of plasma steroid titers in fish exposed to inhibitors of steroidogenesis seemingly involves up-regulation of key ovarian genes. For example, past work in our laboratory with the fathead minnow, as well as research with other fish species (medaka, zebrafish), has shown that exposure to a variety of steroid synthesis inhibitors (fadrozole, prochloraz, ketoconazole) causes up-regulation of any of a number of ovarian transcripts of proteins involved in steroidogenesis, such as *fshr*, *star*, *cyp11a*, *cyp17,* and *cyp19a1a* [14,15,30–33]. In our present study, expression of both *fshr* and *cyp191a1* was up-regulated in trilostane-exposed females. Patterns in up-regulation of the ovarian transcripts in response to exposure to inhibitors of steroidogenesis appear to reflect compensation within the

HPG axis, presumably in response to decreased sex steroid levels/signaling. In time-course studies, up-regulation of the genes closely tracks temporal changes in plasma E2 levels occurring, for example, within hours of exposure (see Skolness et al. [30]) and persisting until after chemical treatment is terminated [14,15]. The gene most commonly affected across the studies listed—and generally exhibiting the greatest fold-change—is *cyp19a1a*, which highlights the key role aromatase activity plays in influencing T and E2 balance.

An interesting change in gene expression observed in the current study in males was an up-regulation in testicular *fshr*. This response occurred in both treatment groups during the 8-d trilostane exposure. Possibly *fshr* messenger RNA up-regulation is indicative of compensation to decreased androgen synthesis in the males, because follicle-stimulating hormone has been shown to stimulate androgen production in fish [34–36]. The veracity of this explanation in the context of the current study is uncertain, however, because trilostane did not cause significant decreases in plasma concentrations or ex vivo testicular T production.

Results of the present study with trilostane were inconsistent with our past work in one notable regard—the linkage between ex vivo steroid production and plasma concentrations of sex steroids. Although it is sometimes a relatively variable endpoint, we have consistently found ex vivo data to be a robust predictor of the status of in vivo steroid levels in fish exposed to a variety of HPG-active chemicals. For example, in time-course studies with fadrozole and prochloraz, changes in ex vivo ovarian production of E2 were accompanied by, and sometimes even preceded, alterations in plasma E2 concentrations in fathead minnows [14,15]. This is entirely consistent with expectations, because circulating sex steroids are derived largely from production in the gonad. In the case of trilostane, however, no discernible, consistent effects on ovarian production of E2 were found, although marked decreases in plasma concentrations of E2 occurred in exposed females. This observation is particularly counterintuitive in light of studies by Villeneuve et al. [7], which demonstrated that trilostane does effectively inhibit E2 production when the chemical is added directly to ovarian slices from control fish, and incubated under the same conditions as used for the ex vivo assay. What could cause this apparent disconnect is uncertain. One possibility is that trilostane, which is a reversible inhibitor of 3β-HSD, is not present at sufficient concentrations in ovaries of exposed animals to continually inhibit steroid production over the course of the 12-h ex vivo assay, perhaps because of metabolism of the chemical [37,38]. Further work is needed to ascertain the degree to which this might explain differences in the effects of trilostane on ex vivo versus in vivo indications of the status of E2 production.

Data from this study increase our understanding of the dynamic nature of changes in the HPG axis of animals exposed to chemicals that inhibit steroidogenesis. Although some variations exist from study to study, basic responses of fathead minnows to the 3β-HSD inhibitor trilostane are remarkably similar to those observed when fish are exposed to inhibitors of steroidogenic CYPs, such as fadrozole and prochloraz. Exposure of fathead minnows to trilostane produced relatively rapid (within hours) effects on HPG function relative to plasma E2 concentrations, which resulted in depressed VTG production in females. Inhibition of steroid synthesis resulted in changes in ovarian gene expression indicative of compensation in females and, perhaps, in males. Once the chemical stress was removed, indications of normal HPG function in the fish were observed within days. From a toxicological perspective, effects caused by trilostane are also very similar to those observed in response to chemical inhibitors of steroidogenic CYPs. Specifically, although trilostane, prochloraz, and fadrozole depress sex steroid synthesis via inhibition of different enzymes, the net result of this inhibition is the same for all three chemicals: decreased circulating E2 and VTG in females and, ultimately, depressed egg production. This work reinforces the utility of adverse outcome pathways based on decreased vitellogenesis in fish for predicting impacts on individuals and populations of specific classes of endocrine-active chemicals [39].

SUPPLEMENTAL DATA

Figure S1. Effects of an 8-d trilostane exposure/recovery on ovarian expression in fathead minnows of: (a) *3βhsd*, (b) *11βhsd*, (c) *20βhsd*, (d) *cyp11a*, (e) *cyp17*, (f) *cyp19a1a*, and (g) *fshr*. Vertical dashed line indicates cessation of chemical delivery. Data points indicate the mean ± standard error of the mean fold-change, relative to controls from the same time point, expressed as log 2 transformed units. Different letters indicate statistically significant differences between treatments, within a given day. Figure S1 corresponds to Figure 3 in the Results, except data from 11 suspect samples were included (see text for details).

Figure S2. Principal components analysis of the seven QPCR endpoints analyzed in the ovaries of fathead minnows exposed to trilostane (control, 300 and 1500 μg/l) for 1, 2, 4, and 8 d, followed by a recovery phase of 1, 2, 4, and 8 d. The plot includes data from all three treatments and all time points (exposure and recovery period), normalized to control means within each sampling time and endpoint. Numbers indicate our internal sample identification code. Square markers indicate samples identified as extremes, which were excluded from subsequent analyses. (49 KB PDF)

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