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USE OF GENE EXPRESSION, BIOCHEMICAL AND METABOLITE PROFILES TO ENHANCE EXPOSURE AND EFFECTS ASSESSMENT OF THE MODEL ANDROGEN 17β-TRENBOLONE IN FISH

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Abstract—The impact of exposure by water to a model androgen, 17β-trenbolone (TRB), was assessed in fathead minnows using an integrated molecular approach. This included classical measures of endocrine exposure such as impacts on testosterone (T), 17β-estradiol (E2), and vitellogenin (VTG) concentrations in plasma, as well as determination of effects on the hepatic metabolome using proton nuclear magnetic resonance spectroscopy. In addition, the rates of production of T and E2 in ovary explants were measured, as were changes in a number of ovarian gene transcripts hypothesized to be relevant to androgen exposure. A temporally intensive 16-d test design was used to assess responses both during and after the TRB exposure (i.e., depuration/recovery). This strategy revealed time-dependent responses in females (little impact was seen in the males), in which changes in T and E2 production in the ovary, as well as levels in plasma, declined rapidly (within 1 d), followed shortly by a return to control levels. Gene expression measurements revealed dynamic control of transcript levels in the ovary and suggested potential mechanisms for compensation during the exposure phase of the test. Proton nuclear magnetic resonance spectroscopy revealed a number of hepatic metabolite changes that exhibited strong time and dose dependence. Furthermore, TRB appeared to induce the hepatic metabolome of females to become more like that of males at both high test concentrations of TRB (472 ng/L) and more environmentally relevant levels (33 ng/L). Environ. Toxicol. Chem. 2011;30:319–329.
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INTRODUCTION

Aquatic ecosystems receive a variety of potentially harmful chemicals and their byproducts through point and nonpoint source inputs. Some of these chemicals possess endocrine activities that may produce adverse effects in exposed fish and other aquatic animals. Many reports have been made of xenoestrogens and their impacts in the environment. However, recent studies also have highlighted the occurrence of androgenic environmental contaminants. For example, Parks et al. [1] reported androgen receptor (AR) binding and induction of androgen-dependent gene expression in in vitro systems exposed to water from sites downstream of a pulp and paper mill plant on the Fenholloway River in Florida, USA. As part of the same study, masculinized female mosquitofish (*Gambusia holbrooki*) also were collected from sites downstream of the discharge.

Another source of androgenic chemicals, particularly in the United States, is concentrated animal feeding operations. These facilities often employ suites of steroid hormones to stimulate growth of muscle mass to rapidly increase yield or prevent loss during transport. Once excreted by the animals, these chemicals and their metabolites/degradates can pollute nearby rivers and streams, thus potentially affecting resident organisms. For example, Orlando et al. [2] reported in vitro androgenic activity in samples from a water body adjacent to a beef concentrated animal feeding operation in Nebraska and found decreased gonad sizes in fathead minnows (*Pimephales promelas*) collected at the site. The potent synthetic steroid, trenbolone acetate (17β-hydroxy-estra-4,9,11-trien-3-one-17-acetate), is the principal androgenic compound used in beef feeding operations. The more active isomer (likely attributable to higher AR binding affinity), 17β-trenbolone (TRB), was identified by Durhan et al. [3] in several water samples collected from a stream near a beef concentrated animal feeding operation in Ohio in which steroid implants were used. Using in vitro assays, water samples collected from discharge at the feedlot also were shown to display significant androgenic activity.

The extensive use of TRB in the United States and other countries, as well as its recognized potency, has stimulated research with the androgen in fish. For example, a 14-d water exposure of Japanese medaka (*Oryzias latipes*) to 0.2 μg/L TRB resulted in reduced fecundity [4]. Ankley et al. [5] found that exposure of female fathead minnows to 0.027 μg/L waterborne TRB for 21 d produced male secondary sexual characteristics (dorsal nuptial tubercles) as well as reduced plasma vitellogenin (VTG) and sex steroid concentrations, and ultimately, egg production. In mosquitofish (*Gambusia affinis*), Sone et al. [6] reported masculinization effects such as the development of a gonopodium from the female anal fin associated with water exposure concentrations ranging from 1 to 10 μg TRB/L.

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Although these earlier studies have reported effects of TRB exposure on a variety of potentially useful exposure indicators (e.g., gene transcripts, proteins, whole organism endpoints), to our knowledge the impact of the androgen on endogenous cellular metabolites has not been evaluated. Collection of these types of data would enhance researchers' abilities to accurately define the impacts of exposure to environmental androgens such as TRB. In addition, the potential for defining adverse outcome pathways when integrating exposure response data from various biological levels of organization (gene transcripts, proteins, and metabolites) is considerable, because each level provides different insights into biological responses [7]. Finally, metabolite data often help to bridge observed changes in transcript and protein levels with previously reported histopathological or whole organism studies [8].

For these reasons, we sought to determine changes in the hepatic metabolite profiles of fathead minnows (FHM) exposed to TRB. As part of these studies we conducted measurements of steroid (testosterone, T; 17β-estradiol, E2) production in gonads as well as effects of TRB on T, E2, and VTG concentrations in plasma. These measurements were included both to assess the temporal nature of steroid levels in gonads and plasma during and after depuration exposure to TRB and to confirm the occurrence of androgen-induced effects as a basis for anchoring and interpreting the metabolomic analyses. Finally, we also determined changes in levels of select gene transcripts in the ovary that should reflect direct effects of, and compensation for, androgen exposure in fish. Evaluation of changes in gene expression, biochemical function, and metabolite profiles in the fish enables a more integrated assessment of overall function of the reproductive endocrine system of the fish rather than isolated measurements of just a few components of the system.

Finally, that organisms' responses to chemical exposure often possess a significant time dependency is well established. Therefore, an intense sampling time course was employed to assess the temporal dependence of these endpoints both during and after exposure to TRB. Such temporal information is essential for determining how quickly responses occur after an exposure event, as well as the extent of an organism's ability both to compensate for the effects of an exposure and to recover once the exposure has ceased.

MATERIALS AND METHODS

Experimental design and sample collection

Sexually mature (5–6-month-old) male and female FHM obtained from an on-site culture facility at the U.S. Environmental Protection Agency laboratory in Duluth, MN, were maintained in glass aquaria in a continuous flow of Lake Superior water. The fish were held at 25°C with a 16:8 photoperiod throughout the duration of the exposure (as well as the depuration/recovery phase). The basic experimental design used for this work was similar to that described by Villeneuve et al. [9]. Briefly, exposures were conducted using 16 replicate tanks per treatment group (0, 50, 500 ng TRB/L [nominal]). The TRB concentrations were chosen both to bracket those known to produce significant alterations in classical biochemical, histological, and whole animal measures, while still reflecting (in the low treatment) levels that could occur in the environment [3]. Fish were fed adult brine shrimp (*Artemia*) twice daily, and breeding tiles (four per tank) were provided for cover. A stock solution of TRB (Sigma) was dissolved in ultraviolet-filtered Lake Superior water without the use of a carrier solvent and delivered continually after appropriate dilution to the test tanks at a flow rate of approximately 45 ml/min. Twenty-four hours after starting chemical delivery, exposures were initiated by randomly assigning four male and four female FHMs to each tank. The time of fish addition and sampling was staggered by replicate so that all samples were collected within 1 h of the intended exposure duration. All of the fish from two replicate tanks per treatment group were sampled at 1, 2, 4, and 8 d of the exposure and at 1, 2, 4, and 8 d after the chemical delivery ceased (i.e., depuration/recovery). Fish were anesthetized in a buffered solution of tricaine methanesulfonate (MS-222; 100 mg/L buffered with 200 mg/L $NaHCO_3$/L; Finquel, Argent). Blood was collected from the caudal artery/vein with a microhematocrit tube, and plasma was isolated by centrifugation for 3 min at 15,000 relative centrifugal force. Fish were weighed, and livers and gonads were removed, transferred to preweighed microcentrifuge tubes, flash-frozen in liquid nitrogen, and stored at −80°C until extracted or analyzed. A portion of the gonad samples also was used immediately for the ex vivo steroidogenesis assay (see later discussion). All laboratory procedures involving animals were reviewed and approved by the United States Environmental Protection Agency Mid-Continent Ecology Division Animal Care and Use Committee in accordance with Animal Welfare Act regulations and Interagency Research Animal Committee guidelines.

Exposure verification

Water samples (1 ml) were collected from the control and treatment tanks four times during the course of the exposure period as well as at the beginning of the depuration/recovery period. These samples were analyzed for TRB by reverse-phase high-pressure liquid chromatography using an Agilent model 1100 high-pressure liquid chromatography device equipped with a capillary pump, chilled auto sampler (4°C), heated column compartment (35°C), and a fluorescence detector. A 500-μl aliquot of sample was injected onto a Zorbax (Agilent) SB-C18 column (2.1 × 75 mm) and eluted isocratically with 70% methanol/water at a flow rate of 0.15 ml/min. The TRB concentrations were determined using the response at an excitation wavelength of 364 nm and emission wavelength of 460 nm, with an external standard method of quantitation.

Routine quality assurance analyses (procedural blanks, spiked matrix, and duplicate samples) were conducted with each sample set and constituted approximately 10% of all samples. The TRB standards were prepared in 10% methanol/water, and spiked matrix samples were prepared by diluting an aliquot of a TRB solution (prepared in methanol) with Lake Superior water (resulting concentration of approximately 100 ng/L). The TRB was obtained from Sigma (>99% pure), and all solvents were chromatography- and high-pressure liquid chromatography–grade or better.

Biochemical analyses, real-time quantitative polymerase chain reaction

Plasma VTG concentrations were determined using an enzyme-linked immunosorbent assay with a polyclonal antibody to FHM VTG [10,11]. Purified FHM VTG protein was used as a standard. Ex vivo production of T (testis, ovary) and E2 (ovary) was determined using an adaptation of the method of McMaster et al. [12], as described by Villeneuve et al. [9], using radioimmunoassay to measure steroids released into the culture medium over a 12-h incubation period. Plasma concentrations of T and E2 in the fish were also determined by radioimmunoassay [13].

The relative abundance of ovarian messenger RNA transcripts coding for fathead minnow aromatase A (CYP19A), cytochrome P45011A (CYP11A), vitellogenin receptor, and AR was determined by quantitative real-time polymerase chain reaction. Ovary samples were extracted, DNAse treated (DNA-free; Applied Biosystems/Ambion), and then 250 ng total RNA was reverse-transcribed to complementary DNA, using methods described by Biales et al. [14]. Assays were conducted as described previously, using Taqman-based methods for analysis of CYP19A, CYP11A, and AR [15] and a SYBR Green–based method for vitellogenin receptor [15]. Relative transcript abundance was estimated based on standard curves generated by analyzing multiple dilutions of a gene-specific amplicon without correction of amplification efficiency (see Ankley et al. [15] for details on standard preparation). Expression of CYP19A, CYP11A, AR, and vitellogenin receptor were evaluated at all treatments and time points, with the exception of samples from the low concentration of TRB on d 16, which were excluded because of poor complementary DNA quality.

Differences in VTG levels between treatments were assessed using analysis of variance (ANOVA) followed by Duncan's multiple range test. Analyses were performed with SYSTAT 9.0 for Windows, and differences were considered significant at $p < 0.05$. The quantitative real-time polymerase chain reaction and radioimmunoassay measurements were conducted such that for any given sampling day, all treatment groups were analyzed in the same assay. This limited the degree to which interassay variability might confound interpretation of treatment effects, but was not ideal for making comparisons across time. Hence, initial statistical analyses were focused on comparisons within, rather than between, time points. Data normality and homogeneity of variance were assessed by using Kolmogorov-Smirnov and Levene's tests, respectively. Parametric data were analyzed using one-way ANOVA with chemical treatment as the independent variable. Duncan's multiple range test was used to determine differences between treatment groups. Data that did not meet parametric assumptions were either transformed (log 10) and analyzed by ANOVA or analyzed using the Kruskall-Wallis test followed by Dunn's post-hoc test. Results were considered significant at $p < 0.05$. Ex vivo steroid data were analyzed as described previously; however, before applying a one-way ANOVA, a general linear models ANOVA was used to evaluate whether steroid production varied significantly as a function of ex vivo tissue mass (mean $\pm$ SD, n; ovary 12.1 ± 7.1 mg, 183; testis 5.1 ± 2.6 mg, 188). Because no significant effects of tissue mass were detected, standard single factor analyses were applied to determine treatment-related effects on ex vivo steroid production. To facilitate presentation of the results, VTG, quantitative real-time polymerase chain reaction, and steroid radioimmunoassay data are presented as fold-change (log 2) relative to control values. Statistical analyses were conducted before fold-change calculation.

Preparation and nuclear magnetic resonance analysis of livers

Liver samples were extracted using a dual-phase procedure adapted from Viant [16]. This procedure generates both a polar and lipophilic metabolite phase. For the current study, only the polar phase was analyzed. All liver samples were processed as described by Ekman et al. [17], with the exception that phase lock gel (Brinkmann Instruments) was used to separate the polar and lipophilic phases after homogenization. Samples were processed in a randomized fashion batchwise. A processing error resulted in the loss of a batch (approximately 15% of the samples). Before nuclear magnetic resonance (NMR) analysis, solvents were removed from the samples by drying using a vacuum concentrator (Thermo Scientific). Each sample was then reconstituted in 260 μl 0.1 M sodium phosphate buffered deuterium oxide (pH 7.4) containing 25 μM sodium 3-(trimethylsilyl) propionate-2,2,3,3-d_4, vortexed briefly, and centrifuged at 10,600 relative centrifugal force for 15 min at 4°C to remove any insoluble components. The resulting supernatant was then pipetted into a standard 3-mm NMR tube.

For each spectrum, 256 scans were collected, using a spectral width of 7,200 Hz and an acquisition time of 2 s at 20°C. Suppression of the residual water resonance was achieved by a 1-s presaturation delay at a field strength of 40 Hz. To confirm metabolite identities, two-dimensional NMR experiments were conducted. Spectral assignments were made using the results of these experiments in conjunction with previously reported values of metabolites in various biological media [18].

NMR data analysis

Spectra were processed using ACD/1D NMR Manager (Advanced Chemistry Development). Specifically, spectra were zero-filled to 32,768 points, and exponential line broadening of 0.3 Hz was applied before Fourier transformation. An automated routine was used to phase and baseline-correct each spectrum, which was then referenced to sodium 3-(trimethylsilyl) propionate-2,2,3,3-d_4 (at 0.0 ppm). The spectra (0.50–10.00 ppm) were then segmented into 0.005-ppm-wide bins, after omitting the regions of 3.34 to 3.37 ppm (peak from an unexplained methanol contamination) and 4.70 to 5.10 ppm (the residual water resonance). The bins were then normalized to achieve unit total intensity for each spectrum and imported into Microsoft Excel.

Next, the Excel spreadsheet of binned spectra was imported into SIMCA-P+ (Umetrics) for principal components analysis (PCA), which was conducted on Pareto-scaled bins. Initially, PCA was used to identify outliers—less than 4% of the data fell outside of the Hotelling's T^2 ellipse at the 95% confidence interval in a scores plot of the first two components, and were removed from the dataset before subsequent analysis. Furthermore, PCA scores plots helped establish and visualize the extent of impact of a given treatment, and the relative impacts of different treatments. These plots were also used to visualize the degree to which the female hepatic metabolome differed from that of the male, both before and after treatment.

The Excel spreadsheet of binned spectra was also used to construct t test–filtered difference spectra, which helped to identify specific metabolites that were affected by chemical treatments. In addition, these difference spectra were used to compare the relative impacts of different treatments at different time points. To generate these difference spectra, an average class spectrum was first calculated by averaging the binned spectra across all class members for each bin. Class was defined by sex, chemical exposure level, and time point. Next, the difference spectrum for each exposed class was generated by subtracting the averaged bins of the relevant control class from those of the exposed class. Then, a t test was conducted on each bin to determine whether the average for the exposed class differed significantly from that of the control class, using a p value of less than 0.05. If not, the bin value in the difference spectrum was replaced with a zero. To reduce false positives, any single isolated bin that passed the t test without an adjacent bin also passing was replaced with a zero, the rationale being that legitimate metabolite peaks span more than one bin at this bin size. To further reduce false positives, any occurrences of two and only two adjacent bins with opposite arithmetic signs

were replaced with zeros, because this outcome is incompatible with NMR peak shapes. The resulting *t* test–filtered difference spectrum for each exposed class exhibits peaks above the baseline that correspond to metabolites that increase with statistical significance on exposure, and peaks below the baseline that correspond to metabolites that decrease.

Once a metabolite change was identified using the *t* test filtered difference spectrum, a final validation step was conducted. This consisted of integrating the area of a given metabolite's most abundant or most isolated peak for each exposure- and control-class member, followed by a univariate *t* test, using a *p* value of less than 0.05. All metabolites discussed here passed this univariate test.

RESULTS AND DISCUSSION

Exposure verification

Measured TRB concentrations were reasonably stable over the exposure period. Mean (standard deviation [SD], *n*) concentrations in the low TRB treatment group were 28 (2.1, 7), 35 (6.7, 16), 32 (6.1, 14), and 31 (5.0, 11) ng/L on test days 1, 2, 4, and 8, respectively. This yielded an overall mean (SD) of 33 (6.0) ng/L. Mean (SD, *n*) concentrations in the high TRB treatment group were 490 (37.6, 17), 487 (38.2, 18), 452 (30.1, 18), and 447 (39.3, 17) ng/L on days 1, 2, 4, and 8, respectively. This yielded an overall mean (SD) of 472 (48.7) ng/L. No TRB was detected in the control tanks or procedural blanks ($n = 68$). By day 9 of the experiment (day 1 of the depuration/recovery period), TRB concentrations were below method detection limits (<26 ng/L) in all tanks. The mean (±SD) recovery of TRB in the spiked matrix samples was $94 \pm 8.6\%$ ($n = 10$), and the mean (±SD) percentage agreement among duplicate samples was $95 \pm 5.4\%$ ($n = 8$).

Steroid and VTG measurements

Fish survival was high over the course of the exposure and depuration/recovery phase of the experiment. Of 384 fish in the experiment, the only mortality was a female that died in the low TRB treatment on the final day of the depuration/recovery period. All other fish survived until sampled and were not notably stressed in appearance or behavior. However, at the biochemical level, TRB produced a marked response in the rate(s) of steroid production by ex vivo ovary explants very early in the exposure. Specifically, a significant decrease in T production was apparent after just 24 h in both TRB treatments (Fig. 1A). This decrease continued for an additional 24 h (i.e., through day 2) but was statistically significant only at the low TRB exposure level ($p < 0.1$ at the high TRB level). Production of E2 was also negatively impacted early in the exposure. However, unlike T production, a significant reduction was not observed until after 2 d of exposure to TRB (Fig. 1B). This delay is consistent with the known steroid biosynthetic pathway for androgens and estrogens, in which the enzyme aromatase (CYP19) uses testosterone as a precursor for the synthesis of E2. Subsequent to the relatively rapid decline in E2 and T, no significant effect on ex vivo production of either of these steroids was detected as of day 4. (Note, however, that the lack of a statistically significant decline relative to controls at this time point may be attributable to increased variability among exposed animals). During the 8-d depuration/recovery phase of the test, only the production of E_2 was impacted, and this occurred only on the final day of the test (day 16).

Because the ovary is the primary site of production for these steroids, declines in the rate of steroid production by ovary tissue should be reflected in altered plasma concentrations. Indeed, plasma E2 concentrations declined significantly after TRB exposure, particularly at the higher concentration of TRB (Fig. 2A). In a previous study, Ankley et al. [5] reported a decline in plasma VTG concentrations coinciding with a decrease in circulating E2 and T after TRB exposure. This is consistent with the role of E2 in stimulating VTG production via activation of the estrogen receptor. In the current study, the high concentration of TRB produced a significant decline in VTG from days 4 through 8 of the exposure period, which persisted over the first 48 h of depuration (days 9 and 10) (Fig. 2B). The VTG concentrations in females exposed to the low concentration of TRB declined more slowly (significant decrease on day 8 only) and recovered over the first 24 h of depuration.

We also measured ex vivo T production for testis and plasma T concentrations in male fish. Beyond a small decline in ex vivo T production after 24 h of exposure (Fig. 3A) and a small increase in plasma T after 24 h depuration (Fig. 3B), TRB

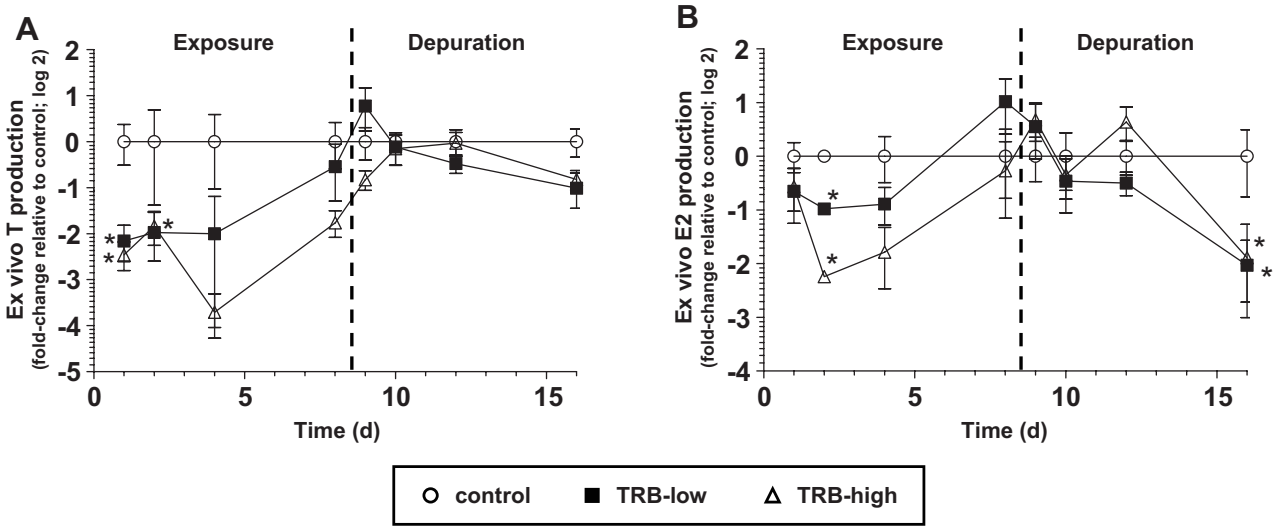

Fig. 1. Fluctuations in (**A**) testosterone (T) and (**B**) 17β-estradiol (E2) production as measured using ovary explants (ex vivo) both during and after exposure to 17β-trenbolone (TRB). $^*p < 0.05$ compared with control. The waterborne concentrations of TRB in the low (TRB-low) and high (TRB-high) treatments were 33 and 472 ng/L, respectively.

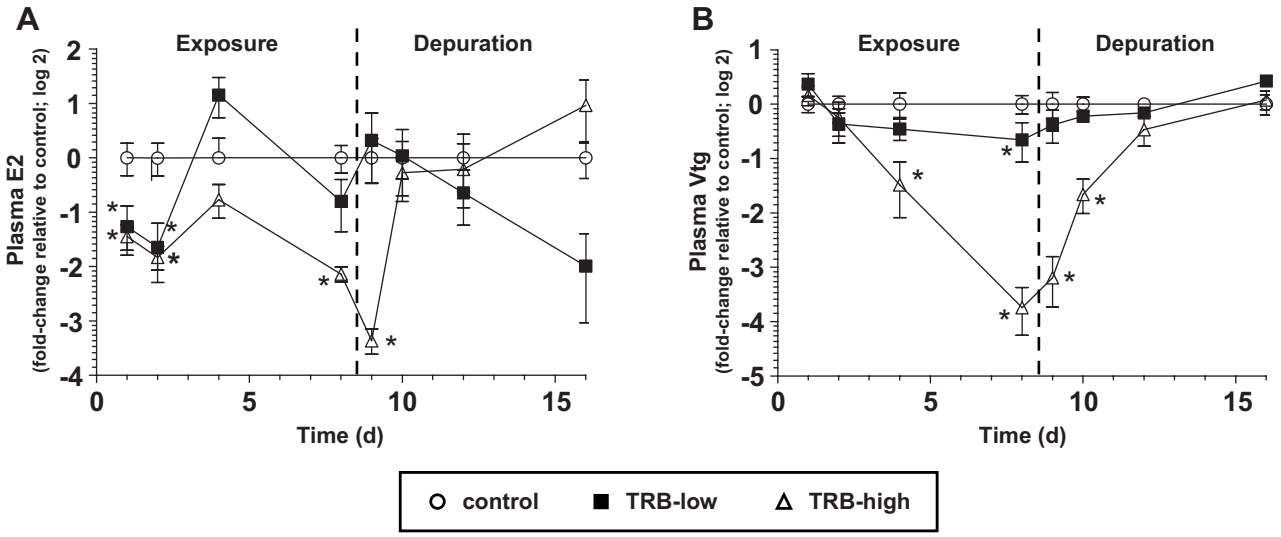

Fig. 2. Fluctuations in (**A**) 17β-estradiol (E2) and (**B**) vitellogenin (VTG) levels in plasma of females both during and after exposure to 17β-trenbolone (TRB). Data are expressed as fold-change (log 2) relative to the control mean measured on a given day; error bars indicate the standard error for seven to eight replicate fish. $^{*}p < 0.05$ compared with control. The waterborne concentrations of TRB in the low (TRB-low) and high (TRB-high) treatments were 33 and 472 ng/L, respectively.

had little effect on steroidogenesis. This lower sensitivity to TRB exposure in the males was similar to that observed by Ankley et al. [5], who reported no significant effects on plasma T, E2, and VTG at TRB concentrations identical to those used for the current study.

Ovarian gene expression

Targeted gene expression analyses for select transcripts were conducted to help understand the integrated nature of the female fishes' responses to the androgenic stressor. This approach revealed dynamic control of transcript levels in the ovary and suggests potential mechanisms for compensation to TRB exposure. For example, exposure of females to TRB could be hypothesized to stimulate a response similar to what would be elicited by a perceived excess concentration of endogenous T. For this case, one might expect the ovary to decrease the synthesis of T as a compensatory measure. Given its role as a primary rate-limiting enzyme in steroid biosynthesis [19], the reduction in CYP11A transcripts that was observed early in the exposure (Fig. 4A) would be a plausible route to achieve or help sustain a reduction in T synthesis. Consistent with the decreased CYP11A transcript measurements, ex vivo production of T by the ovary declined during the earliest time points of the exposure (Fig. 1A). Consequently, both the reduction in ex vivo T production and the decrease in CYP11A transcription would be consistent with a negative feedback response to the androgenic stressor.

Examination of E2 production and the abundance of transcripts coding for CYP19A (aromatase), the rate-limiting enzyme responsible for the conversion of T to E2, suggest some additional complexities in the dynamic response to TRB. Specifically, the level of transcripts for CYP19A declined sharply early in the exposure and then rebounded to control levels by day 4. After 8 d of exposure, the high TRB treatment had induced CYP19A transcript expression to a level above that of the controls (Fig. 4B). The temporal nature of this response paralleled the trends in E2 production rates (measured ex vivo) and plasma E2 concentrations (at the low exposure concen-

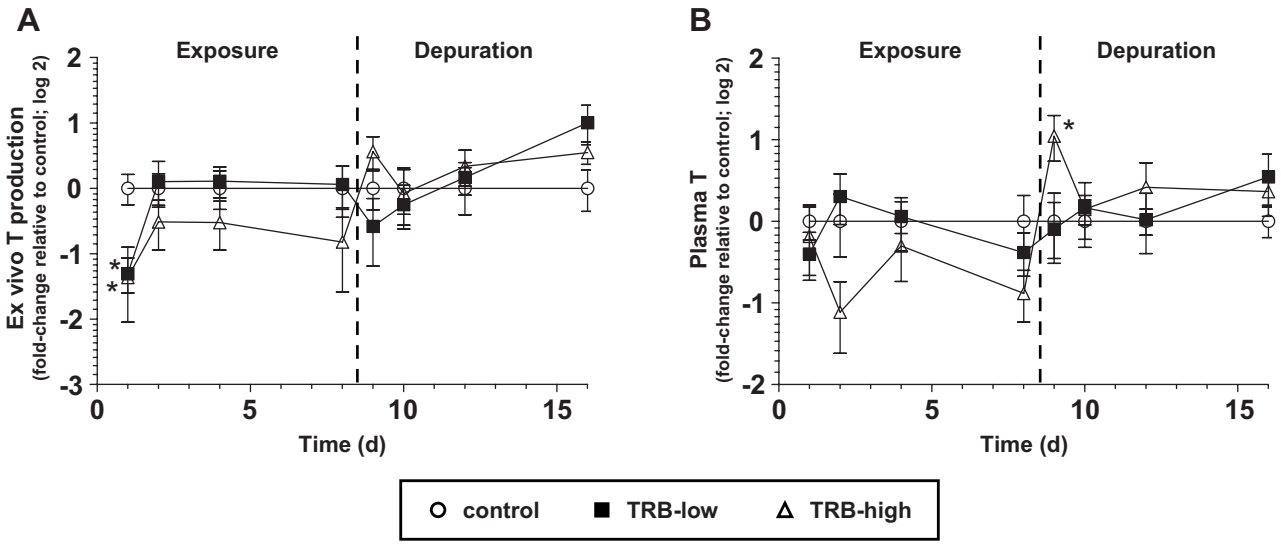

Fig. 3. Fluctuations in (**A**) testosterone (T) production in testis and (**B**) plasma T levels in males both during and after exposure to 17β-trenbolone (TRB). Data are expressed as fold-change (log 2) relative to the control mean measured on a given day; error bars indicate the standard error for seven to eight replicate fish. $^{*}p < 0.05$ compared with control. The waterborne concentration of TRB in the low (TRB-low) and high (TRB-high) treatments was 33 and 472 ng/L, respectively.

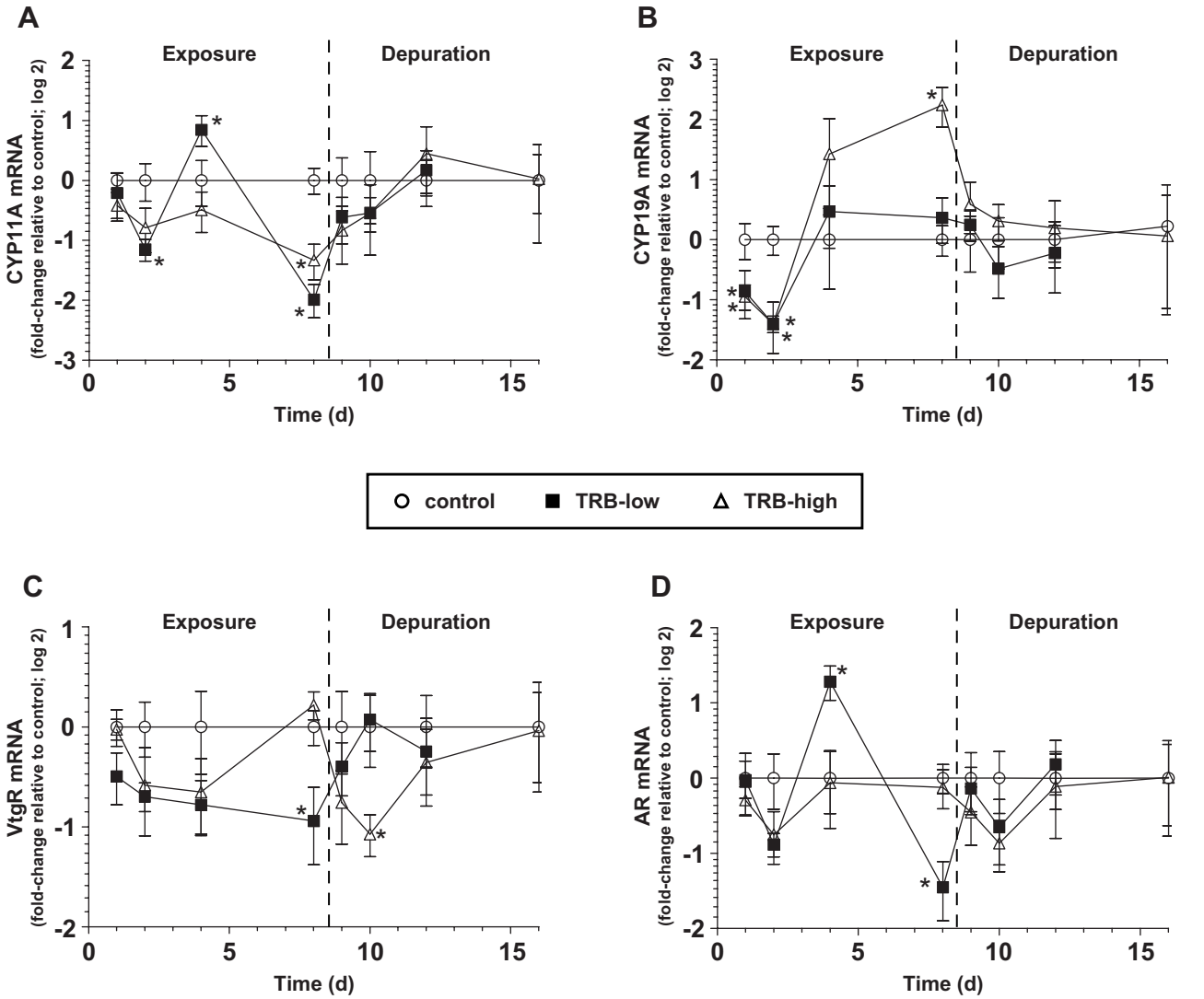

Fig. 4. Relative abundance of messenger RNA transcripts coding for (**A**) cytochrome P450 11A (CYP11A), (**B**) aromatase A (CYP19A), (**C**) vitellogenin receptor (VtgR), and (**D**) androgen receptor (AR) measured in ovary from female fathead minnows exposed to 0 (control), 33 ng/L 17β-trenbolone (TRB-low), or 472 ng/L 17β-trenbolone (TRB-high) and sampled on days 1, 2, 4, or 8 of the exposure period or on days 9, 10, 12, or 16 of the recovery period. Data are expressed as fold-change (log 2) relative to the control mean measured on a given day; error bars indicate the standard error for seven to eight replicate fish. $^{*}p < 0.05$ compared with control.

tration), which showed early declines at days 1 and 2, followed by recovery at exposure days 4 and 8 (see Fig. 1B and Fig. 2A, respectively). Given the lack of evidence that TRB acts as a direct inhibitor of aromatase, the initial declines in E2 production are hypothesized to be the result of decreased T production, the precursor for E2, as a result of an initial feedback response to the stressor. However, the CYP19A transcript data suggest that the decline in E2 may be exacerbated also by reduced CYP19A transcription. Whether reduced CYP19A expression may have resulted from the same feedback response (i.e., activation/deactivation of the same transcription factors/repressors that altered CYP11A transcription) aimed at modulating T production, or whether it may reflect a direct paracrine or autocrine effect of TRB on ovarian gene expression, is not clear. However, regardless of mechanism, the overall result is an inappropriate reduction in E2, because, although TRB can activate the AR like T, it cannot be converted to E2 by aromatase. Additionally, potential reductions in aromatase activity associated with reduced CYP19A transcription expression would slow the loss of T by decreasing the amount converted to E2, which also would be counterproductive.

The latter portion of the exposure period suggests a potential correction. Specifically, the inappropriate drop in circulating E2 concentrations after the initial feedback response to TRB exposure would, in turn, be expected to initiate a feedback response that would promote a compensatory increase in E2 production. Based on previous studies with the aromatase inhibitors fadrozole and prochloraz, induction of CYP19A expression appears to be a common molecular response to declining E2 concentrations in reproductively mature female fathead minnows [9,15]. Consequently, the up-regulation of CYP19A expression observed on day 4 (nonsignificant trend) and day 8 (statistically significant increase) (Fig. 4B) would seem to reflect an attempt to return E2 concentrations to a physiologically optimal level, after an initial incorrect decline in response to the androgenic stressor (Figs. 1B and 2A). The CYP11A transcripts transiently returned to control levels in the high TRB treatment group and increased relative to controls in the low TRB treatment group on day 4, before returning to a significantly reduced abundance on day 8. Previous time-course experiments from our laboratory with aromatase inhibitors showed increased CYP11A transcription in response to declining E2 production/concentrations [9,15]. Consequently, we

hypothesize that the dynamics of the transcriptional responses of CYP19A and CYP11A reflect the interplay of the fishes' attempt to compensate for the perceived excess of circulating androgens caused by exogenous TRB, while maintaining E2 concentrations in a range appropriate for normal vitellogenesis. Because the primary sites of CYP11A and CYP19A activity in the ovary are spatially isolated in theca and granulosa cells, respectively [20], through appropriate cell-specific signaling, achieving a more balanced response to TRB by both reducing T production, in part through reduced CYP11A transcription, and increasing the degree of T to E2 conversion, in part through increased CYP19A transcription, should be possible. This appears to be the state achieved by day 8 of the exposure period.

In addition to measuring transcription of messenger RNAs coding for key rate-limiting enzymes in steroidogenesis, we also examined the dynamic expression profiles for two receptors. Because TRB exposure reduced plasma VTG concentrations, we hypothesized that an increase in expression of VTGR transcripts in the ovary might occur as a compensatory response. However, VTGR transcript levels in the ovaries of TRB-exposed fish were generally lower than that in controls, and with the exception of the high TRB group on day 8, appeared to parallel circulating VTG concentrations (Fig. 4C and Fig. 2B). We also hypothesized that AR expression might be down-regulated in response to the perceived excess androgen. However, AR transcript levels in the ovary revealed an increase at day 4 for the low concentration of TRB followed by a steep decline by day 8 (Fig. 4D). No significant effect on this transcript was seen for the fish exposed to the high concentration of TRB. Thus, although some dynamic changes occurred in AR expression, they were not concentration dependent, nor were they clearly associated with the other measurements made in the study. Subsequent analyses of ovarian RNA samples from the current study using a fathead minnow microarray are expected to provide a more comprehensive understanding of the role of AR and other features of the ovarian transcriptional network in responding to TRB exposure [21].

With the exception of E2 production in the ovary, once TRB was removed from the water, both transcript and steroid production levels returned to control levels. This suggests that the fish are able to recover once the chemical is no longer present in the water. This may have significance for assessing potential long-term risk.

Metabolite profiling of liver

Through our plasma steroid and VTG measurements, we confirmed that TRB produced responses consistent with results reported in an earlier FHM study with the androgen that assessed changes in endocrine function at one time point [5]. Moreover, steroid production in the ovary and testis was assessed, as well as an analysis of expression of select genes in the ovary, revealing a strong time dependence of responses, and suggesting a potential compensatory mechanism for TRB exposure. Because the liver is known to play a significant role in the endocrine function of teleost fishes and vertebrates in general, and is also the primary site for the metabolism of xenobiotics, we pursued a metabolomic analysis of the liver, anticipating the discovery of additional time-dependent and compensatory responses. Moreover, the liver was selected for metabolite profiling, in part because of the existence of previous toxicogenomic studies, both transcriptomic and proteomic, focused on the hepatic effects of TRB [22–24].

Use of the intensive temporal sampling for the TRB exposure and depuration phases afforded the opportunity to observe time-dependent metabolite changes for each exposure level. This is made evident through the use of a series of *t* test filtered difference spectra that revealed time- and TRB concentration–dependent changes in endogenous metabolite levels of exposed females (Fig. 5A, B). Immediately apparent from these difference spectra is the general lack of commonality between

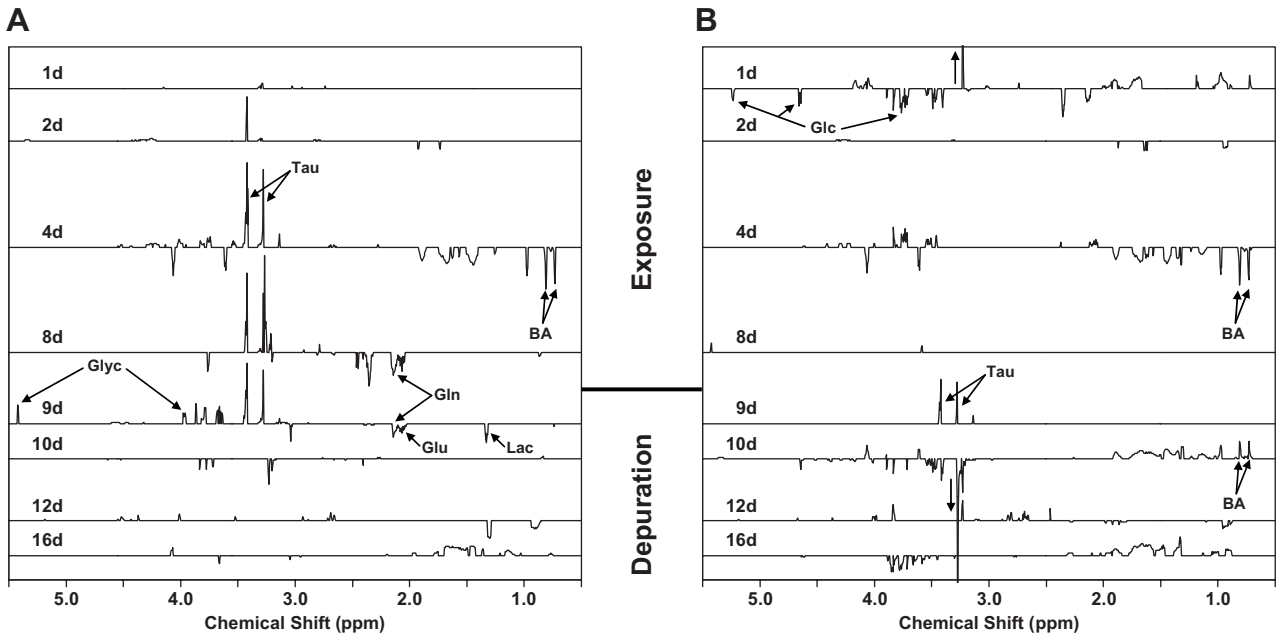

Fig. 5. Average difference spectra (exposed minus control) generated to determine statistically significant metabolite changes in liver metabolite profiles over the course of the exposure and depuration phases for female fathead minnows exposed to either (**A**) 472 ng/L 17β-trenbolone (TRB-high) or (**B**) 33 ng/L 17β-trenbolone (TRB-low). Peaks above the baseline represent metabolites that increased (relative to controls) on exposure, whereas peaks below the baseline represent those that decreased. Only those peaks for which differences were determined to be statistically significant by *t* test ($p \leq 0.05$) were included. Resonances for specific metabolites mentioned in the text are labeled as follows: Glc, glucose; Tau, taurine; BA, bile acids; Glyc, glycogen; Gln, glutamine; Glu, glutamate; Lac, lactate. Not all resonances belonging to a given metabolite are indicated.

responses to TRB for the two exposure levels. With the exception of day 4, in which the metabolite changes seem comparable for both exposure levels, an overall dissimilarity exists both in terms of the metabolite changes observed and in terms of the time dependence of these changes during exposure. Furthermore, once the chemical was removed from the water, a greater level of recovery may have occurred at the higher level of TRB, because the difference spectra generated for the final days of the depuration period (days 12 and 16) generally show fewer features than those of the lower treatment group. Why such differences were observed between the treatment groups in relation to the timing of responses is not clear. However, this serves to highlight the importance of collecting temporally intensive data both during and after exposures for these types of studies.

An analysis of the metabolite changes associated with each time point was conducted using the aforementioned *t* test filtered difference spectra. Large dissimilarities between exposure levels and time points were observed. For example, for the first sampling time point (day 1), metabolite changes produced after exposure to the low concentration of TRB were quite different from those observed for the high concentration. Only a few statistically significant metabolite changes were measured in the livers of the females exposed to the high concentration of TRB at this time point (Fig. 5A). Conversely, a number of resonances were identified as changing in the livers of the females exposed to the low concentration of TRB (Fig. 5B). Most of these changes were caused by a decline in hepatic glucose levels, possibly as a result of antagonistic binding of TRB to the glucocorticoid receptor, a characteristic of TRB reported previously by Wilson et al. [25]. Why this impact was not also observed for the females exposed to the high concentration of TRB is uncertain.

After day 1, the females from the high-TRB treatment group displayed a sustained response to exposure. This is most evident from the increase in the amino sulfonic acid taurine seen on days 4 and 8 of the exposure, and on the first day of the depuration phase (i.e., day 9) (Fig. 5A). The sustained change in taurine appears to be a concentration-dependent effect, as a difference from controls for this metabolite was only observed at one time point (day 9) in females exposed to the low concentration of TRB (Fig. 5B). Little is known regarding the role of hepatic taurine in fish, but various studies have reported on the potential hepatoprotective capacity of taurine in rats and humans [26,27]. The mechanism by which taurine elicits this protective effect are not known with certainty. However, among other roles, taurine may act through the modulation of Ca^{2+} levels [28] and by enhancing the stability of membranes [27]. The increase observed in the current study may reflect a similar protective role in the livers of the female FHM exposed to TRB, because androgens have been shown to produce an elevation in intracellular calcium levels via actions on G protein–coupled receptors [29]. In support of this notion, analysis of gene expression of female rainbow trout (*Oncorhynchus mykiss*) exposed to a similar level of TRB (1 μg/L) showed an increase in transcripts related to calcium ion binding. In relation to taurine's proposed ability to stabilize membranes, this action may be necessary subsequent to exposure to hydrophobic steroids such as TRB. Specifically, hydrophobic steroids such as estrogen, progesterone, and T have been shown to disrupt cellular membranes through their ability to interact with the polar head groups of phospholipids [30]. However, whether the TRB concentrations used in the current experiment would be high enough to cause such a disruption is uncertain.

A time-dependent change in the level of bile acids also was observed in livers of the female fish. We suspected that these changes might influence the levels of hepatic taurine, because most vertebrate species (with the exception of mammals) exclusively conjugate taurine with cholesterol in the production of bile acids [31]. Through use of a simple NMR-based approach originally reported by Gowda et al. [32], we confirmed that the FHM also exclusively uses taurine in the production of bile acids (data not shown). However, a decline in bile acids was only observed at day 4 of the TRB exposure, albeit in both treatment groups (Fig. 5A, B), whereas changes in levels of taurine were observed at multiple time points for the high treatment (days 4, 8, and 9) and at two time points for the low treatment group (days 9 and 10). Thus, no clear correlation exists between these metabolite changes. Another possible explanation exists for the decline in bile acids measured at day 4 of the exposure at both concentrations of TRB. The 17α-alkyl–substituted steroids are capable of inhibiting bile formation in various species [33]. Although TRB is substituted in the β configuration at carbon 17, it also may be capable of such inhibition. Regardless, the fish appear to be able to compensate for this effect relatively rapidly such that this response was no longer seen at day 8 of the TRB exposure. Moreover, the females exposed to the low concentration of TRB showed an increase in these same bile acids by the second day of the depuration phase (day 10) of the test, potentially indicating overcompensation.

With regard to the considerable dependence on TRB concentration observed in the metabolite changes, such non-monotonic responses have been reported in previous studies using mammalian test systems exposed to androgens and anti-androgens [34]. These chemicals can cause U-shaped dose/concentration–responses that are likely the result of separate mechanisms of action being initiated at different dosage levels. Such a scenario also may occur in FHMs exposed to TRB. In fact, a U-shaped concentration–response in plasma steroid concentrations was reported for female FHMs exposed to TRB [5].

Relative to other non–taurine-linked metabolites, after 8 d of exposure to the high concentration of TRB a significant decline in the amino acid glutamine was measured (Fig. 5A). Again, some agreement has been seen between this observation and the gene expression analysis reported by Hook et al. [23], in which the authors reported an up-regulation of hepatic transcripts related to glutamine metabolism in TRB-exposed female rainbow trout. The decline in glutamine in the present study continued for 1 d after the exposure was stopped (day 9) and was accompanied by a decline in glutamate and lactate (Fig. 5A). The decline in these metabolites is particularly interesting when considering that glycogen levels increased substantially at day 9. The observed drop in lactate may have occurred because of a decline in glycolytic activity as glucose was being actively sequestered into storage as glycogen, a well-documented effect of androgen exposure in fish [35]. In an attempt to supplement the loss of this energetic resource, the fish may have turned to both glutamate and glutamine as energetic substrates via their conversion to tricarboxylic acid cycle intermediates (e.g., α-ketoglutarate).

The impact of exposure on the liver metabolite profile observed for the males was substantially less than that seen for the females (data not shown). This is consistent with the almost negligible impact of the TRB exposure on the ex vivo and plasma steroid measurements in the males.

NMR-based metabolomics

Beyond determining specific metabolite changes related to TRB exposure, we also sought to assess the suitability of NMR-based metabolomics for identifying the induction of a male-like hepatic metabolite profile in females exposed to a model androgen such as TRB. The ability to identify chemicals with androgenic activity using metabolomics could prove useful for environmental regulation, because it might provide further evidence and additional information to the whole organism studies that are typically used for this purpose.

To this end, we employed PCA to determine the extent of similarity (or difference) of the metabolite profiles of both control and TRB-exposed females with those of the males. The result of this analysis is depicted in Fig. 6A, which presents a scores plot of the first two components of a model for the day 4 control males and females and their corresponding high concentration TRB-exposed cohorts; day 4 was chosen because of both its apparent importance in time-dependent responses to previous endocrine-disrupting chemical exposures we have conducted [17] and the results of the different spectra analyses, which suggest parallel responses between the TRB treatments at this time point. Immediately evident from this plot is the extent of separation (which was statistically significant at $p < 0.05$) of the control and TRB-exposed females along component number one (PC1, which captured 39.1% of variation in the dataset). The extent of this separation is quite large when compared with that observed for the control and exposed males, which was not statistically significant. This observation is in keeping with the result from our difference spectra described previously and with previous work that showed little impact of TRB exposure on male FHMs [5].

That our PCA approach supports the established observation that females are more strongly affected by exposure to androgens than males is encouraging. Moreover, further inspection of this plot with regard to the extent of intersex variation both before and after exposure is instructive relative to determining the induction of a male-like metabolite profile in the females. Both the component number 1 (PC1) and component number 2 (PC2) score values for the females are statistically different ($p < 0.05$) from those of the males before exposure (i.e., the controls). Thus, the variation between male and female controls is encompassed by both of these principal components. As a rule, PC2 accounts for less of the variation in the model than PC1 and thus represents more subtle differences between groups. The situation with controls contrasts with that observed for the exposed females with regard to both of the male classes. Here the differences in the metabolite profiles occur only along PC2, and thus represent a relatively smaller difference than seen between the sexes before exposure. The only class-occupying space in the negative region of PC1 is that of the control females. All other classes lie in the positive region (right of the ordinate) of PC1 (with PC1 score values that are not statistically different, $p > 0.05$), with males and females separating only along PC2.

This same approach using the low-concentration TRB exposure classes provides an opportunity to determine whether comparable responses might be observed at concentrations of TRB more similar to those measured in the environment. The result of this analysis is shown in Figure 6B. Clearly, although some rearrangement of the classes in PCA space has occurred relative to that observed using the high-concentration TRB classes, an analogous set of relationships exists. Again (using statistical significance of $p < 0.05$), the exposed females are separated from both of the male classes solely along PC2, and the control females exclusively occupy the negative region of PC1, indicating greater similarity of male and female metabolite profiles after exposure. This outcome suggests that NMR-based metabolite profiling is capable of detecting the androgenic effect of TRB on females at concentrations approaching those found in aquatic environments (7 ng/L in receiving water and up to 20 ng/L in discharge [3]).

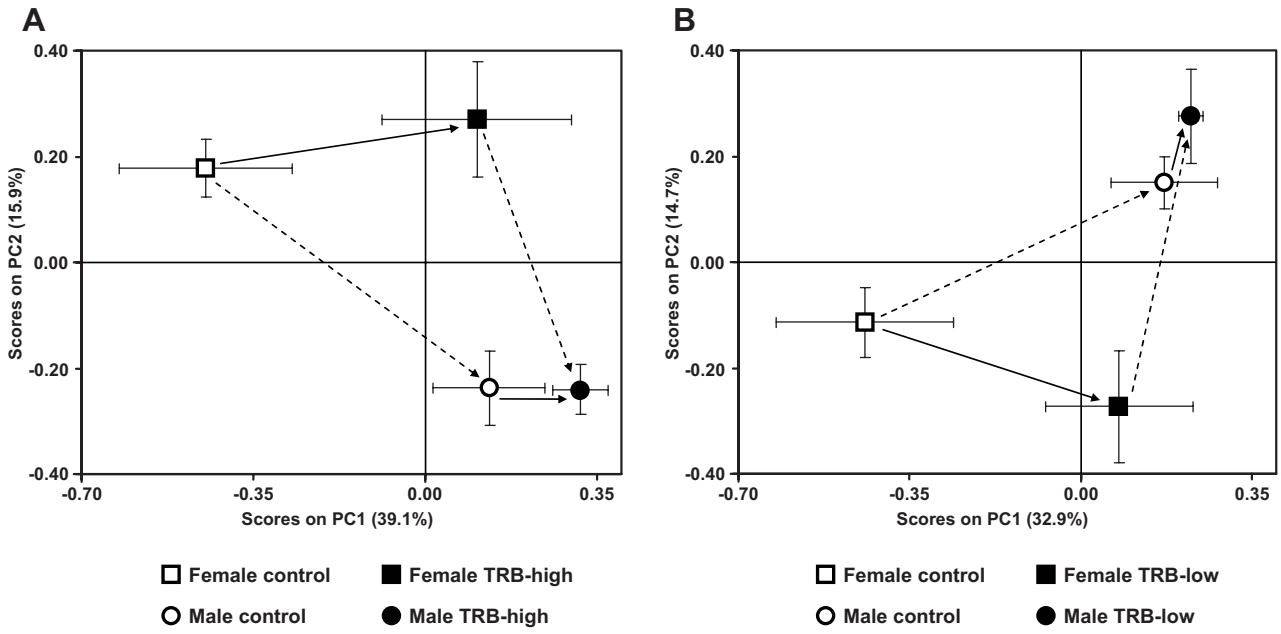

Fig. 6. Two component principal components analysis (PCA) scores plots for male and female fathead minnow controls and those exposed for 4 d to either (**A**) 472 ng/L 17β-trenbolone (TRB-high) or (**B**) 33 ng/L 17β-trenbolone (TRB-low). Each point is the mean score value for a given class, shown with its associated standard error. The amount of variation in the dataset captured by each component is shown parenthetically in the axis titles. For ease of visualization, the solid lines reflect the distance between control and exposed classes for each sex, whereas the dotted lines represent the distance between sexes both before and after exposure.

CONCLUSION

Results of the current study contribute to our ongoing efforts to develop exposure indicators and predictive models of effects based on mechanisms or modes of action [21]. Through a multidisciplinary, highly integrated effort, this larger project is designed to provide innovative approaches to support quantitative risk assessments for endocrine-disrupting chemical through development of cost-effective, predictive tools for monitoring and testing. Furthermore, the project emphasizes the importance of linking molecular indicators to adverse outcomes at the whole organism level to ensure that they are meaningful. For example, assessment of sensitive molecular or biochemical endpoints after short-term in vivo or in vitro exposure would provide a more cost-effective and efficient means to test chemicals or samples for potential endocrine-modulating effects. However, for such endpoints to be viewed as a reliable basis for risk assessment, one must establish and understand the linkages between short-term responses and longer-term outcomes relevant to ecological risk assessment [7]. In the current study, we established that TRB exposure caused rapid effects on plasma steroids and VTG, particularly in females, that were consistent with those observed in longer-term exposures to similar concentrations that were also shown to adversely affect reproduction [5].

We also observed effects on transcription of key rate-limiting steroidogenic enzymes that were consistent with those caused by other endocrine-active chemicals [9,15], but fluctuated up and down dynamically, likely because of an interplay of feedback responses to changes in both perceived T and actual E2 concentrations. These results both point toward the value of developing physiologically based models of feedback control mechanisms employed by the hypothalamic-pituitary-gonadal axis as an important aid to interpreting short-term responses to endocrine-disrupting chemical and provide data to support the development of such models.

In evaluating ecological risks, one also must understand how organisms may respond to fluctuating exposure regimens (i.e., the rapidity, sensitivity, and persistence of effects). Results of the current study suggest that removal of TRB generally resulted in rapid recovery of the steroid-related endpoints back to a control-like state. However, more sustained alterations to hepatic metabolite profiles appeared to occur, suggesting some potential lingering effects, which may or may not be adverse, but may have diagnostic utility. Finally, the current study provides an example of how new analytical technologies such as metabolomics can contribute useful information to support both mechanistic investigations and risk assessments. For example, the observation that the hepatic metabolome of females exposed to a strong androgen become more male-like might provide a useful diagnostic complement to other endpoints traditionally used in evaluating or reconstructing exposures to endocrine-disrupting chemicals. Thus, integrated studies of the dynamics of biological responses to endocrine-disrupting chemicals and other stressors can help advance the science of risk assessment in the long term.

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