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# Toxicokinetic, Toxicodynamic, and Toxicoproteomic Aspects of Short-term Exposure to Trenbolone in Female Fish

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**The toxicokinetics of trenbolone was characterized during 500 ng/l water exposures in female rainbow trout (*Oncorhynchus mykiss*) and fathead minnows (*Pimephales promelas*). Related experiments measured various toxicodynamic effects of exposure. In both species, trenbolone was rapidly absorbed from the water and reached peak plasma levels within 8 h of exposure. Afterwards, trenbolone concentrations in trout (66–95 ng/ml) were 2–6 times higher compared with minnows (15–29 ng/ml), which was attributable to greater plasma binding in trout. During water exposures, circulating levels of estradiol (E2) rapidly decreased in both species to a concentration that was 25%–40% of control values by 8–24 h of exposure and then remained relatively unchanged for the subsequent 6 days of exposure. In trout, changes in circulating levels of follicle-stimulating hormone were also significantly greater after trenbolone exposure, relative to controls. In both species, the pharmacokinetics of injected E2-$d_3$ was altered by trenbolone exposure with an increase in total body clearance and a corresponding decrease in elimination half-life. The unbound percentage of E2 in trout plasma was 0.25%, which was similar in pre- or postvitellogenic female trout. Subsequent incubation with trenbolone caused the unbound percentage to significantly increase to 2.4% in the previtellogenic trout plasma. iTRAQ-based toxicoproteomic studies in minnows exposed to 5, 50, and 500 ng/l trenbolone identified a total of 148 proteins with 19 downregulated including vitellogenin and 18 upregulated. Other downregulated proteins were fibrinogens, α-2-macroglobulin, and transferrin. Upregulated proteins included amine oxidase, apolipoproteins, parvalbumin, complement system proteins, and several uncharacterized proteins. The results indicate trenbolone exposure is a highly dynamic process in female fish with uptake and tissue equilibrium quickly established, leading to both rapid and delayed toxicodynamic effects.**

***Key Words:*** **androgenic; iTRAQ; gonadotropin; clearance; plasma binding.**

There are persistent concerns regarding the release of pharmaceutical agents into aquatic environments and the potential for harmful effects on fish and other aquatic organisms. A class of veterinary pharmaceuticals used as growth promoters in animal husbandry is sex steroids and synthetic analogs. 17β-Trenbolone (referred to as trenbolone throughout) is an androgenic steroid formed after administration of trenbolone acetate to livestock. Trenbolone acetate is widely used to promote growth in cattle, and it has been identified in runoff and surface waters near feedlot operations (Bartelt-Hunt *et al.*, 2012; Gall *et al.*, 2011; Khan and Lee, 2012). Laboratory studies of trenbolone exposure to sexually mature fathead minnows (*Pimephales promelas*) at exposure rates as low as 26 ng/l have documented profound effects on females, which include significant decreases in circulating estrogen (estradiol-17β; E2) levels, cessation of spawning, atrophy of the ovaries, and morphological changes associated with a more masculine external appearance (Ankley *et al.*, 2003; Jensen *et al.*, 2006). In sexually maturing female rainbow trout (*Oncorhynchus mykiss*), short-term exposure (60–77 days) to 35 ng/l trenbolone caused a significant delay in embryological development in embryos formed from eggs of exposed trout (Schultz *et al.*, 2008). *In vitro* binding studies have also indicated trenbolone has an approximately 10-time greater affinity for the fathead minnow androgen receptor (AR) than testosterone (Ankley *et al.*, 2003). Thus, environmental concerns over the release of growth promoters such as trenbolone appear justifiable, given their potency and potential for significant impacts on reproduction in fish and perhaps other aquatic organisms.

Blood is a unique tissue because it is in direct contact with all other tissues. The extracellular portion of blood is plasma, which is the most complex biological fluid containing both low and high–molecular weight (MW) components originating from regions throughout the body (Anderson and Anderson,

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2002). Because blood is in direct contact with tissues involved in absorption and elimination (eg, gills) and tissues that may be the target site for toxicity or may serve primarily for storage, the concentration of both contaminants and endogenous compounds in plasma represents a balance between environmental exposures, synthesis, elimination, and partitioning into peripheral tissues. Thus, plasma is an important biological fluid for monitoring as it contains components that can be diagnostic for some toxic modes of action (eg, vitellogenin expression in male fish), whereas other components can provide insights into toxicological or compensatory responses occurring elsewhere in the animal. These aspects of plasma can also make it valuable for translating effects measured at the cellular/subcellular level to whole organism effects. The latter is an important research area emphasized in recent reports on the future of human and ecological toxicity testing (Ankley *et al.*, 2010; NRC, 2007). The extrapolation of *in vitro* results for assessment of endocrine disruption and reproductive toxicity can be problematic because of the potential for multiple modes of toxic action and regulatory feedback loops that exist between different tissues (Nichols *et al.*, 2011). A challenge is to recognize how the low- and high-molecular weight constituents of plasma change in response to toxicant exposure and the connection to subcellular effects (eg, AR signaling) and whole animal effects (eg, decreased spawning, morphological changes).

In this study, we sought to understand the effects of trenbolone on female fish from the perspective of plasma monitoring and quantitative evaluation of its toxicokinetics and toxicodynamic effects associated with E2. We specifically hypothesized that E2 clearance is increased during trenbolone exposure and contributes to the reduction in circulating levels of E2 observed in previous studies. Interspecies differences in trenbolone effects were evaluated in trout and minnows because of their past use in trenbolone studies and contrasting mode of gamete development (synchronous vs asynchronous). For most experiments, our focus was on changes occurring during the initial 7–10 days of exposure. The uptake of trenbolone was characterized after water exposure to 500 ng/l in both trout and minnows. Trout were also administered an intravascular dose of trenbolone to permit a more complete understanding of its toxicokinetics. Additional experiments measured other toxicodynamic aspects of trenbolone exposure such as plasma binding of E2 and circulating levels of follicle-stimulating hormone (FSH). Changes in the plasma proteome were analyzed using both intact (top-down) and proteolytic (bottom-up) analysis methods.

## MATERIALS AND METHODS

### *Chemicals*

Trenbolone (> 99% purity), Methyl-N-trimethylsilyl-trifluoroacetamide (MSTFA), iodotrimethylsilane (ITS), and resublimed iodine were purchased from Sigma-Aldrich Chemical Co. (St Louis, Missouri). [16,16,17-$^2H_3$]-Estradiol (E2-$d_3$) and [2,4,16,16-$^2H_4$]-17α-ethinylestradiol (EE2-$d_4$) were purchased from C/D/N isotopes (Pointe-Claire, Quebec, Canada). All other chemicals were of reagent grade or better and were obtained from standard sources.

### *Fish and Water Quality*

All fish were maintained according to the guidelines established by the Institutional Animal Care and Use Committee of Battelle Pacific Northwest National Laboratory. All experiments used adult female rainbow trout or fathead minnows except as noted in the toxicoproteomic section. Female rainbow trout (1+ in age) were obtained from a local commercial trout hatchery (Troutlodge, Inc., Sumner, Washington) 1 day after completing their first spawning cycle. After transfer to Battelle Marine Sciences Laboratory (MSL; Sequim, Washington), trout were placed in circular, 1400-l fiberglass tanks, maintained as a single-pass flow-through freshwater system with water in-flow rates set to 15–20 l/min. The source water was from Battelle MSL's artesian well (well depth = 134 m) and was preaerated before reaching the tanks. During subsequent trenbolone exposures, trout were housed in 370-l fiberglass tanks with water in-flow rates set to 4–5 l/min. Selected water quality parameters routinely measured in all tanks were temperature (mean: 11.8°C; range: 11.0°C–12.4°C during the study), dissolved oxygen (> 9 mg/l), and pH (mean 7.9; range: pH 7.8–8.1 during the study). Other water quality parameters were measured once and are listed in Supplementary Table 1. Trout were maintained under lighting that simulated the natural photoperiod according to the latitude for Sequim, Washington (48.079° N) and included a 12-min graded sunrise/sunset period. Holding tanks were partially covered with a black tarp to provide shading. Light intensity measured (Extech Model 407026 light meter) near the surface of the nonshaded region of holding tanks was between 860 and 1100 lux for larger tanks and 630 and 840 lux for the 370-l tanks. One week after arrival at MSL (8 days postspawn), all trout were weighed and tagged with passive inducible transponders (Biomark HPT8, Biomark Inc., Boise, Idaho) to permit identification during nonlethal sampling. Trout were fed a soft moist feed (Vitalis 6-mm pellet; Bio-Oregon Inc., Longview, Washington, District of Columbia) 3 times a week at a ration level (approximately 0.75% body mass/day) designed to encourage growth and ovarian maturation. The body mass of trout at time of tagging was 1174 ± 155 g (mean ± SD, $n$ = 39), which increased to 2380 ± 341 g (mean ± SD, $n$ = 16) after 48–52 weeks, when a cohort of fish was allowed to complete a second spawning cycle. The experiments described below used trout at various times during their second spawning cycle except for the 27-day trenbolone and E2-$d_3$ exposures, which used the older trout that were approximately 16 and 26 weeks postspawn and weighed 2454 ± 284 g and 3151 ± 509 g (mean ± SD, $n$ = 8), respectively, at the start of experiments. Female trout used in supplemental toxicoproteomic experiments were obtained from Nisqually trout farm (Lacey, Washington, District of Columbia), weighed between 950 and 1300 g, were 1+ in age, and were estimated to be within 12–14 weeks of their first spawn at the time of trenbolone exposure.

Adult fathead minnows approximately 16–21 weeks in age were purchased from Environmental Consulting and Testing (La Crosse, Wisconsin). Minnows were obtained 3–6 weeks prior to initiation of experiments to allow time for acclimation to MSL holding conditions and selection of actively spawning females. During the acclimation period, minnows were initially maintained in 185-l aquaria containing approximately 20–22 females and 15 male minnows. After 1–2 weeks in large holding tanks, pairs of minnows (1 male and 1 female) were placed in a 19-l aquaria with a spawning substrate (4″ clay pot longitudinally sectioned). The spawning substrates were subsequently checked daily for the presence of eggs. The minnows were fed twice daily *ad libitum* with frozen San Francisco Bay brand brine shrimp that was thawed and diluted 1:1 (wt/vol) with sterile filtered seawater to obtain a mixture containing approximately 0.5 g (wet weight) of brine shrimp/ml. Breeding pairs were typically observed for 1 week to establish whether females were actively spawning. Nonspawning females were exchanged with a new female from the larger holding tank. Only females established to be spawning within 7 days of the trenbolone exposures were used. All aquaria were maintained as single-pass flow-through using well water that was heated to 25 ± 1°C (mean ± SD of daily measurements) at flow rates of 0.125 l/min (19-l aquaria) and 1.2 l/min (185-l aquaria). Weekly

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measurements of dissolved oxygen (range: 6.6–7.4 mg/l) and pH (8.1–8.3) were made. Photoperiod was maintained on a constant 16:8 (L:D) cycle. The body mass of minnows used in experiments (excluding those euthanized for plasma binding studies, which were not weighed) was 2.04 ± 1.17 g (mean ± SD, $n$ = 115) measured at termination.

*Water Exposures to Trenbolone*

All trenbolone exposures used a continuous flow protocol. Most experiments used a nominal trenbolone-exposure level of 500 ng/l. This dose level was selected because it appears to cause the most significant decrease in plasma E2 levels observed in minnows (Ankley *et al.*, 2003). Lower exposure levels were used in select instances such as the iTRAQ proteomic experiment where additional treatment levels of 50 and 5 ng/l were included and the 27-day trout exposure, which used a 70 ng/l exposure. These lower treatments were added to either provide dose-response information or avoid the potential for overt toxicity resulting from a longer exposure duration. Exposure water solutions of trenbolone were prepared from a concentrated aqueous stock solution that was slowly added to the exposure tanks using a peristaltic pump. No organic cosolvents were used. All exposure tanks were allowed to equilibrate with the dosing system for 3 days prior to the addition of fish. Nominal exposure values were adjusted based on daily monitoring of water and stock solution in-flow rates. Water samples were removed and analyzed for trenbolone from each exposure tank at the start of the exposure and at various times thereafter, depending on the experiment. The duration of the trenbolone exposure was a maximum of 7 days for minnows and 27 days for trout, with shorter exposure periods also used as indicated for each respective experiment.

*Experimental Procedures—Toxicokinetic Studies of Trenbolone*

The uptake of trenbolone during 500 ng/l water exposures was measured in both minnows and trout. For minnows, a serial euthanasia approach was used whereby groups of 3 minnows were placed in a 19-l exposure tank and sampled at 0.5, 1, 2, 8, 24, 96, and 168 h. At the selected time, the minnows were transferred to an oxygenated 250 mg/l solution of MS-222 until cessation of opercular movements, immediately removed, patted dry, and the caudal vein severed using a scalpel. Blood was then collected using a heparinized microcapillary tube (Fisherbrand No: 22-362-566), pooled from all 3 fish, and plasma was obtained after centrifugation (3000 × g, 3 min) and stored at −80°C until later trenbolone analysis. For trout, a repetitive sampling protocol was used with each trout fitted with a dorsal aortic cannula made from 28-G thin wall Teflon tubing using procedures previously described (Schultz and Hayton, 1993). After a 24-h recovery period, individual trout were carefully transferred to the 370-l trenbolone exposure tank, and 0.2 ml of blood was removed (*via* the cannula) at 0.25, 0.5, 0.75, 1, 1.6, 2, 3, 4, and 8 h and then at selected times until 240 h. The trout were then transferred to a similar tank without trenbolone, and blood was sampled periodically for an additional 75 h to measure the depuration of trenbolone. Each blood sample was centrifuged (3000 × g, 3 min) and the plasma stored at −80°C for later analysis of trenbolone. After removal of each blood sample, an equal volume of a modified Teleost saline (Houston 1990) was injected into the trout. At the time of exposure, the trout were approximately 21 weeks past their previous spawning.

An additional experiment was performed using five cannulated trout to characterize trenbolone toxicokinetics after intra-arterial (IA) injection. After 24 h of recovery from surgery, each trout was administered a 0.5 mg/kg IA bolus injection of trenbolone dissolved in 60% dimethylsulfoxide (DMSO):water (vol/vol). The volume of injectate was restricted to 75 µl/kg. Immediately after injection, the cannula was rinsed three times with blood to remove residual traces of the dose to prevent contamination of subsequent blood samples. Blood samples (0.2 ml) were removed at 0.167, 0.33, 0.66, 1.0, 1.5, 2.0, 2.5, 3.0, 3.5, 4, 4.5, 5, 5.5, 6, 6.5, 7.0, 7.5, and 8.0 h and then at selected times until 99 h. Plasma was obtained and archived as described previously. At the time of IA injection, the trout were approximately 37 weeks past their previous spawning.

***Plasma binding—Trenbolone.*** Female minnows excluded from other studies were euthanized, blood was collected from the caudal vein and pooled in a 2.0 ml microcentrifuge tube on ice during sample collection, and plasma was obtained and stored in two separate 0.6-ml aliquots at −80°C. For trout, five fish that were approximately 16 weeks past their previous spawning were euthanized in an oxygenated MS-222 solution (250 mg/l), and 10 ml of blood was removed from the caudal vein using a heparinized 12-ml syringe and an 18-G needle. Plasma was obtained as described previously and stored in 0.5- to 1.0-ml aliquots at −80°C. The fraction of trenbolone not bound to plasma macromolecules (assumed to be proteins) was determined using the Centrifree micropartition system (MW cutoff 10 000–12 000; Amicon Inc., Beverly, Massachusetts). Before ultrafiltration, the plasma samples were incubated for 1.5 h at 12°C in 2-ml glass vials previously coated with sufficient trenbolone (*via* evaporation of the methanol carrier solvent) to produce a concentration of 600 or 90 ng/ml (trout only). After the incubation, a 10–25 µl aliquot was reserved to determine the total plasma trenbolone concentration and the remainder immediately loaded into the Centrifree units and centrifuged for 0.5 and 25 min at 800 × g at the respective acclimation temperatures. The initial ultrafiltrate from the 0.5-min duration was discarded. The subsequent ultrafiltrate and the plasma aliquot were analyzed for trenbolone as described below. Calculation of the percentage of trenbolone that is unbound was made using the following equation:

$$\%fu = \frac{[\text{trenbolone}]\text{ultrafiltrate}}{[\text{trenbolone}]\text{plasma}} \times 100 \quad (1)$$

In preliminary tests, the binding of trenbolone (dissolved in 0.9% NaCl [wt/vol]) to the Centrifree membrane was determined to be < 5%.

*Experimental Procedures—Toxicodynamic Studies*

Three types of experiments were performed to assess impacts of trenbolone exposure on (1) circulating concentrations of endogenous E2 and FSH, (2) changes in the pharmacokinetics of injected E2-$d_3$, and (3) plasma binding of endogenous E2. Circulating levels of E2 were determined in female minnows continuously exposed to trenbolone over a 168-h (7-day) time period. The minnow experiments used a terminal blood sampling procedure as described previously, except that plasma samples were not pooled. Female minnows were sampled at 0, 1, 2, 4, 8, 24, and 168 h after exposure. Circulating levels of E2 and FSH were determined in female trout using a repetitive sampling procedure with either dorsal aortic cannulated fish (168-h exposure) or caudal vein sampling (27-day exposure). For the latter method, trout were anesthetized in an oxygenated solution of MS-222 (90 mg/l) until onset of stage 3 anesthesia, then quickly removed, and 0.5 ml of blood withdrawn from the caudal vein using a heparinzied 3-ml syringe and 1.5″ 23-G needle. Afterward, the trout were returned to the exposure tanks and gently resuscitated until opercular and swimming motion resumed. The time interval between caudal vein samplings was 7 days or greater. The 168-h exposure used trout that were five weeks past their previous spawning (previtellogenic), thus E2 levels were near the seasonal low of 1 ng/ml. The 27-day exposure used trout that were 16 weeks postspawn at the start of the exposure. Plasma samples were later thawed and the E2 levels measured in minnow plasma using an enzyme immunoassay (Cayman Chemical Estradiol kit no. 582251) and trout plasma using radioimmunoassay (Coat-A-Count Estradiol, Diagnostic Products, Los Angeles, California). FSH levels were measured in trout plasma using a radioimmunoassay as described by Swanson *et al.* (1989).

In separate studies, the pharmacokinetics of E2-$d_3$ was studied in female minnows after a single IC injection of a 1-mg/kg dose. The IC injection procedure was essentially as described by Kahl *et al.* (2001), except that the dosing vehicle was 60% DMSO:water (vol/vol) and the volume injected was 10 µl using a 25-µl Hamilton syringe fitted with a 30-G needle. After injection, individual minnows were returned to a 19-l holding tank, gently resuscitated, and later sampled for whole blood at various times between 0.25 and 20 h. The individual blood samples were added to a tared tube containing 100 µl of deionized water, the mass was recorded, and the sample were stored at −80°C. The sample volume was later calculated assuming a density of 1.05. The pharmacokinetics of E2-$d_3$ was studied in female trout fitted with a dorsal aortic

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catheter and administered an IA bolus dose of 0.1 mg/kg. Other aspects of IA injection and sampling were as described previously. Both unexposed and trenbolone-exposed (500 ng/l) minnows and trout were used in the studies. The trout were approximately 26 weeks postspawn when the experiments began. Injection of the E2-$d_3$ dose occurred after 7 days of trenbolone exposure, which was allowed to continue during sampling.

***E2 binding in trout plasma.*** The plasma protein binding of E2 was measured in trout plasma with and without coincubation of trenbolone. Plasma was collected from three trout that were in previtellogenic or late vitellogenic phases of ovarian maturation (7 and 43 weeks postspawn, respectively). The previtellogenic plasma samples were fortified with E2 to bring the concentration up to 75 ng/ml, comparable to levels measured in the late vitellogenic plasma samples. This was necessary to allow quantification of E2 in the filtrate. Aliquots of each sample were incubated with trenbolone to produce a concentration of 90 ng/ml. The binding studies were then performed as described previously for trenbolone.

*Experimental Procedures—Toxicoproteomic Studies*

For all studies, trout and minnow plasma was used without attempting to deplete specific or abundant proteins. In preliminary studies, a top-down approach consisting of 2-dimensional sodium dodecyl sulfate-polyacrylamide gel electrophoresis (2D-SDS-PAGE) and liquid chromatography-matrix-assisted laser desorption and ionization-time of flight-mass spectroscopy (LC-MALDI-TOF MS) was used. These initial studies (summarized in Supplementary Figures) characterized patterns of differentially expressed proteins, which were used to assess sex and interspecies differences in the plasma proteome with or without trenbolone exposure. For these studies, both male and female fathead minnows were exposed for 168 h to 500 ng/l trenbolone. Each minnow treatment group was exposed in 2 replicate tanks ($n$ = 4 minnows/tank). After the exposure, minnow plasma was collected as described previously, pooled from individuals within a tank, mixed with 4 U/ml aprotinin, and frozen at −80°C in 10- to 25-µl aliquots. In a separate experiment, 3 female trout were individually exposed for 168 h to 500 ng/l trenbolone. Trout plasma was collected from the caudal vein and stored as described previously. Both control minnows and trout were similarly housed as exposed fish and sampled on the same day.

For 2D-SDS-PAGE, 30 µg of plasma protein was initially separated on a broad-range immobilized pH gradient (IPG) using 11-cm IPG 3-10NL strips (ReadyStrip, Bio-Rad) and then on 8%–16% SDS-PAGE (Criterion gels, Bio-Rad). Sample application and SDS-PAGE conditions were as described by Barry *et al.* (2006), except that the urea/thiourea solubilized matrix was passed through a 5 kDa low–molecular weight cutoff membrane. The gels were then fixed and stained with the fluorescent dye SYPRO Ruby as described by Barry *et al.* (2006). The stained gels were scanned using a Bio-Rad Molecular Imager, and protein spots analyzed using PDQuest (Bio-Rad).

For the LC-MALDI-TOF MS analysis, plasma samples were first subjected to ultrafiltration using a 100 kDa MW cutoff membrane (microcon 100 Millipore Corp., Billerica, Massachusetts) as described by Wunschel *et al.* (2005), with the filtrate reserved for subsequent analysis. Plasma proteins were then separated by capillary high-performance liquid chromatography linked with a Probot fraction collector for direct deposition on a MALDI sample plate as described by Wunschel *et al.* (2006). A total of 100 fractions were collected that were analyzed by MALDI-MS using a Perspective Biosystems (Framingham, Massachusetts) Voyager SR TOF MS equipped with delayed extraction and ion reflector. External standards were used to generate an instrument mass calibration each day prior to analysis. Data were collected in linear mode using an extraction voltage of 25 kV, a grid voltage of 90%, a guide wire voltage of 0.15%, and a time delay of 120 ns. A low mass gate of 500 m/z was used to deflect the low m/z matrix ions, and a mass range of 3000–76 000 m/z was monitored for data collection. A total of 64 laser shots were collected and averaged to create each spectrum. The mass measurements associated with a specific LC fraction were imported into Excel and then preprocessed and normalized to make the data amenable for statistical comparisons and principal component analysis (PCA). This consisted of segregating the detected masses into 3 size ranges: < 19 000, 20 000–39 999, and > 40 000. All masses detected within each range were summed for each LC fraction, and the sums used for subsequent analyses. In order to do multifraction comparisons, the fraction sums were normalized by the column range method such that the final measure = (value − column minimum)/column range. Each fraction column presents with values from 0 (lowest sum in the column) to 1 (highest sum in the column). ANOVA was carried out (StatistiXL version 1.8) on the normalized values to identify the most significant fractions between each of the groups. All analytes at the highly significant probability of $p < .01$ were then used for PCA, with the results plotted onto a proximity map for verification of differentiation among the groups. In the proximity map, each point represents a separate sample, and the location of the point is characterized by the pattern of masses, such that samples with similar patterns of masses in the significant fractions are located nearer to each other than samples with different patterns.

***iTRAQ-based quantitative proteomic studies.*** This experiment used 4 groups of minnows exposed for 168 h to 0 (control), 5, 50, and 500 ng/l trenbolone. The exposure was as described previously with 2 tank replicates of 4 fish each (8 minnows total/treatment). At completion of the exposure, blood plasma was collected, pooled within each treatment group, and flash frozen in liquid nitrogen without addition of protease inhibitors.

***Protein digestion, iTRAQ labeling, and LC-ESI-MS/MS analysis.*** Equal volumes (25 µl) of plasma were acetone precipitated; then resolubilized in triethylammonium bicarbonate buffer, pH 8.5, containing 4M urea and 0.2% SDS; and the protein concentration measured using BCA (Sigma-Aldrich). Approximately 100 µg of protein from each treatment was reduced by the addition of 3mM Tris-(2-carboxyethyl) phosphine and incubated at 60°C for 60 min. Cysteine alkylation was achieved using methyl methane thiosulfonate (6mM). Sequencing grade modified trypsin (10 mg, Promega, Madison, Wisconsin) was added to each sample and allowed to digest overnight at 37°C. Labeling of the digested samples with the iTRAQ reagents (Applied Biosystems, Foster City, California) was performed according to the manufacturer's recommendations described by Seshi (2006). The control, 500, 50, and 5 ng/l samples were labeled with the 114, 115, 116, and 117 isobaric tags and then pooled. Next, the pooled sample mixture was subjected to strong cation exchange (SCX) as described by Kuzyk *et al.* (2009). A total of 80 SCX fractions were collected, with 14 subsequently selected for MS analysis based on ultraviolet absorbance at 214 nm. The MS/MS analysis was performed using an Applied Biosystems/MDS Sciex QSTAR Elite quadrupole TOF system equipped with a NanoSpray source and heated interface. The LC configuration and MS data acquisition parameters were as described by Kuzyk *et al.* (2009).

***iTRAQ data analysis.*** Raw data were processed by ProteinPilot and the Paragon search algorithm (Applied Biosystems V4.0.0). Data were searched against the UniProtKB/TrEMBL database (accessed on September 09, 2012) using the "thorough search" option with iTRAQ 4-plex peptide labeled as sample type and biological modification as the ID focus. Protein identification was accepted only if the Protein Pilot Unused Score was > 1.3 (> 95% confidence). The local false discovery rate (FDR) was calculated by the Paragon program using the Proteomics System Performance Evaluation Pipeline algorithm (Tang *et al.*, 2008). The predicted FDR for identified proteins with unused scores > 1.3 was < 1%. The PANTHER protein classification system (Protein ANalysis THrough Evolutionary Relationships; http://www.pantherdb.org/, SRI International, Menlo Park, California) was used to characterize identified proteins according to protein class and biological processes. Identification of differentially expressed proteins between control and trenbolone-exposed groups was based on the following criteria: a 115:114 ratio (control: 500 ng/l treatment) that was ≤ 0.7 or ≥ 1.3, a $p$ value ≤ 0.05 and/or an error factor (EF) score of ≤ 2 and at least 2 peptides were used for quantitation. The EF score defines the 95% confidence interval of the ratio and is a measure of the certainty of the average ratio. A score of ≤ 2 indicates the certainty of the ratio is within a factor of 2.

*Gas Chromatography-MS Quantification of Trenbolone and E2*

Samples (exposure water, plasma or blood, ultrafiltrates) were fortified with an internal standard (E2-$d_3$ or EE2-$d_4$ when both E2 and E2-$d_3$ were present in the sample), mixed with a saturated NaCl solution, and then extracted with

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methyl-tert-butyl ether. The ether extracts were evaporated under N2 and the residue mixed with various amounts of MSTFA with or without catalysts, depending on the measurement. For trenbolone analysis, 25 µl of MSTFA containing resublimed $I_2$ as the catalyst was used (Marchand *et al.,* 2000). For samples spiked with EE2-$d_4$, 20 µl of MSTFA with ITS as the catalyst was used (Schultz *et al*., 2001). For E2 analysis, 10–25 µl of MSTFA was used without a catalyst and heated at 70°C for 30 min prior to analysis. The smallest volume of MSTFA was used with ultrafiltrates to improve sensitivity for E2. The steroid-trimethylsilyl derivatives were then quantified using gas chromatography (GC)-MS (Agilent 6890 GC, 5973inert MS). The analytes were separated on a J&W 30 m DB-5 ms column (0.25 mm I.D., 0.25 µm film thickness) with splitless injection at 90°C with other oven conditions as described by Stanford and Weinberg (2007). The MS was operated in electron impact mode with selective ion monitoring made for 416, 419, 429, and 442 m/z, which were used for E2, E2-$d_3$, EE2-$d_4$, and trenbolone quantification, respectively.

### *Toxicokinetic Analysis*

Both clearance-volume compartmental and noncompartmental analysis methods were used to analyze the blood or plasma concentration-time profiles of trenbolone and E2-$d_3$ after IA or IC injection. Preliminary analysis of the individual trenbolone plasma profiles in trout and E2-$d_3$ profiles in fathead minnows indicated a two-compartment model provided the best fit to the observed data based on several criteria: Akaike information criterion, coefficients of variation, and visual inspection of the fits (Boxenbaum *et al*., 1974; Yamaoka *et al*., 1978). The following biexponential equation was then fit to the observed data using WinNonlin 2.1 (Pharsight Corp., Mountain View, California), a nonlinear least squares regression program:

$$C_p t = Ae^{-\alpha t} + Be^{-\beta t} \quad (2)$$

$C_p t$ is the concentration in blood or plasma; $A$, $\alpha$, $B$, and $\beta$ are the macroconstants describing the intercepts and slopes. The derived model parameters reported are area-under-the-curve extrapolated to infinity ($AUC_{0\to\infty}$), total body clearance ($Cl_b$), the apparent volume of the central compartment ($V_1$), the steady-state volume of distribution ($V_{ss}$), and elimination half-life ($t_{½\beta}$). These parameters were calculated by the WinNonlin program from the macroconstants using standard equations (Gabrielsson and Weiner, 2000). Preliminary analysis of the E2-$d_3$ plasma profiles in trout suggested several compartmental models would be needed to describe all the profiles. For greater consistency, a noncompartmental approach was used instead to analyze the individual profiles. The $AUC_{0\to\infty}$ was estimated using the linear trapezoidal method with the area from the last sampling time to infinity calculated from the slope of the terminal portion of the concentration time profile ($\beta$). The $Cl_b$ and $V_{ss}$ were then calculated using standard equations. The parameters calculated from trout studies are presented as arithmetic mean ± SD based on analysis of the individual profiles. Statistically significant ($p < .05$) differences between parameters calculated for control and trenbolone-exposed trout was determined by the Student's *t* test. For fathead minnows, the model estimated value ± SE associated with the nonlinear least squares fit is presented.

## RESULTS

### *Trenbolone Exposures*

For most exposures, the average measured concentration was within ± 20% of the nominal level. One exception was minnow exposures for proteomic analysis, where the measured concentrations at the 500 ng/l level were within ± 30% of nominal. A summary of all measured trenbolone concentrations along with route of administration and duration is provided in Supplementary Table 2.

### *Toxicokinetics*

*Water exposures.* A summary of the uptake of trenbolone in both trout and minnows during water exposures is presented in Figure 1. An enlarged view of the initial 24 h of uptake and the depuration after the exposure is shown in Supplementary Figure 1A. In both species, trenbolone absorption was rapid with readily detectable levels observed at the first sampling times of 0.25 and 0.5 h. Plasma concentrations of trenbolone steadily increased in both species until approximately 8.0 h, when they appeared to reach a plateau or, in the case with minnows, achieve peak levels that declined afterward. In trout, once the pseudoequilibrium was achieved, mean plasma concentrations varied between 66 and 95 ng/ml, corresponding to a plasma:water concentration ratio of approximately 130–252. In minnows, the peak trenbolone plasma concentration was 29 ng/ml, which declined to 15 ng/ml at 168 h, resulting in plasma:water concentration ratio of 61 and 27, respectively. Thus, plasma concentrations of trenbolone are approximately 2–6 times higher in trout relative to minnows during comparable exposures. The depuration of trenbolone in trout was also rapid, with detectable plasma levels observed until 72 h, at which time levels were near the limits of quantification (Fig. 1;

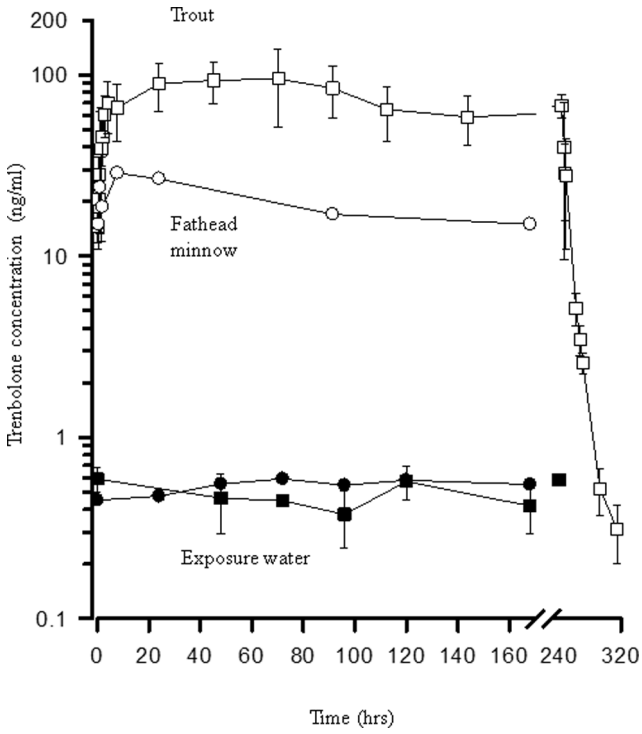


**FIG. 1.** Trenbolone concentration-time profiles in female rainbow trout and fathead minnows continuously exposed to 500 ng/l for 168–240 h in a flow-through exposure system. Measured trenbolone levels in exposure water are shown as corresponding black symbols. The trout plasma profiles (□) were obtained from individual fish fitted with a dorsal aortic cannula and repetitively sampled (mean ± SD; *n*= 3–5). The minnow plasma profiles (○) were obtained from pooled samples collected from a group of three exposed fish/time point.

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Supplementary Figure 1B). The plasma $t_{½}$ of trenbolone during depuration was calculated to be 14.5 h.

*IA injection of trenbolone in trout.* The mean plasma concentration-time profile of trenbolone after administration of a 0.5 mg/kg dose is shown in Figure 2. A summary of the toxicokinetic analysis determined from the individual fish profiles is presented in Table 1. The decline after IA injection appeared biexponential with a terminal elimination $t_{½}$ of 23.8 h (Table 1). The $Cl_b$ was 0.135 ml/h·g, and the $V_{ss}$ was 2.18 ml/g, which is large and indicative of substantial extravascular distribution of trenbolone. The intercompartmental clearance (*Cl*) was 0.303 ml/h·g but highly variable among individuals (Table 1). There was also substantial variability in the plasma concentrations between fish, with the coefficient of variation frequently exceeding 50% at comparable sampling times. In a previous study with EE2, this variability was attributable to multiple plasma peaks caused by glucuronide conjugation, biliary secretion, and subsequent enterohepatic recirculation (Schultz *et al.*, 2001). However, close inspection of the trenbolone profiles during the initial 24 h (see Supplementary Figure 2), when sampling was most intensive, did not clearly indicate multiple peaking was occurring. Thus, a simple 2-compartment model was judged adequate to describe the observed data without the need to include an enterohepatic recirculation component.

*Trenbolone plasma binding.* The unbound percentage of trenbolone in trout and minnow plasma is shown in Figure 3. The binding to trout plasma appeared to be higher compared with minnows, with the unbound percentage being 4.5% versus 18.3%, respectively. Due to experimental design limitations of

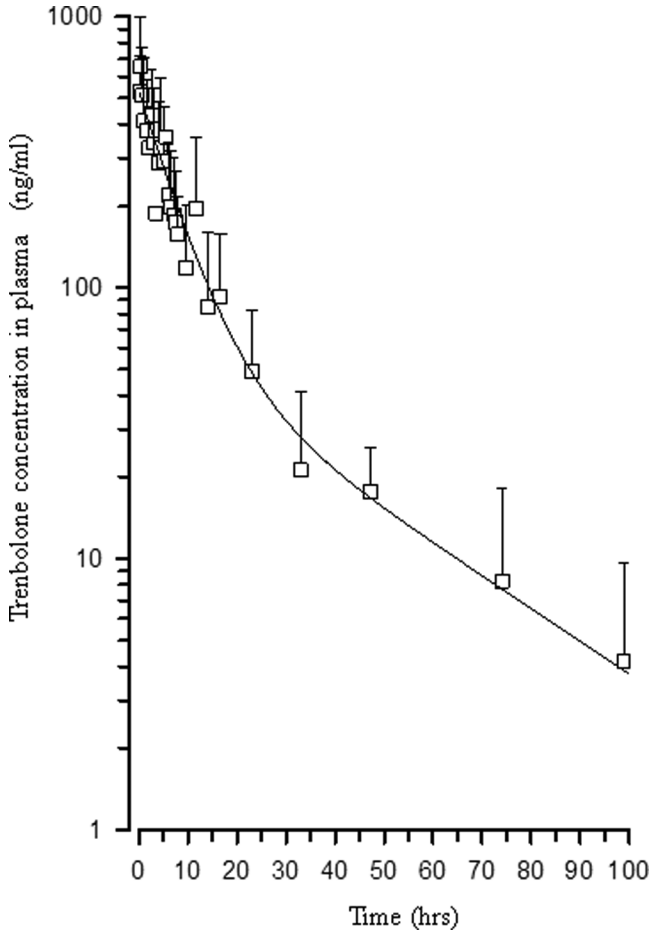


**FIG. 2.** The plasma concentration-time profiles of trenbolone in female rainbow trout after 0.5 mg/kg intra-arterial injection. Values are mean ± SD ($n$ = 3–5). The solid line is the nonlinear least-squares fit of the mean values using a two-compartment model and is provided as a representative example. Parameter estimates shown in Table 1 are based on the individual data collected from each fish, which is shown in Supplementary Figure 2.

**TABLE 1**
**Toxicokinetic Parameters for Trenbolone in Female Trout After IA Administration of a 0.5 mg/kg Dose**

| Parameter | Estimate |
|---|---|
| $AUC_{0\to\infty}$ (ng/ml hr) | 6454 ± 4505 |
| $V_{ss}$ (ml/g) | 2.18 ± 0.34 |
| $V_1$ (ml/g) | 0.92 ± 0.41 |
| $Cl_b$ (ml/h/g) | 0.135 ± 0.095 |
| $Cl_t$ (ml/h/g) | 0.303 ± 0.428 |
| $t_{½,\beta}$ (h) | 23.8 ± 14.3 |

*Note*. Parameter estimates (mean ± SD, $n$ = 5) were calculated from the individual plasma-concentration time profiles after fitting the observed data to a two-compartment model using WinNonlin 3.1. Figure 2 shows the average data for all fish and a representative fit of the data.

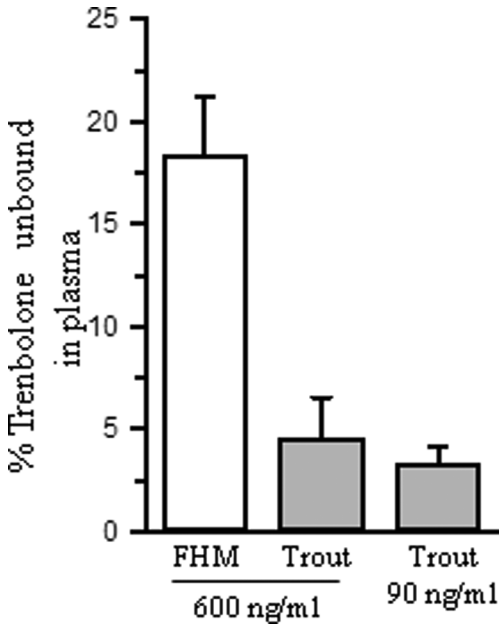


**FIG. 3.** Plasma binding of trenbolone in female rainbow trout and fathead minnows (FHM) determined by ultrafiltration. Unbound percentage of trenbolone in trout plasma (gray bar) and minnow plasma (white bar). Values for trout are mean ± SD ($n$ = 5 trout sampled 16 weeks postspawn). Values for minnows are mean ± SD, determined from a single pool of plasma that was analyzed twice.

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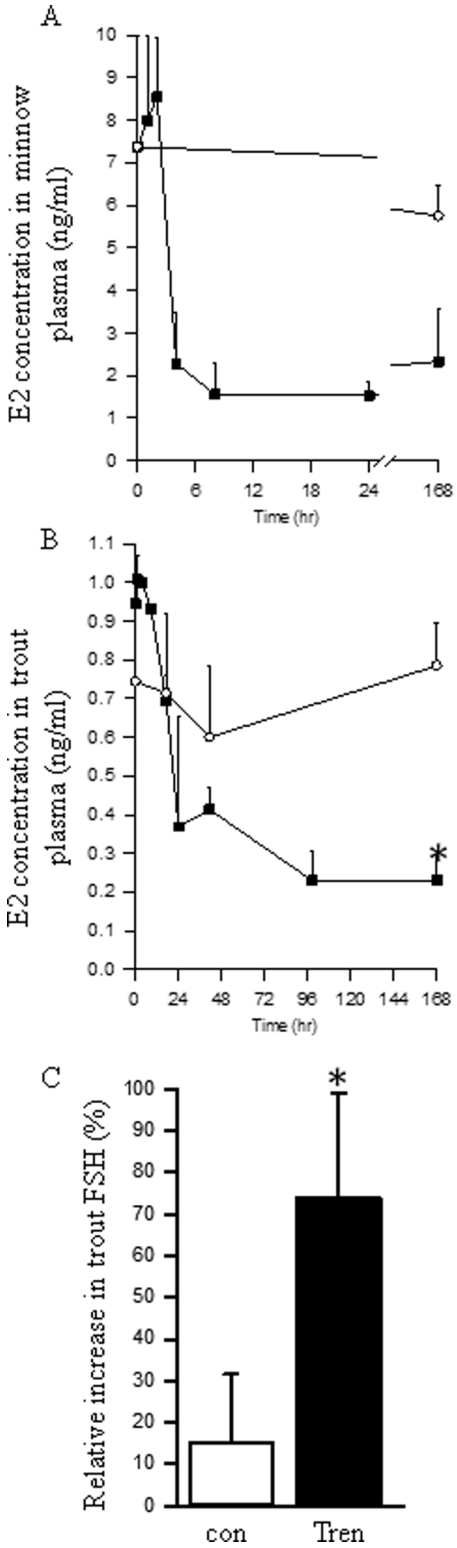


**FIG. 4.** Circulating levels of E2 and FSH in female rainbow trout and fathead minnows during continuous trenbolone exposures. A, E2 concentrations in minnows during 168-h exposure to measured trenbolone concentrations of 878 and 540 ng/l (beginning and end of exposure). Values are mean ± SD ($n$ = 3) for control (○) and trenbolone-exposed (■) minnows and were determined by enzyme immunoassay. *Significantly less than controls ($p$ < .01). B, E2 concentrations in trout during 168-h exposure to a measured trenbolone concentration of 412 ± 91 ng/l (mean ± SD, $n$ = 6 measurements). Values are the mean ± SD from 3 repetitively sampled control (○) and trenbolone-exposed (■) trout fitted with a dorsal aortic cannula. E2 was quantified using RIA. *Significantly less than controls ($p$ < .01). C, Relative increase in FSH concentrations in female trout during the 168-h exposure to trenbolone. Values were determined from the same trout as in (B) and represent the percent increase in FSH plasma concentrations (determined by RIA) relative to values at the start of the exposure. *Significantly greater than controls ($p$ < .04). Abbreviations: E2, estradiol; FSH, follicle-stimulating hormone; RIA, radioimmunoassay.

the minnow measurement (two technical and one pooled biological replicate), no statistical comparisons were made. The unbound percentage of trenbolone was not significantly changed when trout plasma was incubated at the lower concentration of 90 ng/ml.

### *Toxicodynamic Studies*

*Plasma binding and circulating E2 and FSH.* Trenbolone exposure to minnows caused a rapid decline in plasma E2 concentrations after a brief, initial increase (Fig. 4A). The concentration of E2 declined to approximately 2 ng/ml within 4–8 h of exposure and was maintained at that level throughout the remainder of the 168-h exposure, when it was significantly lower than the control minnows ($p$ < .01). In nonexposed female minnows, E2 levels varied between 6 and 8 ng/ml (Fig. 4A).

In trout, 1-week exposure to trenbolone also caused a rapid decline in plasma E2 concentrations, which by 24 h was approximately 40% of the initial concentration (Fig. 4B). After 168 h of exposure, the E2 concentrations were significantly lower than control trout ($p$ < .01). The plasma FSH concentration in these same fish was also measured immediately before and after the trenbolone exposure. In both exposed and control fish, the FSH levels had increased after 168 h; however, the relative increase was significantly greater for the trenbolone-exposed trout compared with control trout (Fig. 4C). The individual before and after FSH values are shown for all fish in Supplementary Figure 3. In the second experiment, trout were exposed to a lower trenbolone concentration (70 ± 19 ng/l mean ± SD, $n$ = 8) for 27 days. The plasma E2 concentrations in these fish were monitored for 60 days before the exposure and then every 7–8 days during the exposure to characterize the E2 concentration-time profiles. The mean ± SD of these measurements for the control and trenbolone-exposed groups is shown in Figure 5 (the individual profiles are shown in Supplementary Figure 4). As observed previously, trenbolone exposure caused E2 concentrations to decline from values measured at the start of the exposure (Fig. 5). The E2 levels in the exposed trout were higher than control trout at the beginning of the exposure (time "0" in Fig. 5), suggesting these fish may have begun to mature earlier than control trout. The decline in E2 levels was not as dramatic as observed previously although it did occur during a maturational time period when E2 levels are normally increasing rapidly as evidenced by control values that increased more than 2-fold during the 27 days (Fig. 5).

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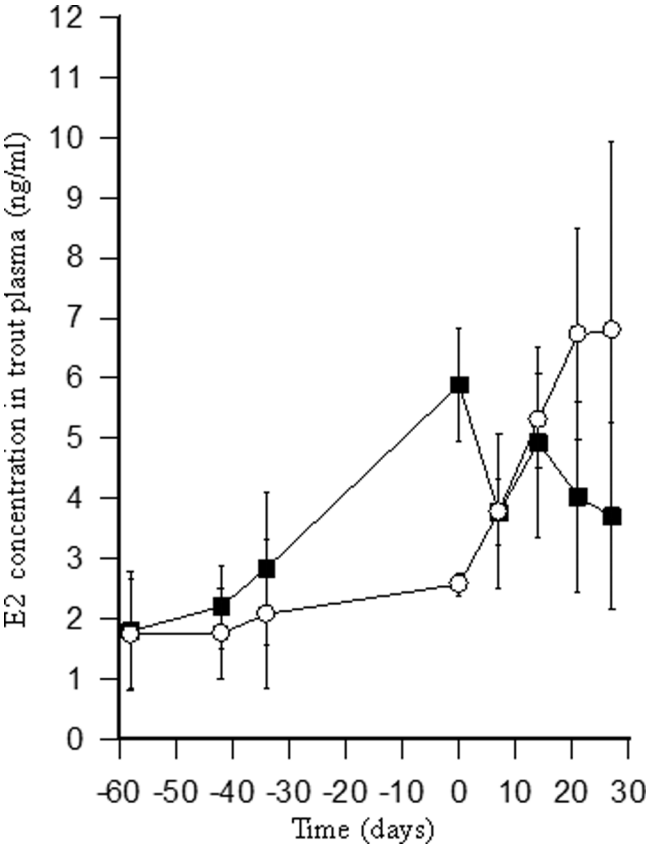


**FIG. 5.** Circulating levels of E2 in female rainbow trout before (indicated by minus days) and during a 27-day trenbolone exposure to measured concentrations of 70 ± 19 ng/l ($n$ = 8 measurements). Values are mean ± SD ($n$ = 4 repetitively sampled trout) for control (○) and trenbolone-exposed (■) trout. E2 was quantified using RIA. Trout were 16 weeks past previous spawning at start of exposure.

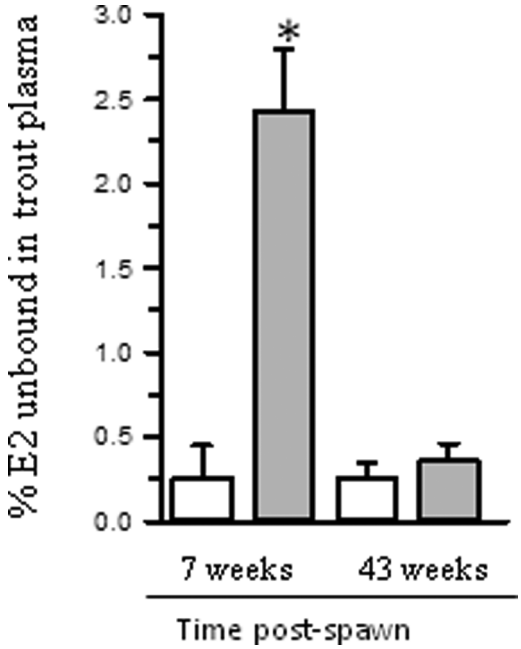


**FIG. 6.** Female rainbow trout plasma binding of E2 determined by ultrafiltration. Unbound percentage of E2 in plasma collected from three fish at two separate times during the annual reproductive cycle (pre- [7 weeks] and postvitellogenic [43 weeks]). Plasma was incubated with (gray bars) or without (white bars) added trenbolone at 90 ng/ml. *Significantly greater than without added trenbolone ($p$ < .02). E2 was measured using GC-MS as described in Materials and Methods section.

The unbound percentage of E2 was observed to be similar in plasma obtained from pre- or postvitellogenic female trout and was 0.25%–0.26% (Fig. 6). Incubation of aliquots of the same plasma with trenbolone caused the unbound percentage to significantly increase to 2.4% in the previtellogenic trout (Fig. 6). Plasma binding of E2 was unaffected by trenbolone incubation in the postvitellogenic trout.

*E2-$d_3$ pharmacokinetics in trout.* The mean plasma concentration-time profiles of IA injected E2-$d_3$ in control and trenbolone-exposed trout are shown in Figure 7. The decline in plasma concentrations after IA injection appeared multiexponential with quantifiable E2-$d_3$ observed until 48–72 h in control fish. In trenbolone-exposed trout, E2-$d_3$ declined more rapidly and could only be quantified until 24–48 h after injection for most trout. A summary of the toxicokinetic analysis determined from the individual fish profiles (which are shown in Supplementary Figure 5) is presented in Table 2. The two most notable differences between treatment groups was the $AUC_{0\to\infty}$, which was significantly lower in the exposed trout, and $Cl_b$, which was significantly increased in these fish (Table 2). The $V_{ss}$ was very similar between groups and approximately 0.26 ml/g. This indicates the primary treatment effect on E2-$d_3$ pharmacokinetics was to increase clearance, which would explain the reduction in plasma $t_{½}$ observed after trenbolone exposure (Table 2).

*E2-$d_3$ pharmacokinetics in minnows.* The mean blood concentration-time profiles of IC injected E2-$d_3$ in control and trenbolone-exposed minnows are shown in Figure 8. The model predicted values are also shown on the graph as solid lines. A summary of the toxicokinetic analysis is presented in Table 3. The overall decline in blood concentrations after injection appeared biexponential, with quantifiable E2-$d_3$ observed in all fish. However, by 20 h several fish had concentrations that were near detection limits. Blood concentrations of E2-$d_3$ were much more variable after 2 h, with % CVs exceeding 100% at some sampling times. The observed concentrations of E2-$d_3$ in exposed minnows frequently appeared to be lower than control minnows, which are reflected in the model predicted profiles (Fig. 8). The toxicokinetic analysis indicated trenbolone-exposed minnows had a much lower $AUC_{0\to\infty}$ largely attributable to a > 2-fold increase in $Cl_b$ (Table 3). The blood elimination $t_{½}$ was also reduced compared with control fish. The volume of the central compartment ($V_1$) was similar between control and exposed minnows; however, the $V_{ss}$ was nearly twice as large in exposed minnows (Table 3). Direct comparisons of

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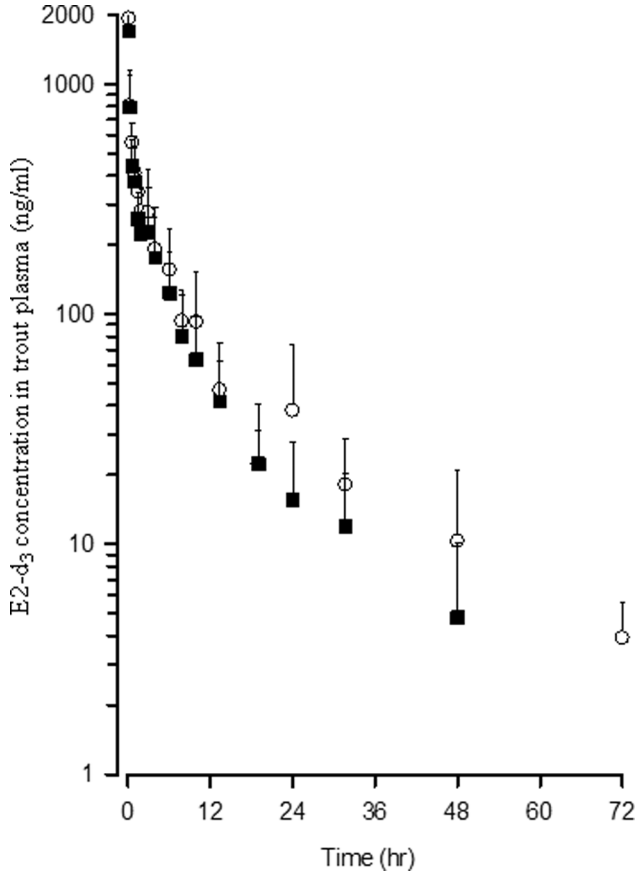


**FIG. 7.** The plasma concentration-time profiles of E2-$d_3$ after 0.1 mg/kg intra-arterial injection in female rainbow trout. Values are mean ± SD ($n$ = 6) for control (○) and trenbolone-exposed (■) trout. The E2-$d_3$ dose was administered on day 7 of the trenbolone exposure and allowed to continue during sampling. Measured trenbolone exposure levels were 463 ± 108 ng/l (mean ± SD, $n$ = 13). Trout were 26 weeks past previous spawning at start of exposure.

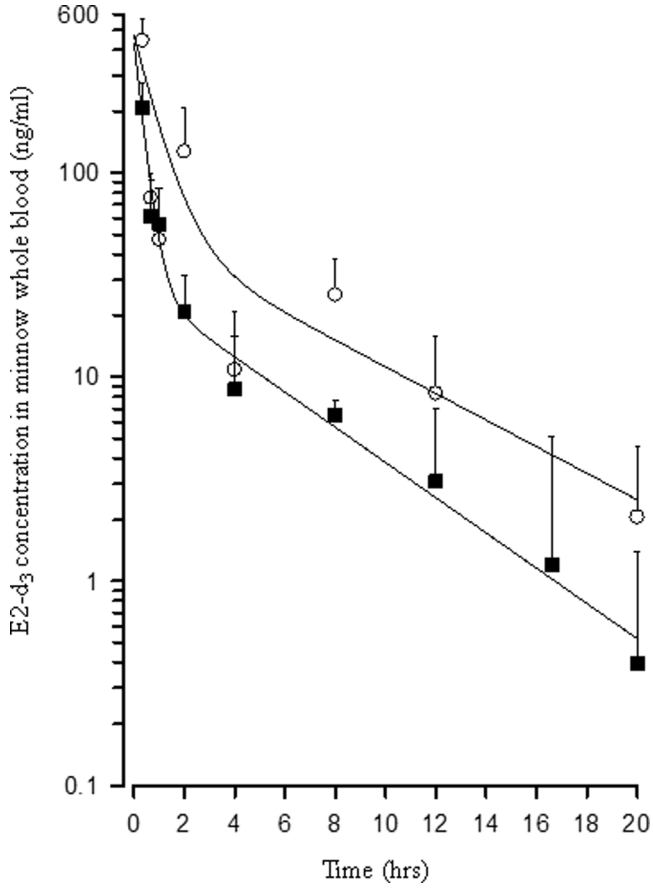


**FIG. 8.** The blood concentration-time profiles of E2-$d_3$ after a 1 μg/g IC injection into female fathead minnows. Groups of 3–6 control (○) and trenbolone-exposed (■) minnows were sampled at each time point. Measured trenbolone exposure levels were 502 ± 123 ng/l (mean ± SD, $n$ = 6). The solid line is the nonlinear least-squares fit of the observed values using a two-compartment model. Parameter estimates from these fits are shown in Table 3.

**TABLE 2**
**Toxicokinetic Parameters for E2-$d_3$ in Female Rainbow Trout After IA Injection (0.1 mg/kg) With or Without 7-Day Exposure to Trenbolone (500 ng/l)**

| Parameter | Control | Trenbolone Exposed |
|---|---|---|
| $AUC_{0\to\infty}$ (ng/ml hr) | 4532 ± 1963 | 3317 ± 1871[a] |
| $V_{ss}$ (ml/g) | 0.261 ± 0.111 | 0.264 ± 0.122 |
| $Cl_b$ (ml/h/g) | 0.022 ± 0.013 | 0.031 ± 0.017[b] |
| $t_{½, β}$ (h) | 17.91 ± 4.69 | 12.87 ± 5.17 |

*Note*. Parameter estimates (mean ± SD, $n$ = 6) were calculated from the individual blood-concentration time profiles using noncompartmental methods.
[a]Significantly less ($p$ < .03) than controls.
[b]Significantly greater ($p$ < .02) than controls.

**TABLE 3**
**Toxicokinetic Parameters for E2-$d_3$ in Female Fathead Minnows After IC Injection (1 mg/kg) With or Without 7-Day Exposure to Trenbolone (500 ng/l)**

| Parameter | Control | Trenbolone Exposed |
|---|---|---|
| $AUC_{0\to\infty}$ (ng/ml h) | 686 ± 207 | 284 ± 47 |
| $V_1$ (ml/g) | 2.13 ± 1.88 | 2.31 ± 1.19 |
| $V_{ss}$ (ml/g) | 5.37 ± 2.98 | 9.35 ± 2.80 |
| $Cl_b$ (ml/h/g) | 1.46 ± 0.44 | 3.52 ± 0.58 |
| $t_{½, β}$ (h) | 4.62 ± 2.41 | 3.49 ± 0.33 |

*Note*. Parameter estimates (± SE) were calculated from the blood concentrations after fitting the observed data to a two-compartment model using WinNonlin. The model predicted values are shown with observed data on Figure 8.

E2-$d_3$ toxicokinetic parameters between trout and minnows are restricted by the 10-fold difference in dose, sampling compartment (blood vs plasma), and route of administration differences. Nonetheless, a common feature in both species was the increase in $Cl_b$ and decrease in $t_{½}$ that occurred after trenbolone exposure.

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*Toxicoproteomic Studies*

Preliminary studies using a top-down approach were able to produce 2D maps of the minnow and trout plasma proteomes and assess the relative influence of interspecies differences and trenbolone exposure. These results are summarized in Supplementary Figures 6–11. The 2D-SDS-PAGE separations of minnow plasma were able to resolve over 400 spots with 87 discrete spots identified as differentially expressed after trenbolone exposure (Supplementary Figure 7). Of these protein spots, 65 appear to be decreased in abundance, whereas 9 are increased in exposed minnows (using Student's *t* test). Three notable clusters of spots that are decreased in the trenbolone-exposed minnows are approximately pI 8–9 with MW 130-170 kDa, pI 9 and 38 kDa, and pI 9.5 with MW 15 kDa. In trout plasma, a similar degree of separation was achieved (Supplementary Figure 8), and 83 protein spots showed significant abundance differences between the controls versus trenbolone-exposed trout, with most spots decreasing in abundance with exposure and a few appearing only in the gels for exposed trout. Differential analysis of the trout gels did not show the same notable clusters as seen with the minnows.

The MALDI-TOF analysis consisted of a total of 26,162 mass measurements between 3 and 76 kDA in the unexposed and exposed fish. Approximately 93% of these measurements were < 20 kDa, 5.3% were between 20 and 40 kDA, and 1.6% were > 40 kDA (Supplementary Table 3). Trenbolone exposure increased the number of mass measurements that were < 20 kDA, with 2 proteins induced after exposure with approximate masses of 7.9 and 14.3 kDa (Supplementary Figure 9). PCA analysis indicated mass measurements in the 20–40 kDA size range from 13 fractions provided the most separation between unexposed female minnows and trout, with PC1 and PC2 accounting for 62% of variance in the data set (Supplementary Figure 10). After trenbolone exposure, interspecies differences were not apparent, and both exposed minnows and trout clustered with unexposed male minnows (Supplementary Figure 10).

*iTRAQ.* The analysis identified a total of 148 proteins in minnow plasma at > 95% confidence based on a total of 1431 distinct peptides that were measured. The complete list of these proteins along with the respective iTRAQ ratios (relative to unexposed fish) for each trenbolone treatment and associated *p* values and EF scores can be found in Supplementary Table 4. Manual inspection of the list indicates the dominance of highly abundant proteins such as lipoproteins, fibrinogen, complement system, and α-2-macroglobulin. The number one ranked protein is fathead minnow vitellogenin (Q9W6I2_PIMPR), which displayed a dose-dependent decrease in its abundance, relative to controls (Table 4). In addition to fathead minnow vitellogenin, fragments of 10 other vitellogenin proteins are listed, presumably due to either protein degradation during processing and/or incorrect grouping of peptides by the ProteinPilot program. Additional characterization of identified proteins was done using the PANTHER program, which uses gene ontology classifications associated with a specific protein. The PANTHER analysis for protein class and biological processes is shown in Figures 9A and 9B. Thirteen different biological processes were recognized from 32 proteins. The process most frequently recognized was metabolic followed by immune system and transport (Fig. 9B). There were 17 different protein classes recognized, also based on 32 proteins, dominated by enzyme modulator, oxidoreductase, hydrolase, and immunity (Fig. 9A). After filtering the list of indentified proteins for differential expression, *p* value, and EF score, a total of 19 proteins were considered downregulated and 18 upregulated. Further reduction was done by selecting the highest rank protein among multiple listings of similar proteins. This subset is presented in Table 4. Downregulated proteins primarily consist of vitellogenin, fibrinogens, α-2-macroglobulin, and transferrin. Upregulated proteins included amine oxidase, apolipoproteins, parvalbumin, complement system proteins, and several uncharacterized proteins. A sex hormone–binding globulin (SHBG-α) was slightly increased in the 500 ng/l treatment.

## DISCUSSION

*Toxicokinetics*

The toxicokinetic properties of trenbolone are similar to other sex steroids, which display among the most rapidly observed uptake and extravascular distribution properties characterized in fish. For example, exposure of adult male rainbow trout to the synthetic estrogen EE2 resulted in achievement of an apparent equilibrium between the exposure water, plasma, and liver within 8–16 h (Skillman *et al.*, 2006). In smaller fishes such as zebrafish, stickleback, and juvenile flatfishes, the uptake and accumulation of endogenous steroids occur even more rapidly with apparent equilibriums achieved within minutes or a few hours (Maunder *et al.*, 2007; Miguel-Queralt *et al.*, 2008; Specker *et al.*, 2003). The kinetic behavior of sex steroids along with their physiochemical properties, notably log octanol-water partitioning between 2.5 and 5, are indicative of the gills as the site of absorption and ventilation volume (volume of water passing over the gills) being the physiological variable that controls absorption rate (Hayton *et al.*, 1990; McKim *et al.*, 1985). An additional factor that influences steady-state plasma concentrations and the plasma:water partitioning ratio is binding to plasma proteins and other macromolecules. The plasma binding results in Figure 3 indicate trenbolone is highly bound to plasma proteins although binding would have been predicted to be even higher, exceeding 99% bound, given the observed plasma:water ratio of 130–252 in trout. In fish as in mammals, it is well established that sex steroids can bind to both high-capacity, low-affinity plasma proteins such as albumin and also a lower capacity, high-affinity protein known as the SHBG-α (Fostier and Breton, 1975; Hammond, 2011). Salmonids such as rainbow trout also produce another SHBG, SHBG-β, which is expressed in nonhepatic tissues

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(Bobe *et al.*, 2008). The lower-than-expected binding of trenbolone may reflect saturation of SHBG or other carrier proteins as a relatively high *in vitro* test concentration of 600 ng/ml trenbolone was initially used for the binding studies. However, a subsequent binding experiment using a lower trenbolone concentration similar to levels observed *in vivo*, did not observe a significant change in trenbolone binding in trout plasma (Fig. 3). Thus, other physiological factors are likely involved, which contribute to maintaining elevated plasma concentrations of trenbolone during prolonged water exposures. One aspect of the trenbolone binding studies that is consistent with the *in vivo* results is the interspecies differences. The 4-fold difference in plasma binding of trenbolone between trout and minnows appears to explain differences in peak or steady-state plasma concentrations during water exposures. This suggests substantial interspecies differences in plasma binding of trenbolone exist in fishes, which may also correspond to differences in plasma concentrations during environmental exposures. Whether the interspecies differences are attributable to differences in specific binding to SHBG-α or the lack of SHBG-β in minnows is unknown but warrants further investigation.

The depuration of trenbolone from trout was also rapid, with near complete loss from plasma within 72 h (Fig. 1). The depuration half-life of 14.5 h is similar to the elimination half-life of 23.8 h calculated after IA injection (Table 1). In some respects, these results are surprising given the rather large $V_{ss}$ determined from the IA dosing (Table 1), which was 2.18 ml/g. A large $V_{ss}$ indicates trenbolone is extensively distributing outside the plasma compartment, with some tissues having substantially higher steady-state concentrations than plasma (eg, tissue:plasma ratios >> 1). However, the intercompartmental clearance ($Cl_t$) is larger than total body clearance ($Cl_b$; Table 1), indicating exchange between tissues and plasma occurs more rapidly than elimination. Thus, the overall toxicokinetic behavior of trenbolone indicates it is in rapid equilibrium between exposure water, plasma, and peripheral tissues. Changes in environmental concentrations are predicted to be rapidly reflected in changes in plasma and tissues levels.

### *Toxicodynamics*

Trenbolone exposure to female fathead minnows has been previously demonstrated to cause a decline in plasma E2 concentrations in a dose-dependent manner up to approximately 500 ng/l (Ankley *et al.*, 2003; Ekman *et al.*, 2011). Higher trenbolone concentrations appear to cause less-pronounced decreases in plasma E2 concentrations (Ankley *et al.*, 2003). Experiments in this study were designed to confirm trenbolone effects on plasma E2, especially during the initial 24 h of exposure and also to investigate whether a similar phenomenon occurs in rainbow trout. Two advantages of including trout in the study are their larger size, permitting catheterization and repetitive blood sampling, and synchronous mode of gamete maturation, permitting experimentation during more discrete periods of oocyte development. The results in Figures 4A and 4B indicate that by 24 h, plasma E2 levels in both species are near their minimum values although the decline is more rapid in minnows. The trout results are interesting in that both experiments, which used trout that were in the previtellogenic (Fig. 4B) and in the vitellogenic phase of maturation (Fig. 5), observed declining plasma E2 concentrations during the exposure. This would suggest that trenbolone effects on circulating E2 are not specific to a particular stage of oocyte development.

It is understood that circulating levels of E2 and other endogenous hormones reflect a balance between synthesis and excretion. The *in vitro* culture of ovarian tissue removed from fathead minnows exposed to 500 ng/l trenbolone for 1 and 2 days has been shown to have significantly decreased secretion of E2 into the culture media (Ekman *et al.*, 2011). The decrease coincided with significant decreases in ovarian gene expression of aromatase A (CYP19A), supporting the hypothesis that decreases in plasma E2 during trenbolone exposure is attributable to reduced synthesis (Ekman *et al.*, 2011). However, other toxicodynamic aspects of trenbolone exposure in minnows suggest additional factors are involved. For example, it was also observed that persistent decreases in plasma E2 occur despite ovarian expression of aromatase recovering and exceeding control values after 4 and 8 days of trenbolone exposure (Ekman *et al.*, 2011). We hypothesized this observation was due in part to increased E2 clearance during trenbolone exposure. The pharmacokinetic analysis of injected E2-$d_3$ in trout supports this hypothesis, where the primary effect of trenbolone exposure was a 50% increase in clearance (Table 2). In minnows, the pharmacokinetic results are complicated by the necessity of using a higher E2-$d_3$ dose and IC injection. However, plasma clearance was increased although the volume of distribution was also substantially increased, partially mitigating effects on the plasma half-life (Table 3). A mechanism for increasing clearance may be associated with changes in E2 plasma binding during trenbolone exposure or perhaps induction of drug-metabolizing enzymes and increased metabolic clearance of E2. The immediate decline in E2 levels observed during exposures (Figs. 4A and B) and the plasma binding results shown in Figure 6 suggest reduced E2 plasma binding is the more likely possibility although it appears dependent on reproductive status of the trout. A decrease in plasma binding would be expected to increase branchial and possibly urinary elimination pathways for E2. Our conclusion is the decline in circulating levels of E2 during trenbolone exposure represents a combination of increased clearance and decrease in synthesis.

An additional observation made in this study was the disproportionate increase in circulating levels of FSH in trenbolone-exposed trout (Fig. 4C). This would appear to be explained by the decrease in E2 and the negative feedback exerted by E2 on plasma levels of FSH in trout (Saligaut *et al.*, 1998). However, the possibility that trenbolone may also exert a direct effect on pituitary synthesis of FSH and/or GnRH release by the hypothalamus cannot be ruled out because these roles for androgens have been documented (Antonopolou *et al.*, 2009; Zohar *et al.*,

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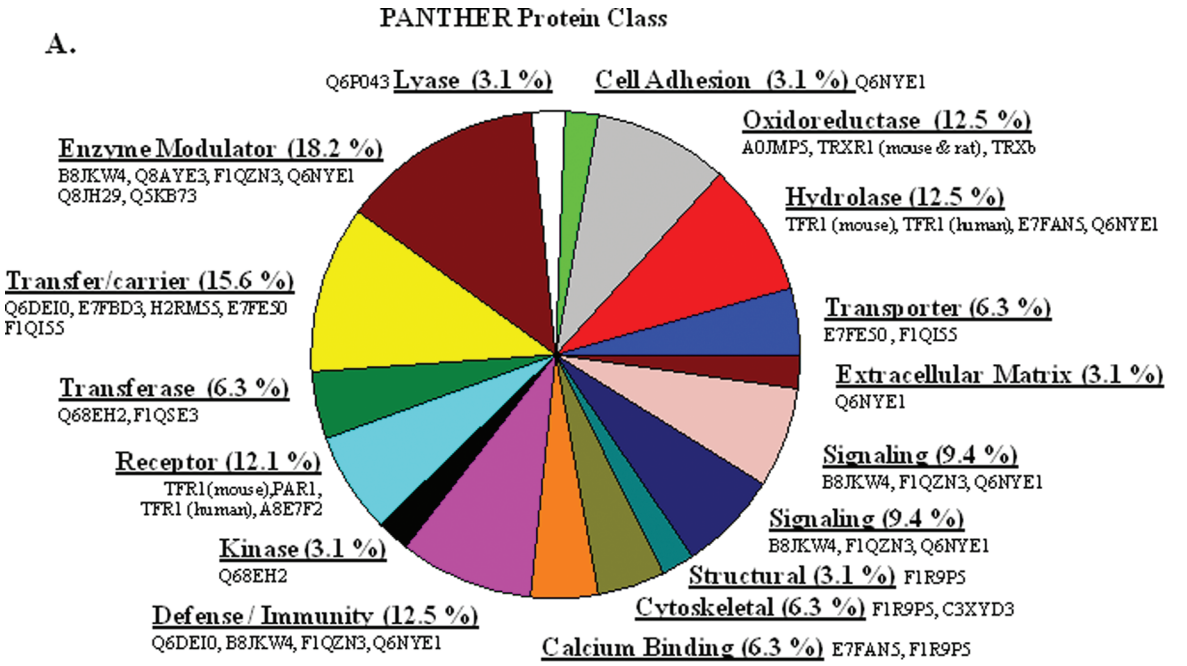


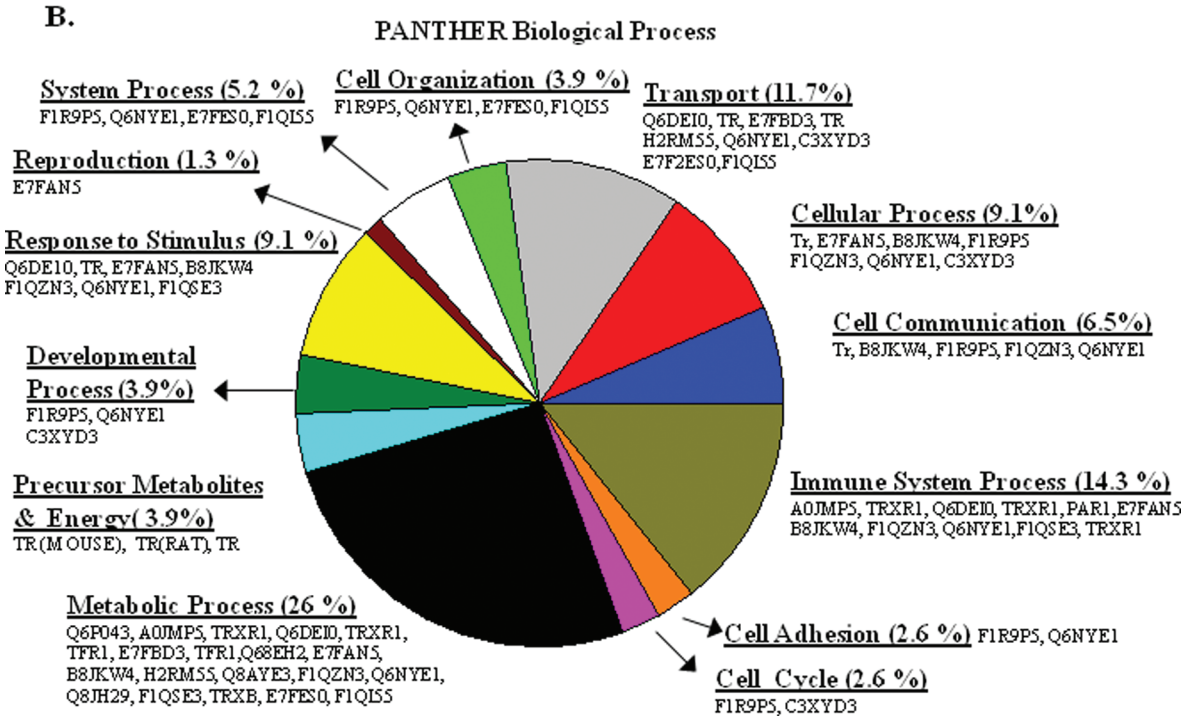


**FIG. 9.** PANTHER database classification of female fathead minnow plasma proteins identified using iTRAQ labeling and LC-MS/MS (Liquid chromatography-tandem mass spectrometry). A, Protein class. Total number of proteins used was 32 with 45 classification hits. B, Biological process, based on gene ontology. Total number of proteins used was 32 with 77 process hits. All proteins with an unused score of >1.3 (95% confidence) were included. The gene name is listed next to or below each category. See Supplemental Table S4 for the full list of identified proteins.

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**TABLE 4**
**Differentially Expressed Proteins in Trenbolone-Exposed Female Fathead Minnows Relative to Unexposed Female Minnows**

| Protein | Accession Number | EF Score | *p* Value | No. of Matched Peptides | Fold Change | | | Functional Annotation[a] |
|---|---|---|---|---|---|---|---|---|
| | | | | | 5 ng/l | 50 ng/l | 500 ng/l | |
| Vitellogenin[b] | Q9W6I2_PIMPR | 1.01 | 8E−09 | 214 | 1.09 | 0.83 | 0.64 | Egg-yolk precursor |
| Fibrinogen γ[b] | D6R707_HYPMO | 1.71 | 0.002 | 8 | 0.82 | 0.54 | 0.21 | Platelet activation/clotting |
| Fibrinogen β[b] | Q6NYE1_DANRE | 1.34 | 0.027 | 5 | 0.85 | 0.63 | 0.31 | |
| U | Q4QRH6_DANRE | 1.53 | 0.073 | 4 | 0.72 | 0.98 | 0.41 | |
| α-2-Macroglobulin[b] | Q9PVU5_CYPCA | 1.33 | 0.22 | 14 | 0.95 | 0.62 | 0.44 | Endopeptidase/coagulation inhibitor |
| Transferrin[b] | Q0PWR9_PIMPR | 1.32 | 0.43 | 14 | 0.16 | 0.48 | 0.51 | Iron transport |
| U | F1SRS2_PIG | 1.29 | 0.63 | 37 | 1.29 | 1.01 | 0.53 | |
| GAPD | Q5NDA0_CYPCA | 1.36 | 0.71 | 2 | 0.43 | 0.76 | 0.69 | Glycolysis |
| Fructose-bisphosphate aldolase | Q6P043_DANRE | 1.61 | 0.32 | 5 | 0.2 | 0.94 | 0.7 | Gluconeogenesis |
| 14 kDA Apolipoprotein | Q0PWR3_PIMPR | 1.63 | 0.49 | 49 | 1.85 | 0.72 | 1.94 | Homolog of Apo-II |
| Sex hormone-binding globulin | Q2WFI3_CYPCA | 1.20 | 0.63 | 4 | 0.98 | 0.94 | 1.37 | Transport of sex steroids |
| Parvalbumin[b] | Q0PWR5_PIMPR | 2.0 | 0.58 | 4 | 0.49 | 3.53 | 4.32 | Calcium binding |
| Complement C3[b] | Q9YIA9_CYPCA | 1.14 | 0.30 | 15 | 0.48 | 0.79 | 1.5 | Immune function, opsonization, chemotaxis |
| U | E7FBD3_DANRE | 1.18 | 0.005 | 8 | 1.8 | 1.96 | 3.25 | |
| Similar ApoB | Q5TZ29_DANRE | 1.72 | 2E−06 | 14 | 3.02 | 2.7 | 6.67 | Lipid/Cholesterol transport |
| Amine oxidase | A0JMP5_DANRE | 1.63 | 0.13 | 3 | 3.44 | 2.97 | 8.8 | Wound healing |

*Note*. EF score and *p* values are associated with the 500 ng/l trenbolone: control group comparison. Supplementary Table 4 lists all detected proteins and treatment ratios, EF scores and *p* values. U = uncharacterized protein.

[a]Functional annotation is from the Uniprot database.

[b]These proteins had multiple listings under different species. Only values associated with the most highly ranked protein (greatest number of matching peptides) are listed.

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2010). Because the decrease in circulating E2 largely occurs during the initial 24 h of trenbolone exposure, it is reasonable to assume it precedes the increase in FSH levels. This could explain the previous findings on aromatase A expression in minnows because FSH is known to stimulate expression in other fishes (Young *et al.*, 2005). The specific roles of gonadotropins with respect to steroidogenesis in asynchronous spawning fishes such as fathead minnows are not as well understood compared to salmonids. However, a recent study in another asynchronous spawning fish, the chub mackerel, indicated FSH could stimulate synthesis of E2 in midvitellogenic follicles (Ohga *et al.*, 2012). Thus, we suggest some of the compensatory responses of minnows observed to occur during trenbolone exposure are the result of increased FSH secretion.

*Toxicoproteomics*

The toxicoproteomic analysis provides a broader perspective on trenbolone effects in female fish relative to the toxicodynamic studies that focus on E2 turnover. Our application of top-down proteomic approaches was limited to characterization of unidentified protein expression patterns but helpful in that it indicated interspecies differences between unexposed female trout and minnows were of greater importance than differences observed after trenbolone exposure. This would suggest trenbolone exposure causes shifts in the plasma proteome that share some commonality among fishes. The iTRAQ analysis produced a list of 148 proteins identified in fathead minnow plasma. Closer inspection of Supplementary Table 4 indicates the iTRAQ analysis using ProteinPilot and the UniProt database was not without problems as a number of identified proteins are likely the same protein selected from different species. This is not uncommon in proteomic studies of nontraditional model organisms (Dowd, 2012) and has been observed in other studies (Martyniuk *et al.*, 2009). Applying more stringent selection criteria primarily identified proteins associated with fathead minnows or related species such as zebrafish and several carp species (Table 4). Using the PANTHER classification system, a diverse group of protein classes and biological processes were identified (Fig. 9), which was expected, given the complexity of plasma and the influence of tissue secretions on its composition.

In general, the iTRAQ results shown in Table 4 do not appear to represent a concerted response to AR activation. Exceptions would be the decrease in vitellogenin and increase in parvalbumin (Table 4). The vitellogenin decrease was expected based on previous trenbolone studies and is a consequence of lowered E2 levels (Ankley *et al.*, 2003; Ekman *et al.*, 2011). Parvalbumin is a $Ca^{2+}$ binding protein found in type II muscle, which has been shown to be induced with androgen treatment in rats (Gentile *et al.*, 2010). The 4-fold increase in parvalbumin during the 500 ng/l treatment would appear to be the result of anabolic effects of trenbolone. Several of the proteins identified as differentially expressed during trenbolone exposure have also been identified in cancer biomarker studies. For example, changes in serum levels of α-2-macroglobulin, apolipoprotein, fibrinogen, glyceraldehyde-3-phosphate dehydrogenase (GAPD), and fructose-bisphosphate aldolase (ALDA) have been associated with prostate cancer (Rehman *et al.*, 2012; van den Bemd *et al.*, 2006). A key distinction is that most of these proteins were upregulated in contrast to observations made in minnows. Many of the proteins listed in Table 4 are primarily synthesized in the liver, so it is also valuable to compare these results with those obtained from hepatic protein expression studies. Using the iTRAQ method, Martyniuk *et al.* (2009) characterized changes in the female fathead minnow liver proteome after a 48-h, 5 µg/l trenbolone exposure. The results identified 166 proteins including GAPD, which was significantly increased (Martyniuk *et al.*, 2009), in contrast to the plasma results from this study. However, decreases in ALDA and transferrin were also observed (Martyniuk *et al.*, 2009). Interestingly, neither fibrinogen nor α-2-macroglobulin was detected in the liver. In a subsequent study using another androgen, 5-alpha dihydrotestosterone, most of the proteins listed in Table 4 were identified in the female minnow liver, but their expression changes were not considered significant (Martyniuk *et al.*, 2012). In some respects, changes observed in the plasma proteome reflect a more generalized response associated with inflammation, cell proliferation, and tissue remodeling (Pitteri *et al.*, 2011, Tung, 2013). The induction of amine oxidase is also interesting as it is known to be associated with wound healing, among other processes (Buffoni *et al.*, 1993; Fogelgren *et al.*, 2005). Because trenbolone exposure is well established to induce morphological changes in female minnows, it seems reasonable to conclude that plasma proteins associated with tissue remodeling and wound healing would likely be altered after exposure.

There are two concerns with the results presented in Table 4 and Supplementary Table 4 that are important to discuss. First is the aforementioned bias toward highly abundant proteins, and second is the tendency of the iTRAQ method to underrepresent the magnitude of expression changes. The bias toward abundant proteins is a well-recognized problem with either plasma or serum proteomic studies (Jacobs *et al.*, 2005) and attributable to both the dynamic range of protein concentrations in plasma (> 10 orders of magnitude) and the fact that approximately 20–25 proteins make up 99% of the total plasma protein content (Anderson and Anderson, 2002). To minimize this problem, some studies use immunodepletion strategies to selectively remove specific proteins. However, this approach is not always effective due to binding and complexing of many types of proteins with abundant proteins (Linke *et al.*, 2007). We chose not to employ a depletion strategy in part because fathead minnow–specific antibodies for abundant proteins are not readily available (other than for vitellogenin) and a decrease in vitellogenin is an established effect of trenbolone and intentional depletion of other abundant proteins may introduce other biases toward the results. However, our results would suggest more extensive fractionation methods are needed to reduce protein complexity of individual fractions and improve characterization. The underrepresentation of fold change in iTRAQ studies has been noted in other studies (Ow

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*et al.*, 2009) and best observed in this study from the vitellogenin results. Although vitellogenin was the number one ranked protein detected and was downregulated to a greater extent at higher treatment levels, it still was only 1.6-fold lower than controls (Table 4). Based on previous studies, the expected decrease in vitellogenin levels should be > 10-fold at the 0.5 µg/l treatment (Ankley *et al.*, 2003). The impact of underrepresentation on other proteins is unknown but may have influenced the number of proteins considered differentially expressed and the extent of dose-response observed at the different trenbolone treatment levels.

## CONCLUSION

The results from this study along with those of other recent studies indicate trenbolone exposure is a highly dynamic process in fish that can be separated into a series of temporal steps that contribute to the overall biological effects observed in fish. During the initial few hours of exposure, uptake and tissue equilibrium is established with the potential for increasing the unbound or free fraction of E2. This would contribute to increased clearance of E2, likely across the gills, but other elimination routes such as urinary could also be involved. Next, trenbolone decreases aromatase expression in the ovary, reducing synthesis of E2. The reduced plasma levels of E2 may have caused a compensatory increase in pituitary release of FSH, which eventually stimulates increased expression of aromatase. The toxicoproteomic analysis indicated that large-scale changes in the plasma proteome are also occurring during this time frame. The dose-response relationships for each of these effects may be different (eg, higher exposure levels are likely needed to increase the E2 free fraction). Thus, interpretation of effects data can be challenging over wide dose ranges and exposure durations. Therefore, continued development and refinement of computational models of the fish brain-pituitary gonadal axis such as the recent model by Li *et al.* (2011) for trenbolone should be encouraged as they appear well suited to incorporate effects at multiple target sites.

## SUPPLEMENTARY DATA

Supplementary data are available online at http://toxsci.oxfordjournals.org/.

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