Published in final edited form as:

Environ Toxicol Chem. 2006 November ; 25(11): 2997–3005.

DYNAMICS OF 17 α -ETHYNYLESTRADIOL EXPOSURE IN RAINBOW TROUT (*ONCORHYNCHUS MYKISS*): ABSORPTION, TISSUE DISTRIBUTION, AND HEPATIC GENE EXPRESSION PATTERN

Ann D. Skillman[†], James J. Nagler[‡], Sharon E. Hook[†], Jack A. Small[§], and Irvin R. Schultz^{†,*}

[†]*Pacific Northwest National Laboratory—Marine Sciences Laboratory, 1529 West Sequim Bay Road, Sequim, Washington 98382, USA*

[‡]*Department of Biological Sciences and Center for Reproductive Biology, University of Idaho, Moscow, Idaho 83844-3051, USA*

[§]*Pacific Northwest National Laboratory, MS-K3-61, Richland, Washington 99352, USA*

Abstract

17 α -Ethinylestradiol (EE₂) is a synthetic estrogen identified in sewage effluents. To understand better the absorption kinetics of EE₂ and the induction of vitellogenin (VTG) and estrogen receptor α (ER α) mRNA, we subjected male rainbow trout (*Onchorynchus mykiss*) to continuous water exposures of 125 ng/L of EE₂ for up to 61 d. Trout were either repetitively sampled for blood plasma or serially killed at selected time intervals. Vitellogenin, ER α mRNA, and EE₂ were measured using enzyme-linked immunosorbent assay and using quantitative polymerase chain reaction and gas chromatography–mass spectrometry, respectively. In separate experiments, trout were exposed to EE₂ for 7 d, and hepatic gene expression was assessed using a low- and high-density cDNA microarray. The EE₂ was rapidly absorbed by the trout, with an apparent equilibrium at 16 h in plasma and liver. The ER α mRNA levels also increased rapidly, reaching near-peak levels by 48 h. In contrast, plasma levels of VTG continuously increased for 19 d. After 61 d, tissues with the highest levels of VTG were the liver, kidney, and testes. Microarray-based gene expression studies provided unexpected results. In some cases, known estrogen-responsive genes (e.g., ER α) were unresponsive, whereas many of the genes that have no apparent link to estrogen function or EE₂ toxicity were significantly altered in expression. Of the two microarray approaches tested in the present study, the high-density array appeared to be superior because of the improved quality of the hybridization signal and the robustness of the response in terms of the number of genes identified as being EE₂ responsive.

Keywords

Toxicokinetics; Microarray; Xenoestrogens; Endocrine disruptor

INTRODUCTION

It is becoming increasingly apparent that endocrine disruption in fish adversely affects normal development and maturation. A comparatively large number of environmental pollutants are

* To whom correspondence may be addressed (ir_schultz@pnl.gov).

hypothesized to act as estrogen agonists (xenoestrogens) [1]. Male fish typically have very low levels of circulating estrogen [2] and are particularly sensitive to exposure to estrogenic chemicals [3–5]. Although numerous contaminants have been identified that bind to fish estrogen receptors (ERs), the affinity typically is several orders of magnitude less than that of estradiol (E_2) and synthetic steroid analogues [6]. One of the more potent synthetic estrogens is 17 α -ethynylestradiol (EE_2), a biologically persistent analogue of estrogen that is widely used in oral contraceptives and hormone replacement therapy. The presence of EE_2 in surface waters is attributed to municipal sewage discharges [7,8]. Both EE_2 and E_2 have been implicated as the primary contaminants contributing to the estrogenic activity in surface waters from both the United Kingdom and the United States [9–11]. These findings are of concern, because EE_2 is known to cause a variety of endocrine-disruptive effects in fish, including altered sex steroid levels, impaired gonadal development, and decreased reproductive performance [3–5, 11–15].

The toxicokinetics of EE_2 in fish have been studied only recently. After intravascular injection in trout, EE_2 is slowly eliminated, with biological half-lives of up to 132 h, although at lower doses (<0.1 mg/kg), the half-life is less than 21 h [16]. Elimination appears to occur by a combination of branchial excretion of the parent compound and through biotransformation and conjugation. Based on limited data in trout, EE_2 undergoes extensive conjugation to glucuronide and secretion into bile [16], which is similar to EE_2 metabolism in mammals [17]. Interestingly, glucuronidation in trout does not appear to significantly facilitate excretion of EE_2 , because of extensive enterohepatic recirculation and mass balance studies in trout suggest that most of the EE_2 ($>80\%$) is excreted unconjugated [16].

The predominant effects of estrogen are regulated by intracellular ERs. Similar to mammals, fish have two distinct ER subtypes, ER α and ER β [18,19]. In a few nonsalmonid species, some studies have examined a limited number of genes expressed in response to xenoestrogens, and many of these genes are believed to be regulated by ERs [20–24]. A similar set of genes appears to be induced in fish liver by treatment with EE_2 or E_2 [21]. Many of the estrogen-responsive genes in rainbow trout appear to be under the control of ER α [25]. Studies using other fish species also support the hypothesis that ER α is the principal ER in the liver [26]. A well-known ER-regulated gene product in fish is vitellogenin (VTG). Vitellogenin is the major egg-yolk precursor protein that is synthesized in the liver of fish (as in other egg-laying vertebrates) before its transport to the ovaries and incorporation into developing oocytes. Both male and female fish can be induced to synthesize VTG after exposure to estrogen or xenoestrogen. In male rainbow trout, EE_2 is more potent than E_2 in stimulating VTG synthesis [27].

A need exists to understand better the disposition of important xenoestrogens, such as EE_2 , and the changes in gene and protein expression that occur in fish as a result of exposure. In a previous study, we characterized the kinetics of VTG induction and elimination in trout administered graded intra-vascular injections of EE_2 over a dose range of 0.001 to 10 mg/kg [16]. These studies identified a consistent pattern of VTG induction, which was a 12-h lag time before increase from basal levels, then increasing sharply to maximum levels within 7 to 9 d. At doses of 1 mg/kg or less, VTG was eliminated with a biological half-life of 42 to 49 h [16]. In the present study, we exposed rainbow trout to EE_2 using continuous water exposures, and we repetitively sampled individual fish over time. These studies were performed to characterize the plasma kinetics of EE_2 and VTG under exposure conditions similar to environmental exposures. Separate groups of similarly exposed trout were serially killed, and the EE_2 and VTG concentration–time profiles in various tissues were characterized. We also measured the expression profile of the hepatic ER α mRNA over time. Guided by these results, we conducted separate experiments to characterize the expression of other estrogen-responsive genes in the trout liver using cDNA microarrays. These latter experiments used two different approaches for microarray-based toxicogenomic analysis: A targeted, low-density cDNA array

developed as part of the present study, and a commercially available, high-density salmonid cDNA gene array. The microarray studies characterized hepatic gene expression profiles after a single exposure time, which was chosen as when EE₂ tissue levels had reached an apparent equilibrium with the exposure solution and, thus, better reflected changes in gene expression occurring during steady-state conditions.

MATERIALS AND METHODS

Fish

All experiments used male rainbow trout (*Onchorynchus mykiss*). Kinetic studies and initial cDNA microarray experiments used sexually mature fish (1+ age) averaging 0.71 ± 0.16 kg (mean $\pm$ standard deviation, $n = 27$) in weight. These fish were obtained from the Nisqually Trout Farm (Lacey, WA, USA) and were transported to the Marine Research Laboratory (MRL) in Sequim (WA, USA) for a minimum acclimation period of two weeks before exposure. The fish were maintained in 1,400-L, circular tanks before individual exposure in 370-L, circular tanks. The MRL uses artesian well water (depth, 150 m), which was pre-aerated and maintained at a minimum flow rate of 1.0 L/min/kg. Water-quality parameters were routinely measured in holding and treatment tanks and averaged as follows: temperature, 12°C; dissolved oxygen, >9 mg/L; pH 7.9; total alkalinity, 200 mg/L as CaCO₃; ammonia, <0.05 mg/L; and nitrate–nitrite, <0.01 mg/L. Fish were fed a commercial trout soft, moist, pelleted feed (Bio-Oregon, Warrenton, OR, USA) ad libitum daily. The stocking density was maintained at less than 15 kg/m³. All holding and exposure tanks were maintained under a simulated natural photoperiod regime with graded off- and on-controls.

A second microarray experiment used five juvenile, male, isogenic rainbow trout of the Oregon State University $\times$ Swanson cross [28]. These fish were produced by the Aquaculture Core Facility, Center for Reproductive Biology, Washington State University, and were shipped to MRL at 530 degree-days in age (postfertilization). These fish were held under water-quality conditions, feeding protocol, and photoperiod regime identical to those of the adult fish. At the time of exposure, the isogenic trout were 1,800 degree-days in age (approximately five months post-hatch) and averaged 0.01 kg in weight.

Water exposures

All trout were continuously exposed to EE₂ for up to 1,456 h (61 d) using a flow-through exposure system. All kinetic experiments and initial gene-array experiments used a nominal EE₂ exposure level of 125 ng/L. The second gene-array experiment used a nominal EE₂ exposure level of 50 ng/L. The EE₂ concentrations were measured every 7 to 10 d during the exposures to quantify actual levels. A concentrated stock solution of EE₂ was prepared in methanol and slowly added to the exposure tanks using a peristaltic pump at a flow rate of 0.10 ml/min (0.0005% methanol in the tank water). Control tanks received only methanol as a solvent control. The exposure tanks were allowed to equilibrate with the EE₂ dosing system for 7 d before the addition of trout.

Three separate experiments were performed. Initial experiments used trout fitted with a dorsal aortic cannula [16]. Plasma was repetitively sampled via the cannula for analysis of EE₂ and VTG. Between 0.1 and 0.2 ml of blood was withdrawn and replaced with a similar volume of saline. Other aspects of blood sampling and plasma storage have been well described in previous publications [16,29]. Similarly sized trout in a separate group were killed at various sampling times, and the liver, testes, and kidney were removed and stored at –80°C. A third exposure lasting 7 d used the Nisqually and isogenic trout. At the time of termination, the livers were removed and placed in RNeasy Lysis Buffer (Qiagen, Hilden, Germany) solution and then stored according to the manufacturer protocols for later extraction of RNA and gene-array analysis.

VTG and EE₂ analysis

Plasma, liver, testes, and trunk kidney were analyzed for VTG using a competitive enzyme-linked immunosorbent assay. Details regarding the development and validation of this assay have been provided previously by Schultz et al. [16]. Before the enzyme-linked immunosorbent assay, solid tissue samples were processed using the methods described by Nagler et al. [30]. Free and total EE₂ were calculated based on the difference between measured EE₂ levels before and after treatment with β -glucuronidase [16]. Plasma and tissue analysis of EE₂ was by gas chromatography–mass spectrometry (GC-MS) after extraction using methyl-*tert*-butyl-ether (MTBE) and subsequent derivitization with *N*-methyl-*N*-trimethylsilyl-trifluoroacetamide, as described previously [16]. Before MTBE extraction, tissue samples were homogenized in four volumes of a saturated aqueous NaCl solution.

Hepatosomatic index

Livers were harvested from individual fish, and the hepatosomatic index (HSI) was calculated as follows: (liver weight/total body weight)·100.

Hepatic ER α mRNA

All liver samples for ER α mRNA analysis were flash-frozen in liquid nitrogen and stored at -80°C . Later, the samples were thawed on ice, and the total RNA was extracted in triplicate using a commercially available kit (Qiagen). Total RNA was used in a quantitative reverse transcription–polymerase chain reaction (qRT-PCR) specific for ER α mRNA. This procedure has been described in detail previously by Nagler et al. [31] and was performed without modification.

cDNA microarrays

Two different cDNA microarrays were used in the present study. Initially, we used a targeted, low-density microarray consisting of 32 different fish (mostly rainbow trout) DNA sequences either known to be or suspected of being estrogen-responsive genes. Subsequent to these preliminary gene-array studies, we used a recently developed, high-density salmonid cDNA microarray [32], which was purchased from the Genomic Research on Atlantic Salmon Project (GRASP), University of Victoria (BC, Canada).

Low-density microarray—Selected gene sequences were used to construct primer pairs for RT-PCR reactions. The products of the RT-PCR reactions served as mRNA targets on the planar microarray. Total RNA from liver, testes, and brain of a single EE₂-exposed, male rainbow trout served as template for the RT-PCR reactions. Primers were designed from gene sequences available in the National Center for Biotechnology Information (NCBI; National Institutes of Health) Entrez database using Xpression Primer 3 software (Premier Biosoft International, Palo Alto, CA, USA). The PCR products were designed to have a melting temperature of 60°C and to be 500 to 600 bp in length. Several rounds of RT-PCR were used to generate amplicon concentrations of 400 to 600 ng/ μl . The amplicon was checked for size (as a proxy for identity) by PCR gel electrophoresis. Targets were concentrated using a heated vacuum centrifuge to a concentration of at least 400 ng/ μl (range, 398–600 ng/ μl) and then spotted onto aminosilane-coated microarray slides using a Microgrid robotic microarray printer (Apogent Discoveries, Hudson, NH, USA). Each target was printed five times in the same row. The RNA used in the original RT-PCR reactions was used as the target solution.

High-density array—A high-density salmonid microarray consisting of 16,000 cDNA and expressed sequence tags from Atlantic salmon (*Salmo salar*) and rainbow trout was designed, developed, validated, and custom printed as described previously by Rise et al. [32]. Microarrays were purchased from the GRASP consortium (Ben Koop, University of Victoria,

BC, Canada; <http://web.uvic.ca/cbr/grasp/>) and used within three months of purchase. Cross-species hybridization comparisons and the results of the present study indicate the GRASP arrays can be used successfully with rainbow trout and other salmonid species in addition to Atlantic salmon [32].

RNA extractions

The RNA was harvested from the livers of exposed and control trout using TRIzol reagent (Invitrogen Corporation, Carlsbad, CA, USA) according to the manufacturer's protocol. For work with the high-density microarray, RNA was DNase-treated using TURBO-DNAfree cleanup procedure (Ambion, Austin, TX, USA). SUPRANasin (RNase inhibitor; Ambion) was added, and samples were stored at -80°C to preserve sample integrity. The RNA quality was verified with gel electrophoresis and quantified with Ribogreen reagent (Invitrogen). Total RNA was used for the labeling procedure.

Labeling and hybridization procedures

Low-density microarray—For the low-density microarray, 2 $\mu\text{g}/\mu\text{l}$ of transfer RNA were directly labeled with Alexa fluor dyes (Molecular Probes) using the Ulysis® nucleic acid labeling kit (Invitrogen). Labeled RNA was incubated at 95°C to linearize transcripts, then hybridized to microarrays overnight at 37 to 42°C . Slides were rinsed with decreasing concentrations of sodium chloride–sodium citrate buffer (SSC) and sodium dodecyl sulfate (SDS) to remove unbound material.

High-density microarray—For the high-density microarray, cDNA was indirectly labeled with Cy3 (control) and Cy5 (exposed) fluors (Amersham, Buckinghamshire, UK) using an amino allyl technique (Superscript cDNA Indirect Labeling kit; Invitrogen). Two micrograms of RNA were used for each technical replicate; 6 μg were used in total for each individual fish. The control samples were labeled with Cy3, and the exposed samples were labeled with Cy5. A split-control experiment (which divides the control RNA into two aliquots, one labeled with Cy3 and the other with Cy5 before recombining them) was used to determine if results were influenced by differences in the fluors [33]. Exposed and control samples were normalized to cDNA yield and dye incorporation before combining and reducing the volume to 32 μl in a speed vacuum. The combined labeled cDNA was incubated with 20 μl of transfer RNA, 20 μg of herring sperm DNA (to prevent nonspecific hybridization), and 35 μl of hybridization buffer (Genisphere, Hatfield, PA, USA; 50% formamide, 40% 20 $\times$ SSC, 9% Denhardt's solution, and 1% SDS) at 80°C for 10 min to linearize the template. This material was hybridized to the microarrays overnight at 45°C in a humidified chamber, then washed following Genisphere's protocols. Slides were scanned at optimal laser power and photomultiplier tube gain using a ScanArray Express (PerkinElmer, Wellesley, MA, USA).

Microarray data analysis

Data were extracted using ScanArray Express software (PerkinElmer) and normalized to β -actin expression. Relative expression levels of mRNAs in control versus exposed treatments were determined for each gene, and significance was determined using a *t* test. For the high-density microarray, the mean fluorescence intensity with background subtracted was imported into a Minimum Information About Microarray Experiments (MIAME) compliant database [34], imported into Genespring (Agilent Technologies, Palo Alto, CA, USA), locally weighted least squares (LOWESS) normalized [33], and screened for fold-change. The significance of these screens was determined using a one-way analysis of variance ($p = 0.05$). Genes that were significantly up- or down-regulated were verified by submitting the sequence to BLAST (NCBI, National Institutes of Health, Bethesda, MD, USA) [35].

qRT-PCR validation of microarray results

The RNA was quantified using an ABI 7300 Real-Time System and TaqMan® One-Step RT-PCR kit. Standards were made either from the isolated rainbow trout genes for the low-density microarray (J.A. Small, unpublished data) or from cDNA clones as printed on the high-density GRASP array (kindly provided by Ben Koop, University of Victoria, BC, Canada). Plasmids of genes to be used as standards were in vitro transcribed (Riboprobe system; Promega, Madison, WI, USA) and quantified with the Ribogreen kit (Molecular Probes). The qRT-PCR primers are listed in Table 1. Transcription levels of three replicate control and exposed fish were derived from a dilution series of the standards, and all measurements were made in triplicate. Each qRT-PCR plate contained a standard curve, no reverse transcriptase (identify DNA contamination), and no template (blank) controls.

RESULTS

Uptake and disposition of EE₂

The measured water levels of EE₂ during kinetic experiments were close to the nominal level of 125 ng/L, varying between 117 to 150 ng/L during the present study (Fig. 1A). Before exposure, no EE₂ was detected in plasma. After the trout were transferred to the exposure tank, EE₂ was rapidly absorbed and was detected at the first sampling time of 0.25 h (Fig. 1B). Plasma levels then rose sharply, and by 16 h, plasma levels appeared to reach an apparent equilibrium with the exposure water, which was maintained throughout the remainder of the 1,464-h (61-d) exposure (Fig. 1B). Once the apparent equilibrium was attained, plasma levels varied between 60 to 90 ng/ml, which corresponds to a plasma concentration to water concentration ratio of from 480 to 720.

The concentration of EE₂ in the liver closely paralleled plasma levels (Fig. 1B and C), varying little between 48 and 456 h. The peak levels of EE₂ in the liver were similar to that measured in plasma, indicating the plasma to liver partition ratio is approximately one (Fig. 1C). Analysis of bile for free and conjugated EE₂ also is shown on Figure 1C. The concentration of free EE₂ in bile was comparable to liver and plasma levels (Fig. 1C). However, the concentration of conjugated EE₂ tended to increase until 240 h, at which point 99% of the bile EE₂ was conjugated (Fig. 1C).

HSI, VTG, and ER α mRNA expression

As expected, the HSI increased throughout the exposure, in contrast to the HSI of unexposed fish, which remained unchanged (Fig. 2A). Plasma VTG was measured in aliquots of the same samples used for EE₂ analysis, allowing comparison of the time delay between absorption of EE₂ and appearance of VTG. During the initial 36 h of the exposure, little change occurred in the plasma VTG concentration. Beginning at the 48-h sampling time, plasma VTG levels rapidly increased, before tapering off at 384 h (Fig. 2B). Plasma VTG levels were similar between the 576 and 1,464-h sampling times, indicating that an apparent equilibrium between synthesis, secretion, and elimination of VTG is reached by 576 h. A similar concentration–time profile for VTG was observed in the liver, although the VTG levels were from 30- to 70-fold higher compared to those in plasma (Fig. 2B). It also is interesting to note that the HSI continued to increase throughout the 1,464-h exposure, despite peak levels of VTG occurring by 456 h (Fig. 2A and B). The VTG concentrations also were measured in the trunk kidney (456 and 1,464 h) and testes (1,464-h sample). Interestingly, VTG levels in these tissues were similar to levels measured in the liver (Fig. 2B).

The expression of hepatic ER α mRNA (femtograms; ER α mRNA/ μ g total RNA) after 4, 16, 48, 240, and 456 h of treatment is shown in Figure 2C. Initially, ER α mRNA levels were relatively unchanged compared to control trout. After 48 h of treatment, ER α mRNA was

elevated 10-fold compared to the level in unexposed trout. This overall expression level remained consistently elevated throughout the remainder of the exposure, although a transient decrease in expression was observed at 240 h (Fig. 2C). Variation in the expression of ER α mRNA in EE₂-treated trout also was greater at the later sampling times, with the coefficient of variation as high as 57%.

Hepatic gene expression using low- and high-density microarrays

Low-density microarray—Based on the results from EE₂ disposition studies, a 7-d (168-h) EE₂ exposure period was used for gene expression studies. This exposure duration was chosen to allow equilibrium between the exposure water and liver EE₂ levels to be reached (Fig. 1B and C) but still occur during the time period of active changes in estrogen-regulated gene and protein expression (e.g., Fig. 2B and C). The measured EE₂ water level at termination was 75 ng/L (data not shown). Initial cDNA microarray studies measured hepatic gene expression using the targeted, low-density microarray and fish of similar age and strain as used in kinetic studies. The results from this experiment are shown in Figure 3. Of the 32 genes on the microarray, only four were observed to be altered by more than twofold relative to control fish (Fig. 3). These genes were the thyroid hormone receptor α , apoptosis-related cysteine protease gene caspase 6, VTG precursor-1, and VHSV (retinoic acid-responsive gene). Other genes that were marginally up-regulated included cytochrome P450s (CYP2M1 and CYP1A3), the eggshell zona radiata protein, the transporter/efflux pump MDR4, and heat shock protein 70. In contrast to the PCR-based measurements of ER α mRNA, relatively little change in expression was observed in ER α on this microarray.

High-density microarray—In this experiment, isogenic rainbow trout were used to help minimize interindividual variation in response. The split control experiment did not indicate significant transcript-specific, differential labeling of the Cy3 and Cy5 fluors, and no correction factors were used to account for dye bias (data not shown) [36]. The measured EE₂ water level at the time of fish euthanasia was 37 ng/L (data not shown). The results from these experiments are presented in Table 2. Forty-nine genes were significantly up-regulated, including VTG (gene 1, up-regulated 10.42-fold over control, $p < 0.001$) and vitelline envelope proteins (gene 3, up-regulated 7.4-fold, $p < 0.001$), and an apolipoprotein (gene 4, up-regulated threefold, $p < 0.001$). In all, 140 genes were down-regulated, including many isoforms of cytochrome P450 (down-regulated between 1.5- and 3-fold depending on the form, $p < 0.001$), spermidine/spermine *N*(1)-acetyltransferase (down-regulated approximately threefold, $p < 0.001$), and a fatty acid-binding protein (gene 189, down-regulated 6.9-fold, $p < 0.001$). Many of the genes with altered expression have unknown function or a function that is not clearly related to estrogen exposure (Table 2).

PCR validation of microarray results

Comparison of changes in mRNA expression for nine genes measured by the high-density microarray and separate analysis by qRT-PCR indicates the microarray data were similar to the qRT-PCR data, but with notable exceptions (Table 3). One gene sequence (toxin-1) was determined to be down-regulated by the array (−2.05-fold change), although qRT-PCR analysis indicated that it remained largely unchanged (1.05-fold change) (Table 3). The ER α gene was not found to be altered in expression by the array, although qRT-PCR analysis indicated it was up-regulated by 33-fold. Both the array and qRT-PCR analyses identified significant up-regulation of the two VTG genes, although considerable difference was observed in the measured level of induction between the two techniques (Table 3).

DISCUSSION

In the present study, we simultaneously measured the concentration–time profiles of EE₂ and VTG in various tissues to understand better the dynamics between EE₂ absorption and VTG induction in fish. This experimental approach also allowed characterization of the time delay between EE₂ tissue distribution and VTG synthesis and secretion into plasma. Our results clearly show that within 16 h, an apparent equilibrium exists for EE₂ between the exposure water, blood plasma, and liver. The high EE₂ plasma to water concentration ratio also indicates that EE₂ is more than 99% bound to plasma macromolecules (proteins). This observation, combined with the rapid uptake of EE₂, is consistent with the gills being the primary site for absorption and with gill ventilation volume being the physiological variable controlling the absorption rate of EE₂ in fish [37]. Although plasma and liver levels of EE₂ are at or near their peak values by 16 h, VTG is still near basal levels in plasma and liver and does not begin to increase until 36 to 48 h. This time delay indicates that approximately 24 h are needed for EE₂ to stimulate VTG synthesis in the liver. This result is consistent with those of studies using rainbow trout liver sections, in which significant VTG induction occurs between 24 to 48 h after incubation with 17 β -estradiol [38].

The rapid exchange of EE₂ between blood plasma and exposure water suggests measurement of EE₂ (and, perhaps, of other synthetic steroids) in fish plasma may be useful in environmental monitoring studies. Because EE₂ is concentrated (480- to 720-fold) in plasma relative to water, increased sensitivity in detection may be afforded by monitoring plasma levels in feral fish. During prolonged exposures, the highest concentrations of EE₂ are found in bile in the form of glucuronide conjugates (Fig. 1C) [16]. Thus, plasma levels reflect current or recent environmental exposures, whereas total EE₂ levels in bile are more indicative of cumulative exposures. Combined plasma and bile measurements in environmental monitoring studies would provide important information regarding environmental levels of EE₂, which can be difficult to characterize based solely on water and sediment samples (for review, see Langston et al. [1]).

With regard to VTG levels measured in nonhepatic tissues, it is interesting that both the kidney and testes had levels similar to that of the liver (Fig. 3B). In our previous study of VTG kinetics in rainbow trout, we observed that VTG has a large steady-state volume of distribution (837 ml/kg), which is comparable to the total body water volume of rainbow trout (71–81% of body wt). This is much larger than that of plasma proteins, such as albumin, which have a distributive volume approximately 12% of body weight in rainbow trout [39]. The results in the present study are consistent with this previous kinetic analysis and suggest that besides the liver, other tissues, including the testes and kidney, can sequester VTG, because these tissues had high tissue to plasma VTG concentration ratios during prolonged water exposure to EE₂. These findings are important, because both nephrotoxicity and testicular toxicity in male fish exposed to high doses of E₂ and EE₂ is well established [3,40,41]. Estrogen-induced nephrotoxicity in male fish is thought to be associated with the elevated levels of circulating VTG [40], whereas the effect on the testes generally is assumed to be a direct estrogenic effect. The present results suggest the testes also may sequester VTG from plasma, which may be a contributing factor to testicular toxicity, particularly at exposure levels causing high induction of VTG.

We measured EE₂-induced changes in hepatic gene expression using two different microarrays. Because of differences in the number of cDNA sequences on the microarrays and differences in labeling efficiency between the direct RNA labeling (used for the low-density microarray studies) and the indirect amino allyl-labeling protocols, direct comparisons between the two microarrays are difficult. The amino allyl labeling provides greater fluorescent signal than the direct RNA labeling, making the dynamic range of gene expression as measured on the high-density microarray greater. The greater signal allowed for the measurement of greater changes

in gene expression and for sharper discrimination between unchanged and altered genes. The microarray results also likely differ because of the use of sexually mature and juvenile rainbow trout, the latter of which were used because of the perceived advantages of isogenic animals in microarray studies. It also is worth noting that the low-density microarray was intentionally biased toward genes thought to be estrogen responsive, in contrast to the high-density microarray. Thus, many of the genes that were deemed to have EE₂-altered expression using the high-density microarray do not have an obvious role in gametogenesis or steroid metabolism. Further analysis of these genes may reveal new insights regarding the toxicity pathway of EE₂ in salmonid fish. Finally, different fragments of the same gene often were found to be altered to a different extent. We would speculate that this difference arises because of the semiquantitative nature of cDNA microarrays. Each cDNA printed on the slide will have different optimal hybridization conditions, such that a given hybridization procedure will always measure some genes with more accuracy than others. Consequently, cDNA array results typically are presented as the fold-change from control as opposed to an absolute measure of expression [33]. Because different cDNA fragments do not have the same hybridization efficiencies, some fragments may be measured with cDNA arrays more efficiently than others, resulting in slight differences in the fold-change between different fragments of the same gene. Neither array was able to detect significant changes in ER α . We suggest that this results from the low discriminatory power of the low-density array. Also, the ER printed on the microarray may be the basally transcribed isoform as opposed to the estrogen-induced ER α .

In conclusion, EE₂ is both rapidly absorbed and equilibrated with peripheral tissues in rainbow trout during water exposures. Changes in hepatic gene and protein expression become apparent after 24 h. For some inducible genes, such as ER α , peak levels were observed within 48 h, whereas the VTG protein levels continued to increase in liver and plasma for more than 21 d. Microarray-based gene expression studies provided unexpected results in some cases because of a lack of response from known estrogen-responsive genes (e.g., ER α), whereas many of the genes identified as being significantly altered in expression (Table 3) have no apparent link to estrogen function or EE₂ toxicity. The latter finding is currently being pursued in studies seeking to develop better bioinformatic approaches to assist with interpreting altered patterns of gene expression in fish. Although the low-density array was designed to offer the advantage of genes targeted to endocrine function, the broad-spectrum, high-density GRASP array, used in conjunction with the Cy3 and Cy5 fluors, appears to be superior as a tool for characterizing changes of gene expression in rainbow trout.

Acknowledgements

This research was supported by the U.S. Department of Energy under contract DE-AC06-76RLO 1830 and the U.S. National Institute of Environmental Health Sciences grant 5R01ES012446-03.

References

1. Langston WJ, Burt GR, Chesman BS, Vane CH. Partitioning, bioavailability, and effects of estrogens and xenoestrogens in the aquatic environment. *J Mar Biol Assoc UK* 2005;85:1–31.
2. Jensen KM, Korte JJ, Kahl MD, Pasha MS, Ankley GT. Aspects of basic reproductive biology and endocrinology in the fathead minnow (*Pimephales promelas*). *Comp Biochem Physiol Part C Toxicol Pharmacol* 2001;128:127–141.
3. Lange R, Hutchinson TH, Croudace CP, Siegmund F. Effects of the synthetic estrogen 17 α -ethinylestradiol on the life cycle of the fathead minnow (*Pimephales promelas*). *Environ Toxicol Chem* 2001;20:1216–1227. [PubMed: 11392131]
4. Schultz IR, Skillman A, Nicolas JM, Cyr DG, Nagler JJ. Short-term exposure to 17 α -ethinylestradiol decreases the fertility of sexually maturing male rainbow trout (*Oncorhynchus mykiss*). *Environ Toxicol Chem* 2003;22:1272–1280. [PubMed: 12785584]

5. Segner H, Navas JM, Schafers C, Wenzel A. Potencies of estrogenic compounds in in vitro screening assays and in life-cycle tests with zebrafish in vivo. *Ecotoxicol Environ Saf* 2003;54:315–322. [PubMed: 12651187]
6. Kloas W, Schrag B, Ehnes C, Segner H. Binding of xenobiotics to hepatic estrogen receptor and plasma sex steroid-binding protein in the teleost fish, the common carp (*Cyprinus carpio*). *Gen Comp Endocrinol* 2000;119:287–299. [PubMed: 11017776]
7. Arcand-Hoy LD, Nimrod AC, Benson WH. Endocrine-modulating substances in the environment: Estrogenic effects of pharmaceutical products. *Int J Toxicol* 1998;17:139–158.
8. Larsson DGJ, Adolfsson-Erici MB, Parkkonen JA, Pettersson MB, Berg AH, Olsson PE, Förlin LA. Ethinylestradiol—An undesired fish contraceptive? *Aquat Toxicol* 1999;45:91–97.
9. Desbrow C, Routledge EJ, Brighty GC, Sumpter JP, Waldock M. Identification of estrogenic chemicals in STW effluent. 1. Chemical fractionation and in vitro biological screening. *Environ Sci Technol* 1998;32:1549–1558.
10. Snyder SA, Keith TL, Verbrugge DA, Snyder EM, Gross TS, Kannan K, Giesy JP. Analytical methods for detection of selected estrogenic compounds in aqueous mixtures. *Environ Sci Technol* 1999;33:2814–2820.
11. Metcalfe CD, Metcalfe TL, Kiparissis Y, Koenig BG, Khan C, Hughes RJ, Croley TR, March RE, Potter T. Estrogenic potency of chemicals detected in sewage treatment plant effluents as determined by in vivo assays with Japanese medaka (*Oryzias latipes*). *Environ Toxicol Chem* 2001;20:297–308. [PubMed: 11351429]
12. Folmar LC, Denslow ND, Rao V, Chow M, Crain DA, Enblom J, Marcino J, Guillette LJ. Vitellogenin induction and reduced serum testosterone concentrations in feral male carp (*Cyprinus carpio*) captured near a major metropolitan sewage treatment plant. *Environ Health Perspect* 1996;104:1096–1101. [PubMed: 8930552]
13. Zillioux EJ, Johnson IC, Kiparissis Y, Metcalfe CD, Wheat JV, Ward SG, Liu H. The sheepshead minnow as an in vivo model for endocrine disruption in marine teleosts: A partial life-cycle test with 17 α -ethinylestradiol. *Environ Toxicol Chem* 2001;20:1968–1978. [PubMed: 11521823]
14. Weber LP, Balch GC, Metcalfe CD, Janz DM. Increased kidney, liver, and testicular cell death after chronic exposure to 17 α -ethinylestradiol in medaka (*Oryzias latipes*). *Environ Toxicol Chem* 2004;23:792–797. [PubMed: 15285374]
15. Balch GC, Mackenzie CA, Metcalfe CD. Alterations to gonadal development and reproductive success in Japanese medaka (*Oryzias latipes*) exposed to 17 α -ethinylestradiol. *Environ Toxicol Chem* 2004;23:782–791. [PubMed: 15285373]
16. Schultz IR, Orner G, Merdink JL, Skillman A. Dose–response relationships and pharmacokinetics of vitellogenin in rainbow trout after intravascular administration of 17 α -ethinylestradiol. *Aquat Toxicol* 2001;51:305–318. [PubMed: 11090892]
17. Guengerich FP. Metabolism of 17 α -ethinylestradiol in humans. *Life Sci* 1990;47:1981–1988. [PubMed: 2273938]
18. Nilsson S, Makela S, Treuter E, Tujague M, Thomsen J, Andersson G, Enmark E, Pettersson K, Warner M, Gustafsson JA. Mechanisms of estrogen action. *Physiol Rev* 2001;81:1535–1565. [PubMed: 11581496]
19. Tchoudakova A, Pathak S, Callard GV. Molecular cloning of an estrogen receptor β subtype from the goldfish, *Carassius auratus*. *Gen Comp Endocrinol* 1999;113:388–400. [PubMed: 10068500]
20. Denslow ND, Lee HS, Bowman CJ, Hemmer MJ, Folmar LC. Multiple responses in gene expression in fish treated with estrogen. *Comp Biochem Physiol Part B Biochem Mol Biol* 2001;129:277–282.
21. Denslow ND, Bowman CJ, Ferguson RJ, Lee HS, Hemmer MJ, Folmar LC. Induction of gene expression in sheepshead minnows (*Cyprinodon variegatus*) treated with 17 β -estradiol, diethylstilbestrol, or ethinylestradiol: The use of mRNA fingerprints as an indicator of gene regulation. *Gen Comp Endocrinol* 2001;121:250–260. [PubMed: 11254367]
22. Larkin P, Sabo-Attwood T, Kelso J, Denslow ND. Gene expression analysis of largemouth bass exposed to estradiol, nonylphenol, and *p,p'*-DDE. *Comp Biochem Physiol Part B Biochem Mol Biol* 2002;133:543–557.

23. Larkin P, Folmar LC, Hemmer MJ, Poston AJ, Denslow ND. Expression profiling of estrogenic compounds using a sheepshead minnow cDNA macroarray. *Environ Health Perspect* 2003;111:839–846.
24. Brown M, Davies IM, Moffat CF, Robinson C, Redshaw J, Craft JA. Identification of transcriptional effects of ethynyl estradiol in male plaice (*Pleuronectes platessa*) by suppression subtractive hybridization and a nylon macroarray. *Mar Environ Res* 2004;58:559–563. [PubMed: 15178082]
25. Pakdel F, Delaunay F, Ducouret B, Flouriot G, Kern L, Lazennec G, Le Drean Y, Petit F, Salbert G, Saligaut D, Tujague M, Valotaire Y. Regulation of gene expression and biological activity of rainbow trout estrogen receptor. *Fish Physiol Biochem* 1997;17:123–133.
26. Socorro S, Power DM, Olsson PE, Canario AVM. Two estrogen receptors expressed in the teleost fish, *Sparus aurata*: cDNA cloning, characterization, and tissue distribution. *J Endocrinol* 2000;166:293–306. [PubMed: 10927619]
27. Sheahan, DA.; Bucke, D.; Matthiessen, P.; Sumpter, JP.; Kirby, MF.; Neall, P.; Waldock, M. The effects of low levels of 17 α -ethynylestradiol upon plasma vitellogenin levels in male and female rainbow trout, *Oncorhynchus mykiss*. In: Muller, R.; Lloyd, R., editors. *Sublethal and Chronic Effects of Pollutants on Freshwater Fish*. Fishing News Books; London, UK: 1994. p. 99–112.
28. Young WP, Wheeler PA, Fields RD, Thorgaard GH. DNA fingerprinting confirms isogenicity of androgenetically derived rainbow trout lines. *J Hered* 1996;87:77–81. [PubMed: 8742822]
29. Schultz IR, Hayton WL. Influence of body fat on trifluralin toxicokinetics in rainbow trout (*Oncorhynchus mykiss*). *Environ Toxicol Chem* 1997;16:997–1001.
30. Nagler JJ, Ruby SM, Idler DR, So YP. Serum phospho-protein phosphorus and calcium levels as reproductive indicators of vitellogenin in highly vitellogenic mature female and estradiol-injected immature rainbow trout (*Salmo gairdneri*). *Can J Zool* 1987;65:2421–2425.
31. Nagler JJ, Krisfalusi M, Cyr DG. Quantification of rainbow trout (*Oncorhynchus mykiss*) estrogen receptor- α messenger RNA and its expression in the ovary during the reproductive cycle. *J Mol Endocrinol* 2000;25:243–251. [PubMed: 11013350]
32. Rise ML, von Schalburg K, Brown GD, Devlin RH, Mawer MA, Kuipers N, Busby M, Beetz-Sargent M, Alberto R, Gibbs AR, Hunt P, Shukin P, Zeznik JA, Nelson C, Jones SRM, Smailus D, Jones S, Schein J, Marra M, Davidson WS, Koop BF. Development and application of a salmonid EST database and cDNA microarray: Data mining and interspecific hybridization characteristics. *Genome Res* 2004;14:478–490. [PubMed: 14962987]
33. Draghici, S. *Data Analysis Tools for DNA Microarrays*. Chapman Hall/CRC; Boca Raton, FL, USA: 2003.
34. Brazma A, Hingamp P, Quackenbush J, Sherlock G, Spellman P, Stoeckert C, Aach J, Ansorge W, Ball CA, Causton HC, Gaasterland T, Glenisson P, Holstege FCP, Kim IF, Markowitz V, Matese JC, Parkinson H, Robinson A, Sarkans U, Schulze-Kremer S, Stewart J, Taylor R, Vilo J, Vingron M. Minimum information about a microarray experiment (MIAME)—Toward standards for microarray data. *Nature Rev Gen* 2001;29:365–371.
35. Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ. Basic local alignment search tool. *J Mol Biol* 1990;215:403–410. [PubMed: 2231712]
36. Rosenzweig BA, Pine PS, Domon OE, Morris SM, Chen JJ, Sistare FD. Dye-bias correction in dual-labeled cDNA microarray gene expression measurements. *Environ Health Perspect* 2004;112:480–487. [PubMed: 15033598]
37. Hayton WL, Barron MG. Rate-limiting barriers to xenobiotic uptake by the gill. *Environ Toxicol Chem* 1990;9:151–157.
38. Shilling AD, Williams DE. Determining relative estrogenicity by quantifying vitellogenin induction in rainbow trout liver slices. *Toxicol Appl Pharmacol* 2000;164:330–335. [PubMed: 10799344]
39. Bushnell PG, Conklin DJ, Duff DW, Olson KR. Tissue and whole-body extracellular, red blood cell, and albumin spaces in the rainbow trout as a function of time: A reappraisal of the volume of the secondary circulation. *J Exp Biol* 1998;201:1381–1391. [PubMed: 9547319]
40. Herman RL, Kincaid HL. Pathological effects of orally administered estradiol to rainbow trout. *Aquaculture* 1988;72:165–172.

41. Weber LP, Hill RL, Janz DM. Developmental estrogenic exposure in zebrafish (*Danio rerio*): II. Histological evaluation of gametogenesis and organ toxicity. *Aquat Toxicol* 2003;63:431–446. [PubMed: 12758007]

Fig. 1. 17α-Ethinylestradiol (EE₂) concentration–time profiles in water and rainbow trout (*Onchorynchus mykiss*) that were continuously exposed to 125 ng/L of EE₂ for up to 1,456 h (61 d) in a flow-through exposure system. **(A)** Measured EE₂ levels in exposure water. **(B)** EE₂ plasma profile in fish fitted with a dorsal aortic cannula and repetitively sampled (mean ± standard deviation [SD], $n = 3–5$, error bars not shown fit within the data point). **(C)** EE₂ in liver (open squares, solid line) and bile (circles, dashed line). Conjugated EE₂ in bile also is shown (triangles, dotted line). Liver and bile measurements were made in fish serially killed at 0, 4, 16, 48, 240, and 456 h of exposure (mean ± SD, $n = 3$, error bars not shown fit within the data point).

Fig. 2. Hepatosomatic index (HSI), vitellogenin (VTG) plasma and tissue concentration–time profiles, and hepatic estrogen receptor α (ER α) mRNA expression in rainbow trout (*Onchorynchus mykiss*) continuously exposed to 125 ng/L of 17 α -ethynylestradiol (EE₂) for up to 1,456 h. (A) HSI (mean $\pm$ standard deviation [SD], $n = 3-5$). (B) VTG plasma and tissue profiles. Plasma VTG (squares, solid line) was measured in aliquots of the same plasma samples used for EE₂ analysis in Figure 1B. Liver VTG (triangles, dashed lines), trunk kidney (circles, dotted line), and testes (filled diamond, single measurement at 1,456 h) were measured in trout serially killed at times indicated on the graph (mean $\pm$ SD, $n = 3-5$, error bars not shown fit within the data point). (C) ER α mRNA expression (squares) measured by quantitative reverse

transcriptase–polymerase chain reaction (mean $\pm$ SD, $n = 3$). The ER α mRNA values for control trout were measured at 0 and 48 h and are shown on the graph as open circles. (Note that tissue–time data presented in Figures 1C and 2 were determined from tissue samples collected from the same fish.) fg = femtogram.

Fig. 3.

Hepatic gene expression in sexually mature male rainbow trout (*Onchorynchus mykiss*) measured using a low-density cDNA microarray. Three trout were individually exposed for 7 d to a measured 17 α -ethynylestradiol (EE₂) concentration of 75 ng/L. A total of 32 cDNA sequences were on the microarray and are listed below the figure. Results are expressed as the mean fold-change relative to control trout that received only the methanol carrier.

Table 1Primers used for quantitative reverse transcriptase polymerase chain reaction^a

Gene	Primer		
	Forward	Reverse	Fluorescently labeled
Androgen receptor	AGCGCCAACCTGGTCGAA	CACATGCAGATTCCGAAAAC	TGGTCAAGTGGGCAAAGGCATG
Apolipoprotein CII	GCAGGATGCCAGCGTAAGTC	AGGCCACGAATGTCCTCAGT	TCGCTGCCACGGTGGTGT
β-Actin	ACGGCCAGAGGCGTACAG	TTC AACCTGCCATGT	ACAACACGGCCTGGATGGCCA
Cytochrome P4501A3	CCCCTTCCGCCATATTGTC	CGGCCGAAGCACATTCC	TATCGGTGGCCAAGTCATCTG
δ-6-Dehydrogenase	GGGAAGTCCATGTTTCTACACA	CATGCCCCGTCATAACTACCA	AGCACGGACCAGAGGAGCCACCA
Estrogen receptor	GCAGGACCAAACTCCGTAGTG	TGGCCAACGCGAGGTA	TACCCAGAGGCAAAGTCGCTGCAGA
GADPH	TGACCGTCCGTCTGGAGAA	TCGGCAGCAGCCTTAACAA	CCTGCCAGCTATGATGCCATCAAGAAG
Toxin-1	GCATTGCAGCGGTTCTGAT	GGCTGTGCTAGGCTGGAGTT	CAGCACTTCCAGCCTGTTTCGAGAGC
Vitellogenin	CTTCTGAACCCTGAGATC	GCAGCTGGGACGAAAGG	TTGAGTACAGTGGTGTGTGGCCCAAAGA
Vitelline envelope	GCCGGTTCCTCCTCCAAAT	TCCGCTGCCCAGTCTGA	CTGATATAGCTCCTGGGCCCTCATAGTTG

^a All primers are listed from 5' to 3'. Fluorescently labeled primers have a 6-FAM fluor on the 5'-end and a TAMRA fluor on the 3'-end.

Table 2
Summary of gene expression changes in trout liver using the Genomic Research on Atlantic Salmon Project (GRASP) high-density salmonid cDNA microarray^a

Gene ID	Accession no.	Identity	Fold-change ^b	p	Taxon ^c	e-Value ^d
CA051633	[P] AAL29923	Vitellogenin	10.42	1.48E-05	<i>Salmo salar</i>	3.0E-31
CB509947	[P] NP_031475	Angiogenin-related protein	9.67	0.0191	<i>Mus musculus</i>	2.0E-15
CB490454	[P] AAF71260	Vitellogenin-related protein gamma	7.22	1.01E-05	<i>Oncorhynchus mykiss</i>	5.0E-39
CB506103	[P] BAB40965	28-kDa-1e apolipoprotein	3.07	6.64E-05	<i>Anguilla japonica</i>	1.0E-19
CB497763	[P] P98093	Complement C3-1 (contains C3A anaphylatoxin)	-3	0.00879	<i>Oncorhynchus mykiss</i>	2.0E-36
CA038612	[P] L20313	Transferrin	-3.06	0.00855	<i>Salmo salar</i>	1.0E-130
CB491157	[P] BAB62189	Glyceraldehyde 3-phosphate dehydrogenase	-3.07	0.00794	<i>Oncorhynchus mykiss</i>	8.0E-39
CB492575	[P] NP_033147	Diamine acetyltransferase (spermidine/spermine N(1)-acetyltransferase)	-3.1	0.00763	<i>Mus musculus</i>	4.0E-41
CA037616	[P] S46199	Probable complement regulatory plasma protein SB1	-3.18	0.00698	<i>Paralabrax nebulifer</i>	4.0E-33
CA038163	[P] P98093	Complement C3-1 (contains C3A anaphylatoxin)	-3.19	0.00879	<i>Oncorhynchus mykiss</i>	1.0E-06
CB510301	[P] AAM21198	Toxin-1	-3.21	0.00693	<i>Oncorhynchus mykiss</i>	4.0E-29
CA042983	[P] AAO16805	Diamine acetyltransferase (Spermidine/spermine N(1)-acetyltransferase)	-3.41	0.00763	<i>Rana ridibunda</i>	1.0E-72
CB510761	[P] BAB03526	Fulectin-4	-3.44	0.00704	<i>Anguilla japonica</i>	1.0E-27
CB497396	[P] CAA67764	Pentraxin	-3.44	0.00688	<i>Oncorhynchus mykiss</i>	5.0E-79
CA038790	[P] CAB64714	Antithrombin	-3.56	0.00849	<i>Salmo salar</i>	1.0E-106
CB509867	[P] AAR06589	α_2 macroglobulin-1	-3.66	0.00584	<i>Sparus aurata</i>	6.0E-35
CA038730	[P] CAG04877	Unlabeled protein product	-3.83	0.00592	<i>Tetraodon nigroviridis</i>	9.0E-31
CB496876	[P] AAH74056	Unknown protein	-3.86	0.00568	<i>Danio rerio</i>	1.0E-64
CA041519	[P] NP_956878	Glutathione-S-transferase, θ^3	-4.02	0.00563	<i>Danio rerio</i>	4.0E-47
CB491279	[P] BAC23057	Complement component C5-1	-4.17	0.00539	<i>Cyprinus carpio</i>	1.0E-08
CA057214	[P] AAH74056	Unknown protein	-4.28	0.00503	<i>Danio rerio</i>	1.0E-63
CA042004	[P] NP_991280	Solute carrier family 31 (copper transporters)	-4.51	0.00537	<i>Danio rerio</i>	4.0E-43
CB493973	[N] CAA57449	Apolipoprotein B	-4.72	0.00382	<i>Salmo salar</i>	9.0E-09
CB505935	[P] NP_840082	Selenoprotein Pa	-4.78	0.0047	<i>Danio rerio</i>	1.0E-04
CB492868	[P] AY785950	Zonadhesin gene	-5.05	0.00435	<i>Oryzias latipes</i>	2.0E-13
CB510002	[P] CAA67765	Pentraxin	-5.61	0.00391	<i>Salmo salar</i>	3.0E-45
CB497097	[P] AAA92556	Probable complement regulatory plasma protein SB1	-6.04	0.00372	<i>Paralabrax nebulifer</i>	6.0E-23
CB509924	[P] NP_694492	Liver basic fatty acid-binding protein	-6.94	0.00287	<i>Danio rerio</i>	8.0E-57

^aJuvenile isogenic trout were exposed for 7 d to a measured ethynylestradiol concentration of 37 ng/L. The table lists only identified genes that were altered threefold. The full list of the 188 genes can be found in the Supplementary Material (SETAC Supplemental Data Archive, Item ETC-25-11-002; <http://etc.allenpress.com>). The accession number and gene identity refers to the gene to which the salmonid expressed sequence tags (EST) have the highest homology as provided by the GRASP consortium and BLAST search, respectively. The annotation [P] denotes that a BLAST search was done against the translated database; the annotation [N] denotes that a nucleotide-nucleotide search was run.

^bThe mean fold-change for a given spot.

^cTaxon corresponds to the species with which the salmonid EST had the greatest homology in the annotation.

^de-Values give a measure of homology between the EST and the most closely corresponding gene.

Table 3Comparison between the high density microarray data and quantitative polymerase chain reaction (qPCR) data^a

Gene	Array		qPCR	
	Fold-change	<i>p</i>	Fold-change	<i>p</i>
Cytochrome P4501A3	-1.81	0.02	-3.68	<0.001
Estrogen receptor	1.11	0.223	33	<0.01
Vitellogenin	10.42	<10 ⁻³	65,000	<10 ⁻⁵
Vitelline envelope	7.22	<10 ⁻⁵	36,500	<10 ⁻⁶
Glyceraldehyde phosphate dehydrogenase	-3	<0.01	-2.5	0.06
Androgen receptor	NA	NA	2.73	<0.005
Apolipoprotein CII	-2.6	<0.01	-1.5	0.16
δ-6-desaturase	2.8	<10 ⁴	2.5	<10 ⁻⁵
Toxin-1	-2.15	0.02	1.05	0.42

^a Mean fold-change was determined by comparing expression levels in treated fish with those in methanol-only controls, as described in the text. The *p* values were determined via a Student's *t* test assuming equal variance, compared to a fold-change of one for the array or versus control trout (qPCR data). The qPCR data are normalized to actin expression levels, except as noted, as described in the text. The androgen receptor was not measured on the array and is noted as not applicable (NA).