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Brain Cytochrome P450 Aromatase Gene Isoforms and Activity Levels in Atlantic Salmon After Waterborne Exposure to Nominal Environmental Concentrations of the Pharmaceutical Ethynylestradiol and Antifoulant Tributyltin

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In this study, the effects of two environmental endocrine disruptors, the synthetic pharmaceutical estrogen (ethynylestradiol, EE2) and antifoulant (tributyltin, TBT) representing two different modes of action on the endocrine system, were studied on brain steroidogenic pathway of juvenile Atlantic salmon (*Salmo salar*). Neurosteroidogenesis was studied using brain aromatase gene isoforms and enzyme activity, in parallel with typical xenoestrogen responses, such as brain estrogen receptor (ERα) and plasma vitellogenin (Vtg) levels. Fish were exposed to nominal waterborne EE2 (5 and 50 ng/l) and TBT (50 and 250 ng/l) concentrations dissolved in dimethyl sulfoxide (DMSO), singly and in combination. Gene expressions were quantified using real-time PCR with gene-specific primers, aromatase activity was analyzed using the tritiated water-release assay, and plasma Vtg was analyzed using competitive ELISA. Our data show that EE2 induced a concentration-specific modulation of P450aromA, P450aromB, and aromatase activity in addition to ERα and plasma Vtg levels in juvenile salmon at day 3 postexposure. TBT exposure caused both the elevation and inhibition of P450aromA, P450aromB, and aromatase activity levels, depending on concentration, at day 7 postexposure. TBT elevated and inhibited ERα and plasma Vtg and also antagonized EE2-induced expression of the studied variables at day 7 postexposure. Interestingly, the carrier vehicle DMSO modulated the receptor-mediated and non–receptor-mediated estrogenic responses at day 7 postexposure, compared to day 3. In general, these findings suggest that the exposed animals are experiencing impaired steroidogenesis and modulations of receptor-mediated endocrine responses. Given the integral role of neurosteroids in homeostatic process, growth, metabolism, reproduction, and development of central nervous system and function, these effects may have serious impact on this endocrine pathway and potentially affect organismal reproductive performance and health. In conclusion, the regulation of steroidogenesis is a fundamental mechanism involved in the biosynthesis of important biological compounds, irrespective of organ; therefore, the search for the molecular targets of xenoestrogens, given singly and also in combination, in these pathways will increase our understanding of organismal endocrine disruption and potential consequences.

Key Words: **neurosteroidogenesis; aromatase genes; pharmaceutical; antifouling agent; endocrine disruption; fish.**

Human and wildlife data indicate that the reproductive system, including its associated endocrine and neural controls, can be very susceptible to alterations by occupational or environmental exposures to a variety of chemical and physical agents (Arukwe and Goksøyr, 1998, 2003; Colborn *et al.*, 1993). Chemical compounds known to mimic the effects of endogenous estrogens in laboratory and field studies include synthetic steroids such as those used in contraceptive pills (Nash *et al.*, 2004), many pesticides (Donohoe and Curtis, 1996), phytoestrogens (Pelissero *et al.*, 1991), alkylphenol polyethoxylates (Kwak *et al.*, 2001; Meucci and Arukwe, 2005), and antifouling agents (McAllister and Kime, 2003).

Ethynylestradiol (EE2) is a pharmaceutical used in birth control pills and a potent endocrine modulator known to be present in the aquatic environment at biologically active concentrations (Nash *et al.*, 2004). In sewage treatment work effluents, steroidal estrogens are believed to be responsible for, partly, the feminized responses in some wild fish species (Desbrow *et al.*, 1998; Jobling *et al.*, 2002). The concentration of EE2 reported in effluents and surface waters from Europe range between 0.5 and 7 ng/l (Desbrow *et al.*, 1998; Larsson *et al.*, 1999), and concentrations of up to 50 ng/l have been reported by Aherne and Briggs (1989) in aquatic environments. In the United States, a survey of 139 streams showed that several rivers had concentrations > 5 ng/l with an extreme EE2 concentration up to 273 ng/l reported at some riverine sites (Kolpin *et al.*, 2002). Despite the lower EE2 concentrations in surface waters compared to natural steroidal estrogens, its estrogenic potency in fish *in vivo* studies is 10- to 50-fold higher than that of 17β-estradiol (E2) and estrone (E1) (Segner

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et al., 2003) most likely due to its longer half-life and tendency to bioconcentrate (650- and 10,000-fold in whole-body tissues and bile, respectively) (Lange *et al.*, 2001; Larsson *et al.*, 1999).

Tributyltin (TBT) is an organotin compound used primarily as a biocide in antifouling paints for ships, boats, and fishing nets (Horiguchi 1994). The use of TBT as antifouling paint has been banned for small boats and fishing nets in most countries as it was shown to be toxic to aquatic life and as an endocrine-disrupting chemical that causes severe reproductive effects in aquatic organisms (Horiguchi 1994). TBT was shown to exert masculinizing effect in zebra fish (*Danio rerio*) (McAllister and Kime, 2003) and induce imposex that was first described for dogwhelk, *Nucella lapillus* L. (Babler, 1970), and later reported in the female whelks (*Buccinum undatum* L.) from the North Sea (Borghi and Porte, 2002). Other effects of TBT include the inhibition of aromatase activity in fish (McAllister and Kime, 2003). The mechanisms by which TBT induces the modulation of the endocrine systems are not well understood, although several possible mechanisms have been proposed. These include the regulation of gonadotropin concentrations and receptor binding, regulation or modification of transcription factors, and regulation of transcription/translation or direct inhibition of the enzymes involved in steroid hormone biosynthesis (Ohno *et al.*, 2005; Yamazaki *et al.*, 2005). All these mechanisms are typical of non–receptor-mediated antiestrogenicity.

Studies on endocrine disruption, particularly with regard to steroidogenesis, have focused mainly on gonadal reproductive steroids, and little is known about the effects and mechanisms of xenoestrogens on reproductive neurosteroids. In vertebrates, the cytochrome P450 aromatase (CYP19) is a crucial steroidogenic enzyme catalyzing the final step in the conversion of androgens to estrogens (Callard *et al.*, 2001; Kishida and Callard, 2001). In teleost there are two structurally distinct CYP19 isoforms, namely, P450aromA and P450aromB. The P450aromA is predominantly expressed in the ovary and plays important roles in sex differentiation and oocyte growth, while P450aromB is expressed in neural tissues such as brain and retina and is speculated to be involved in the development of the central nervous system and sex behaviors (Callard *et al.*, 2001; Kishida and Callard, 2001). Research on endocrine toxicology has mainly focused on estrogenicity that involves direct estrogen receptor (ER)–mediated effects. Therefore, the present study was designed to investigate the effects of EE2 and TBT, singly and in combination, on neurosteroidogenic pathway using the aromatase activity and gene isoforms as model end points, in addition to direct ER-mediated end points such as the egg yolk protein (vitellogenin, Vtg) and brain ERα gene expressions. The ERα isoform was chosen as opposed to ERβ because our recent data showed that its expression in the brain showed a direct relationship with plasma Vtg levels in salmon after exposure to a xenoestrogen (Meucci and Arukwe, in press). Our hypothesis is that exposure of juvenile salmon to environmentally relevant EE2 and TBT concentrations, singly and in combination, will modulate brain aromatase isoform genes and activity in a concentration-dependent and time-dependent manner and that these effects will parallel direct ER-mediated responses and represent a new and quantitative measure of effect biomarker and response toxicity on the endocrine system.

MATERIALS AND METHODS

Chemicals. EE2, tributyltin chloride, ^{3}H-androstenedione bovine serum albumin (BSA), and *o*-phenylenediamine dihydrochloride (OPD) were purchased from Sigma-Aldrich Chemical Company (St. Louis, MO). Trizol reagent and oligo(dT)$_{18}$ primer were purchased from Gibco-Invitrogen Life Technologies (Carlsbad, CA). DNA ladder, RNase-free DNase, RevertAid First Strand cDNA Synthesis Kit (FERMENTAS GMBH, Germany), and deoxynucleotide triphosphates were purchased from Stratagene (La Jolla, CA). SYBR Green REAL-TIME PCR Master mix was purchased from BioRad Laboratories (Hercules, CA). Microtiter plates (MaxiSorp) were purchased from Nunc (Roskilde, Denmark). Polyclonal Arctic charr Vtg antibody was purchased from Biosense Laboratories, (Bergen, Norway). All other chemicals were of the highest commercially available grade.

Fish and experiment. Immature Atlantic salmon (*Salmo salar*, mean weight and length 10 ± 2.5 g and 9 ± 2 cm, respectively) were obtained from Lundamo Hatcheries (Trondheim, Norway) and kept in 70-l tanks at 7 ± 0.5°C and for a 14:10-h photoperiod at the Department of Biology, Norwegian University of Science and Technology (NTNU) animal-holding facilities. The experiment was performed after a 24-h acclimation period.

This study was designed to test two hypotheses in parallel under similar experimental conditions. Firstly, we tested the hypothesis that EE2, in addition to inducing classical ER-mediated responses, will modulate the expression of brain P450arom isoform genes and enzyme activity after short-term (3 days) waterborne exposure at different concentrations. For this experiment, three groups of 12 fish were exposed once to waterborne EE2 at 5 and 50 ng/l. Secondly, we tested the hypothesis that TBT, in addition to affecting EE2-induced classical ER-mediated responses, will antagonize EE2-induced modulation of brain P450arom isoform genes and enzyme activity and that this antagonistic effect will depend on TBT and EE2 concentrations. For this experiment, six groups of 12 fish were exposed once for 7 days to TBT at 50 and 250 ng/l singly and in combinations with EE2 at 5 and 50 ng/l. In addition, an EE2-positive control group and one group serving as solvent carrier control were exposed to the carrier vehicle dimethyl sulfoxide (DMSO; 7.5 ppb or 0.00075%). The final concentration of DMSO was similar in all exposure groups. The EE2 and TBT concentrations were chosen because they represent environmentally relevant concentrations.

During the experimental period, fish were starved. For both experiments, replicate sampling of six fish each per exposure group was sacrificed for gene expression and aromatase activity. Blanks were collected at the start of the experiments by sampling brain tissue of six individuals each for gene expression and aromatase activity analysis. Samples were collected from each exposure group after the fish were anesthetized with benzocaine (5 mg/l), and blood was collected before sacrifice. After sacrifice, the brain was excised and weighed and then processed as explained below. Blood was taken from the caudal vein using heparinized syringes, centrifuged immediately at 5000 × g for 5 min and stored at −80°C until analyzed.

RNA purification and cDNA synthesis. Total RNA was purified from brain tissues homogenized in Trizol reagent according to the manufacturer's protocol. Total cDNA for the real-time PCRs was generated from 0.2 μg DNase-treated total RNA from all samples using poly-T primers from iScript cDNA Synthesis Kit as described by the manufacturer (BioRad).

Primer optimization, cloning, and sequencing. The PCR primers for the amplification of 96– to 216–base pairs gene-specific PCR products were designed from the conserved regions of the studied genes. The primer

sequences, their amplicon size, and the optimal annealing temperatures are shown in Table 1. Prior to PCRs, all primer pairs were used in titration reactions in order to determine the optimal primer pair concentrations and their optimal annealing temperatures. All chosen primer pair concentrations used at the selected annealing temperatures gave a single band pattern for the expected amplicon size in all reactions. PCR products from the genes to be investigated were cloned into pCRII vector in INV αF′ *Escherichia coli* (Gibco-Invitrogen Life Technologies, Carlsbad, CA). Each plasmid was sequenced using ABI-prism 3100 Genetic Analyzer (Applied Biosystems, Foster City, CA) at the Department of Biology, NTNU, Norway. Sequences were confirmed using NCBI nucleotide BLAST software (http://www.ncbi.nlm.nih.gov/BLAST).

Quantitative (real-time) PCR. Quantitative (real-time) PCR was used for evaluating gene expression profiles. For each treatment, the expression of individual gene targets was analyzed as described by Arukwe (2005), using the Mx3000P REAL-TIME PCR SYSTEM (Stratagene). Each 25-μl DNA amplification reaction contained 12.5 μl of iTAQSYBR Green Supermix with ROX (BioRad), 1 μl of cDNA, and 400nM of each forward and reverse primers. The three-step real-time PCR program included an enzyme activation step at 95°C (5 min) and 40 cycles of 95°C (30 s), 55–60°C depending on target gene (see Table 1; 30 min), and 72°C (30 s). Controls lacking cDNA template were included to determine the specificity of target cDNA amplification. Cycle threshold (Ct) values obtained were converted into nanogram of DNA using standard plots of Ct versus log ng DNA standards. We generated standard plots for each target sequence using known amounts of plasmid containing the amplicon of interest. Target cDNA amplifications were averaged and expressed as nanogram per microliter of RNA. The use of the standard curves was based on equal amplification efficiency between the target gene and the plasmid containing the gene of interest (Arukwe, 2005).

Quantitative ELISA analysis of Vtg. A quantitative Vtg ELISA was performed using a polyclonal Arctic charr Vtg antibody as previously described by Meucci and Arukwe (2005). Purified Vtg protein was used to coat the plates and for the preparation of the standard curve. Briefly, purified salmon Vtg was serially diluted to obtain standard concentrations between 3 and 1000 ng/ml. Standards and diluted samples were incubated for 1 h at 37°C with an equal volume of the primary antibody (diluted 1:5000). Triplicate aliquots of standards and samples (200 μl) were added to 96-well microtiter plates previously coated with Vtg (100 ng/ml overnight at 4°C) and incubated for 1 h at 37°C. The plates were washed with Tween–phosphate-buffered saline, and a 1:2000 dilution of goat anti-rabbit peroxidase-conjugated secondary antibody (BioRad) was added and incubated for 1 h at 37°C. Levels of Vtg in the samples were measured colorimetrically at 492 nm using OPD as substrate with an Ultra microplate reader Elx 808 from Bio-Tek Instruments Inc. (Winnoski, VT). Vtg ELISA absorbance values (expressed as optical density, OD) were converted to the proportion of antibody bound (B) expressed as a percentage in the zero standard by the following equation: $B\ (\%) = (\mathrm{OD} - \mathrm{NSB}/\mathrm{OD}_0 - \mathrm{NSB}) \times 100$ (where OD is the absorbance of a given sample or standard, OD_0 is the absorbance of the zero standard, and NSB is the nonspecific binding absorbance value). Binding percentage values were logit transformed as shown below: $\mathrm{logit}\ B = \log 10\ (B/1 - B)$ and plotted against log dose to achieve a linear transformation of standard and plasma dilution curves. In evaluating the detection limit of the ELISA assay, the minimum amount of Vtg that produced a response significantly different from OD_0 was 2 ng/ml with 90% binding. The range of the standard curve was between 2 and 500 ng/ml, with 50% of binding around 35 ng/ml. ELISA values for Vtg obtained from control and exposed fish are expressed as mean ± SEM.

Aromatase activity assay. Aromatase activity was determined by the tritiated water release from the C-1 carbon atom of labeled androstenedione ((1,2,6,7-^{3}H)-androst-4-ene-3,17-dione) during its conversion to estradiol (Thompson and Siiteri, 1974) and was assayed according to the standard protocol described by Ankley *et al.* (2002) with some modifications. Briefly, brain tissue from individual fish (20–40 mg) was homogenized in 100 μl of 10mM phosphate buffer (containing 100mM EDTA, 1mM dithiothreitol at pH 7.4) per milligram of tissue and centrifuged at 10,000 × g for 20 min at 4°C. A 50-μl volume of the supernatant was incubated in a water bath at 25°C for 1 h together with 4nM of ^{3}H-androstenedione and 1mM NADPH in a total volume of 200 μl. The samples were assayed in duplicates with a blank containing buffer instead of sample for each set. The reaction was stopped by adding 300 μl of diethyl ether on ice to the reaction tubes. Tubes were then transferred to −80°C for 10 min to freeze the lower aqueous phase. Thereafter, the ether phase containing the steroids was discarded. The solution was then mixed thoroughly with 2:1 (vol/vol) of 0.25% dextran-coated (2.5%) charcoal and centrifuged at 2500 × g for 30 min at 4°C to remove residual aromatics. A 500-μl volume of the supernatant was added in a scintillation vial containing 5 ml of scintillation cocktail, and ^{3}H was measured as disintegrations per minute using a liquid scintillation counter. Aromatase activity was then expressed as femtomoles enzyme activity per hour per milligram of total protein content. Total protein concentration was measured using the Bradford assay (Bradford, 1976), with BSA as standard.

Statistical analysis. Comparison of different concentrations of EE2- or TBT-treated groups (singly and in combination) and control groups was performed using Dunnett's method. Statistical differences among treatment groups were tested using ANOVA and the Tukey-Kramer method. For all the tests the level of significance was set at $p < 0.05$, unless otherwise stated.

RESULTS

Modulation of P450arom Isoform Genes and Aromatase Activity

Quantitative RT-PCR analyses revealed that juvenile salmon relatively expressed both P450aromA and P450aromB genes in the brain of control and exposed individuals. The P450aromB mRNA expression was induced by EE2 in an apparent concentration-dependent manner after 3 days of exposure (Fig. 1A). Exposure to 50 ng EE2/l caused a 6.2-fold significant increase of brain P450aromB gene expression, compared to control (Fig. 1A). Exposure to 50 and 250 ng TBT/l caused,

TABLE 1
Primer Pair Sequences Used for Real-Time PCR

Target gene	Primer sequence[a] Forward	Reverse	Amplicon size (nucleotides)	Annealing temperature (°C)
P450aromB	CTGACCCCTCTGGACACG	TCTCGTTGAGAGGCACCC	96	55
P450aromA	GGGCACTGTCTGATGATGTC	GGGCTTGAGGAAGAACTCTG	104	60
ERα	GCTCCTGCTGCTGCTCTC	CCCTATGCTGGAGCCTGT	216	55

[a]Sequences are given in the 5′–3′order.

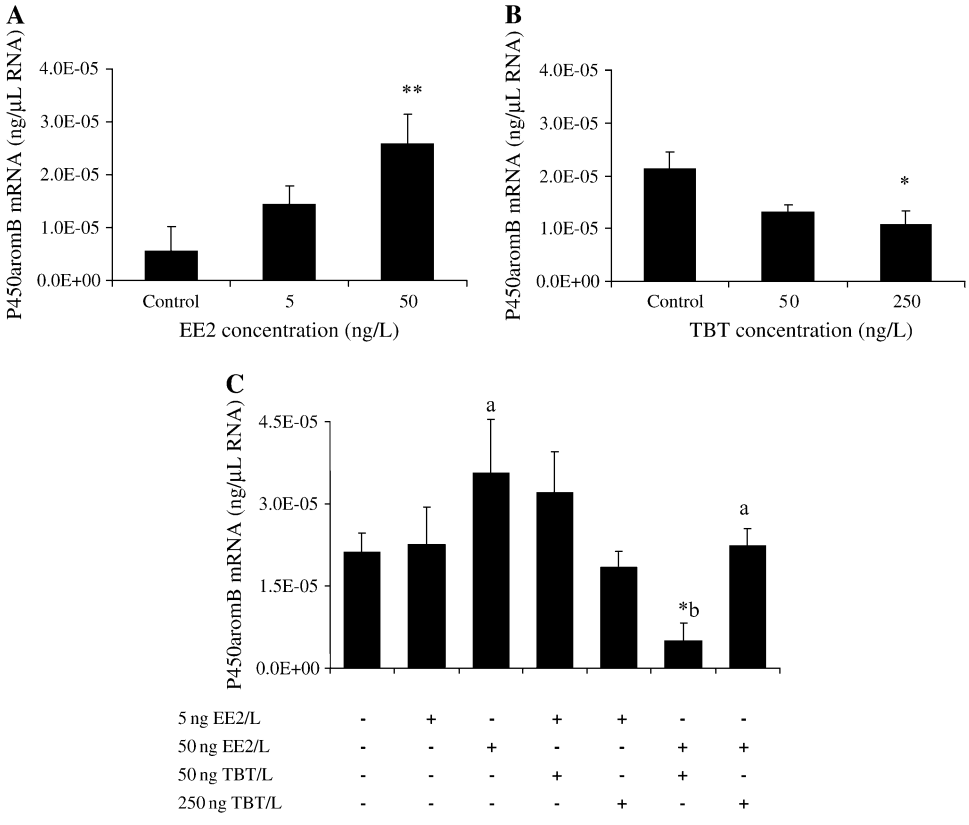

FIG. 1. Transcriptional changes of brain P450aromB mRNA levels in juvenile Atlantic salmon exposed to waterborne EE2 and TBT. (A) The P450aromB mRNA expression after 3 days of exposure to 5 and 50 ng EE2/l. (B) The P450aromB mRNA expression after 7 days of exposure to 50 and 250 ng TBT/l. (C) The P450aromB mRNA expression after 7 days of exposure to 5 and 50 ng EE2/l singly and in combination with 50 and 250 ng TBT/l. All values represent the mean ($n = 6$) ± SEM quantified using real-time PCR with specific primer pairs. $*p < 0.05$, $**p < 0.01$ compared to solvent control. Different letters denote combined exposure groups that are significantly different ($p < 0.05$), analyzed using ANOVA.

respectively, 0.4- and 0.5-fold nonsignificant decreases in brain P450aromB gene expression after 7 days of exposure, compared to the corresponding day 7 control group (Fig. 1B). The effect of EE2 after 7 days of exposure on the transcription abundance of the brain P450aromB gene, although elevated, was not significant in any of the exposures (Fig. 1C). The P450aromB gene expression showed a 1.7-fold increase in 50 ng EE2/l–exposed group compared to carrier control (Fig. 1C). When EE2 and TBT were given in combination, TBT antagonized, depending on concentration, the effect of EE2 on P450aromB expression (Fig. 1C). When compared to the 50 ng EE2/l group alone, combined exposure with 50 ng TBT/l significantly inhibited brain P450aromB gene expression (Fig. 1C).

The gene expression of brain P450aromA was significantly induced 4- and 3.6-fold, respectively, after 3 days of exposure to 5 and 50 ng EE2/l, compared to the control (Fig. 2A). In the second experiment, while exposure to 50 ng TBT/l did not have any effect, 250 ng TBT/l resulted in a 0.4-fold significant decrease in brain P450aromA gene expression after 7 days of exposure, compared to the control group (Fig. 2B). Overall, combined EE2 and TBT exposures did not cause any significant changes in brain P450aromA gene expression (Fig. 2C). The abundance of P450aromB was generally higher than P450aromA in the brain of controls and exposed animals.

Aromatase activity was measured in brain samples from control and exposed fish after 3 and 7 days of exposure. Exposure to 50 ng EE2/l resulted in a 2.4-fold significant increase, compared to control, of aromatase activity at day 3 postexposure (Fig. 3A). Similar to the P450arom isoform gene expression, aromatase activity was apparently inhibited after 7 days of exposure to TBT concentrations (Fig. 3B). Exposure to EE2 caused an apparent concentration-dependent decrease in aromatase activity at day 7 postexposure (Fig. 3C). In particular, 50 ng EE2/l significantly decreased aromatase activity 0.6-fold at day 7 postexposure, compared with the control (Fig. 3C). Coexposure of fish to TBT concentrations with 5 ng EE2/l sustained the EE2-inhibited aromatase activity (Fig. 3C). Interestingly, combined exposure of TBT concentrations with 50 ng EE2/l significantly increased brain aromatase activity, compared to the 50 ng EE2/l–exposed group alone (Fig. 3C).

Modulation of ERα Gene Expression

Exposure of fish to 5 and 50 ng EE2/l for 3 days caused, respectively, 8.5- and 3.5-fold significant induction of ERα

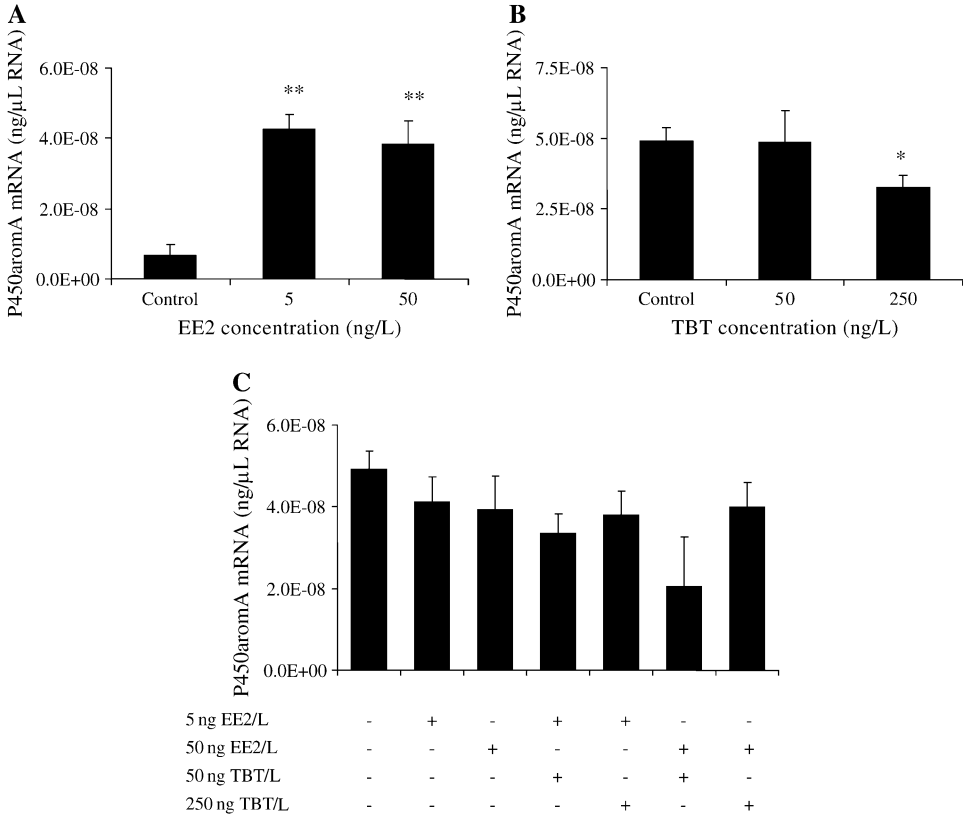

FIG. 2. Transcriptional changes of brain P450aromA mRNA levels in juvenile Atlantic salmon exposed to waterborne EE2 and TBT. (A) The P450aromA mRNA expression after 3 days of exposure to 5 and 50 ng EE2/l. (B) The P450aromA mRNA expression after 7 days of exposure to 50 and 250 ng TBT/l. (C) The P450aromA mRNA expression after 7 days of exposure to 5 and 50 ng EE2/l, singly and in combination with 50 and 250 ng TBT/l. All values represent the mean ($n = 6$) ± SEM quantified using real-time PCR with specific primer pairs. *$p < 0.05$, **$p < 0.01$ compared to solvent control, analyzed using ANOVA.

mRNA expression, compared to the control (Fig. 4A). Exposure to TBT concentrations in the second experiment caused no significant elevation of ERα mRNA expressions, resulting in, respectively, 1.6- and 1.7-fold at 50 and 250 ng TBT/l (Fig. 4B). After 7 days of EE2 exposure, ERα gene expression was still induced 1.8- and 1.5-fold in the 5 and 50 ng EE2/l exposure groups, respectively, but this induction was not significant (Fig. 4C). Combined exposure of fish to 5 ng EE2/l and 50 ng TBT/l resulted in a 8.1-fold increase in ERα gene expression, compared to the 5 ng EE2/l exposure group alone (Fig. 4C). When 5 ng EE2/l was given in combination with 250 ng TBT/l, the 5 ng EE2/l–induced ERα gene expression was inhibited (Fig. 4C). Coexposure of 50 ng EE2/l with 50 ng TBT/l significantly inhibited the EE2-induced ERα gene expression, reaching a value equal to 34% of the control (Fig. 4C).

Modulation of Plasma Vtg Levels

Exposure of fish to EE2 for 3 days caused the increase of plasma Vtg levels in a concentration-dependent manner with a 2.2-fold significant increase at 50 ng EE2/l, compared to the control (Fig. 5A). After 7 days, while TBT concentrations did not significantly affect plasma Vtg levels (Fig. 5B), exposure to 50 ng EE2/l resulted in a significant decrease of Vtg, compared to the control (Fig. 5C). Combined exposure of fish to 5 ng EE2/l and TBT did not significantly alter plasma Vtg levels, while coexposure of 50 ng EE2/l with 50 or 250 ng TBT/l significantly restored the 50 ng EE2-inhibited Vtg levels to almost control levels at day 7 (Fig. 5C).

Overall, it is important to note that the carrier vehicle (DMSO) used in the present study caused significant elevations of P450aromB (Fig. 1A vs. 1C), P450aromA (Fig. 2A vs. 2C), aromatase activity (Fig. 3A vs. 3C), ERα (Fig. 4A vs. 4C), and plasma Vtg (Fig. 5A vs. 5C) levels after 7 days (see C-figures) of exposure compared to 3 days of (see A-figures) exposure.

DISCUSSION

The present study has investigated the effects of EE2 and TBT (given singly and also in combination), representing two different modes of action on the endocrine system, on salmon brain P450arom gene isoforms and aromatase enzyme activity levels, in parallel with typical xenoestrogen responses (ERα and Vtg). Our data, based on nominal exposure concentrations,

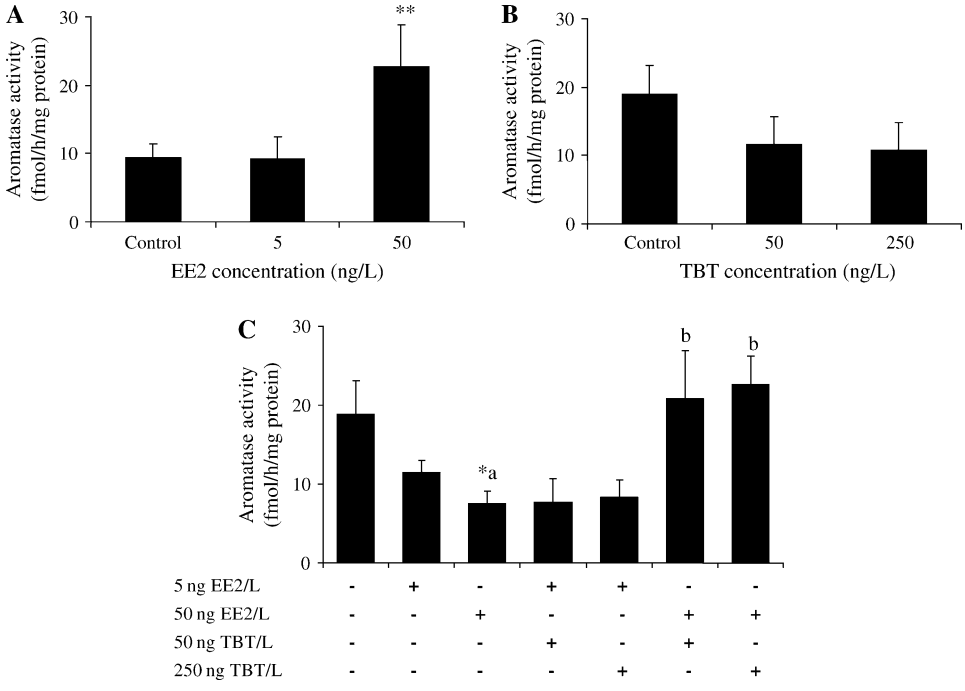

FIG. 3. Modulation of brain aromatase activity levels in juvenile Atlantic salmon exposed to waterborne EE2 and TBT. (A) Aromatase activity levels after 3 days of exposure to 5 and 50 ng EE2/l. (B) Aromatase activity levels after 7 days of exposure to 50 and 250 ng TBT/l. (C) Aromatase activity levels after 7 days of exposure to 5 and 50 ng EE2/l, singly and in combination with 50 and 250 ng TBT/l. All values represent the mean ($n = 6$) ± SEM quantified using real-time PCR with specific primer pairs. *$p < 0.05$, **$p < 0.01$ compared to solvent control. Different letters denote combined exposure groups that are significantly different ($p < 0.05$), analyzed using ANOVA.

show that EE2 induced a concentration-specific modulation of the P450arom isoforms and activity, ERα gene, and plasma Vtg levels in juvenile salmon. TBT caused variable effects, depending on concentration, on P450arom isoforms and activity levels, ERα gene, and plasma Vtg and also antagonized EE2-induced expression levels of the studied variables.

Modulation of P450arom Gene and Enzyme Activity

The present study shows that the brain P450arom genes were differentially affected by the pharmaceutical endocrine disruptor, EE2, in juvenile salmon with P450arom isoforms showing a unique expression pattern that was dependent on concentration and time of exposure. In parallel, the brain aromatase activity was significantly increased after 3 days of exposure to EE2. Recently, several studies have shown that P450aromB mRNA levels were upregulated after steroid hormone exposure (Gelinas *et al.*, 1998; Lee *et al.*, 2000; Tsai *et al.*, 2000). In the brain of adult sea bass (*Dicentrarchus labrax*), it was shown that aromatase activity was significantly higher than that in the gonads (Gonzalez and Piferrer, 2002, 2003). In view of these studies and the unique and comparable expression pattern between brain P450aromB mRNA and activity observed in the present study, we speculate that the brain aromatase activities reported in the present study might be due to the P450aromB isoform as opposed to P450aromA.

The finding that P450aromB has higher abundance than P450aromA is in accordance with previous studies demonstrating the expression of P450aromB in the brain with high levels of mRNA (Meucci and Arukwe, in press). The P450aromA is highly expressed in ovary with relatively low mRNA expression and enzyme activity (Callard *et al.*, 2001). In goldfish, these two P450arom genes are nonequivalent in their tissue-specific expression, indicating distinct promoters and regulatory mechanisms (Callard *et al.*, 2001). In accordance with the present study, brain P450arom expressions showed a differential expression pattern in neural and nonneural tissues but revealed a degree of overlap (Callard *et al.*, 2001). Elsewhere, Kazeto *et al.* (2004) reported that P450aromA mRNA levels in whole-body samples of zebra fish juveniles did not show any alteration after a short-term exposure to environmental concentrations of EE2, except at high (100nM) concentration, while significant EE2 concentration-dependent inductions in P450aromB mRNA levels were observed. Similarly, continuous exposure of zebra fish fry to 170nM of EE2 for a period of up to 10 days postfertilization, significantly elevated the expression of P450aromB gene in whole-body tissue, and the expression of the P450aromA gene was not affected by the exposure (Trant *et al.*, 2001).

Although the overlap between brain P450arom genes has been suggested as a result of an evolutionary remnant, it could be speculated that these differences are a result of the

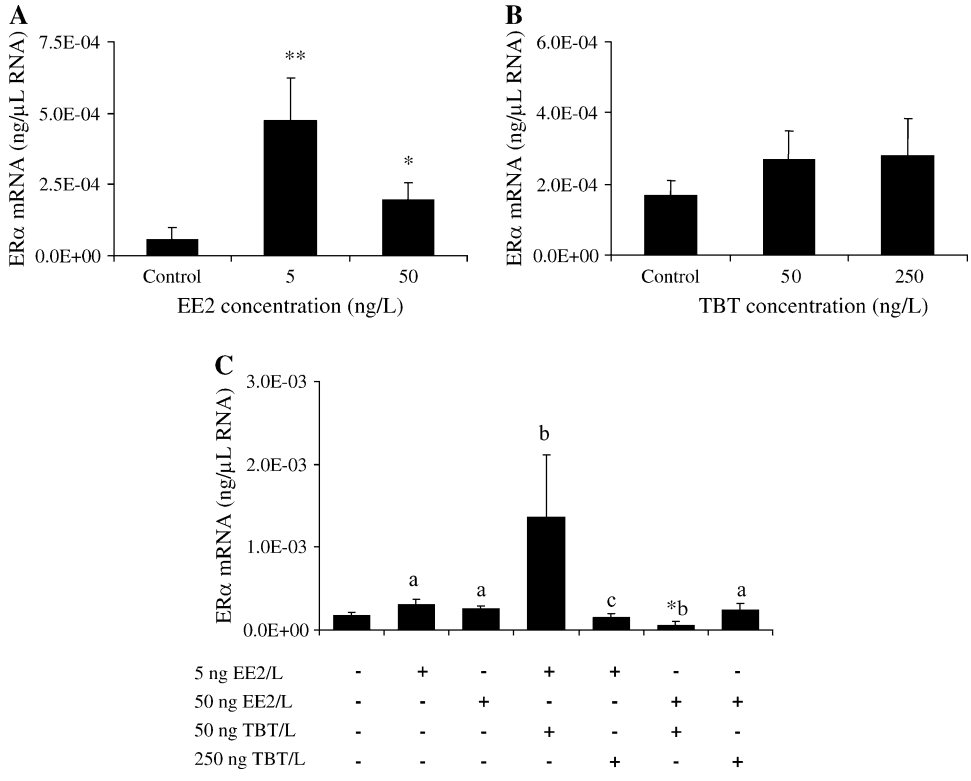

FIG. 4. Transcriptional changes of brain ERα mRNA levels in juvenile Atlantic salmon exposed to waterborne EE2 and TBT. (A) The ERα mRNA expression after 3 days of exposure to 5 and 50 ng EE2/l. (B) The ERα mRNA expression after 7 days of exposure to 50 and 250 ng TBT/l. (C) The ERα mRNA expression after 7 days of exposure to 5 and 50 ng EE2/l, singly and in combination with 50 and 250 ng TBT/l. All values represent the mean ($n = 6$) ± SEM quantified using real-time PCR with specific primer pairs. *$p < 0.05$, **$p < 0.01$ compared to solvent control. Different letters denote combined exposure groups that are significantly different ($p < 0.05$), analyzed using ANOVA.

P450aromA function in the brain, in addition to an E2 negative feedback control mechanism. These speculations are supported by the fact that the teleost brain P450aromA isoform lacks the consensus estrogen response element (ERE) in the promoter region (Kazeto *et al.*, 2001; Tchoudakova *et al.*, 2001). TBT exposure inhibited both P450arom isoforms, but only the EE2-induced brain P450aromB was significantly antagonized by combined TBT exposure. These findings suggest that P450aromB could be a candidate and a sensitive exposure gene biomarker for environmentally relevant estrogenic compounds. An autoregulative loop for P450aromB expression has been described for adult goldfish (Callard *et al.*, 2001; Pasmanik *et al.*, 1988) and in zebra fish embryos (Kishida and Callard, 2001; Kishida *et al.*, 2001). The identification of EREs in the promoter region of the P450aromB gene of the zebra fish (Kazeto *et al.*, 2001; Tchoudakova *et al.*, 2001) points to a direct estrogen responsiveness of the P450aromB gene, and our study suggests that environmental estrogens, including synthetic estrogens such as EE2, affect the P450aromB in a similar way as the natural estrogen. The masculinizing effects of TBT are well documented in invertebrates, especially in gastropods where TBT at a concentration as low as 1–2 ng/l causes the imposition of male sex organs including a penis and vas deferens (Bryan *et al.*, 1986; Depledge and Billinghurst, 1999; Gibbs and Bryan, 1994). It has been suggested that the mechanism of TBT-induced imposex is due to the inhibition of aromatase functions, resulting in subsequent accumulation of testosterone that could otherwise be metabolized (Matthiessen and Gibbs, 1998; Spooner *et al.*, 1991). In fish, relatively low levels (11.2 ng/l) of TBT exposure for 3 weeks decreased sperm counts in adult guppies (*Poecilia reticulata*) by 40–75% (Haubruge *et al.*, 2000), and administration of 1 µg of TBT/g bodyweight to male Japanese medaka significantly decreased fertilization success and suppressed sexual behavior (Nakayama *et al.*, 2004). Juvenile zebra fish exposed to TBT concentrations for up to 70 days posthatch showed a male-biased population and production of sperm lacking flagella (McAllister and Kime, 2003). In the study by McAllister and Kime (2003), the lowest observable effect concentration (LOEC) found was below 1 ng/l. Several field studies have demonstrated a correlation between TBT concentration in water and incidence of imposex with determined LOEC of 5 ng/l. An autoradiographic study demonstrated that a substantial amount of labeled TBT accumulated in the nervous tissue of the dogwhelk, thus supporting the hypothesis that imposex is caused through TBT action on some neurohormones

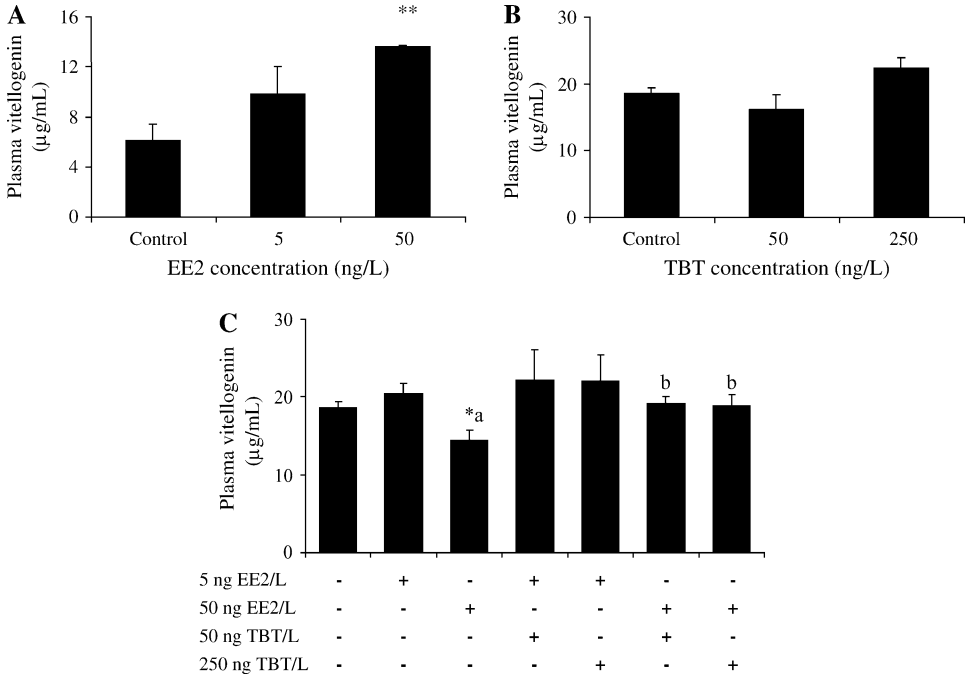

FIG. 5. Modulation of plasma Vtg levels in juvenile Atlantic salmon exposed to waterborne EE2 and TBT. (A) The Vtg levels after 3 days of exposure to 5 and 50 ng EE2/l. (B) The Vtg levels after 7 days of exposure to 50 and 250 ng TBT/l. (C) The Vtg levels after 7 days of exposure to 5 and 50 ng EE2/l, singly and in combination with 50 and 250 ng TBT/l. All values represent the mean ($n = 6$) ± SEM quantified using real-time PCR with specific primer pairs. *$p < 0.05$, **$p < 0.01$ compared to solvent control. Different letters denote combined exposure groups that are significantly different ($p < 0.05$), analyzed using ANOVA.

(Feral and LeGal, 1983). In a recent study by Yamazaki *et al.* (2005), it was shown that TBT at concentrations higher than 100nM suppressed all steroid biosynthesis in bovine adrenal cells, and this suppression was closely correlated to the decrease in steroidogenic acute regulatory (StAR) protein. In our study, exposure of juvenile salmon to TBT for 7 days resulted in the inhibition of brain P450arom isoform genes expression and enzyme activity and is in agreement with the theory that TBT is an aromatase inhibitor, in addition to other effects involving different mechanisms of action. Nevertheless, the TBT-induced activation of aromatase expression observed in the present study is novel and needs to be investigated in detail, and the mechanism involved should be studied in a differently designed study. These studies are currently underway in our laboratory.

Modulation of ERα Gene and Plasma Vtg Levels

In this study, we have also demonstrated that EE2 induced ERα gene expression in the brain and plasma Vtg levels. The molecular basis for Vtg gene and protein expression shows that the Vtg gene activations are receptor-mediated responses that are ligand structure–dependent interactions with ER, probably involving all isoforms, in addition to other coactivators. Maintenance of Vtg synthesis and concentration in oviparous species, including fish, is achieved through activation of ERs by E2 (Specker and Sullivan, 1994). Therefore, the induction of Vtg and Zr-protein synthesis in response to E2 and their mimics has been described in several fish species (Arukwe *et al.*, 2001; Flouriot *et al.*, 1997; Yadetie *et al.*, 1999). Although TBT inhibited aromatase gene expression and activity in the brain, other effects such as induction of ERα gene expression and plasma Vtg levels show that the mode of action for TBT effect is not restricted to aromatase inhibition as earlier anticipated. When adult fathead minnow (*Pimephales promelas*) were exposed to the aromatase inhibitor fadrozole for 21 days at concentrations up to 50 µg/l, aromatase activity was significantly inhibited in both males and females (Ankley *et al.*, 2002), and this inhibition was accompanied by a significant decrease in E2 and Vtg levels in the plasma.

Relevant to the present findings, the aromatizable xenoandrogen, 17α-methyltestosterone (MT) is found to increase Vtg levels in adult fathead minnow (Hornung *et al.*, 2004; Pawlowski *et al.*, 2004). It was suggested that MT was possibly converted into estrogens due to the action of aromatase (Pawlowski *et al.*, 2004; Simpson *et al.*, 1994). The production of Vtg in goldfish by high doses of MT, ethynyltestosterone, and methylandrostenediol has also been reported, but non-aromatizable androgens did not have the same effect (Hori *et al.*, 1979), suggesting a possible role of aromatase activity. In support of the same theory, Zerulla *et al.* (2002) found that estrogenic effects, such as Vtg induction, caused by MT in juvenile fathead minnow could be blocked through co-administration of an inhibitor of CYP19 aromatase to the fish. However, Hornung *et al.* (2004) did not find any

methylestradiol in the plasma of fish, despite the fact that Vtg levels were induced. These findings and the present study suggest a complicated mode of action for putative xenoandrogens and androgens in fish systems.

The induction of steroidogenic enzymes is highly tissue specific and cell-type specific and is controlled by different promoters and second messenger pathways. These pathways provide various targets for interaction with xenobiotics. Given the important roles of aromatase in sexual differentiation, development, reproduction, and behavior, particularly in the gonads and the brain (Callard *et al.*, 2001), interferences with the catalytic activity or expression of aromatase may be expected to result in disruptions of endocrine-regulated processes, such as sperm production and maturation, development of puberty, masculinization and feminization of (sexual) behavior, and the inhibition or stimulation of estrogen-dependent development. Furthermore, estrogens produced from increased aromatase activity have a number of physiological functions that are dependent on the tissue and development stages of the organism. For example, estrogens are involved in the sex-dependent behavior in the brain and control bone development, lipid metabolism, and distribution in peripheral tissue. Therefore, EE2 and TBT effects, singly and also in combination, on the brain P450arom gene expressions and subsequent activity may result in altered estrogen synthesis affecting multiple tissues.

A critical observation in the present study is the fact the carrier vehicle DMSO modulated receptor-mediated and non–receptor-mediated estrogenic responses at day 7 postexposure, compared to day 3. The use of DMSO as carrier solvent as opposed to ethanol was preferred in the present study because of the evidence that alcohol may activate P450arom activity and promote the conversion of testosterone to estradiol in mature female tilapia (*Oreochromis niloticus*) and their ovaries (Kim *et al.*, 2003). To our knowledge, there is no published data reporting possible estrogenic effects of DMSO on fish *in vivo* studies. However, in our experiment, exposure of juvenile salmon to DMSO for 7 days resulted in the upregulation of aromatase isoform genes, enzyme activity, ERα gene, and plasma Vtg, compared to the 3-day exposure levels. Kazeto *et al.* (2003) exposed juvenile zebra fish to EE2 and nonylphenol dissolved in DMSO (0.1% vol/vol) for 3 days and found that EE2 and nonylphenol induced P450aromB gene expression in a dose-dependent manner. Elsewhere, Alberti *et al.* (2005) exposed adult zebra fish to E2 and nonylphenol dissolved in DMSO (0.02% vol/vol) for 11 days and found a strong Vtg gene expression in the liver of male fish at high E2 (500 ng/l) and nonylphenol (250 μg/l) concentrations. For female fish exposed to E2 and nonylphenol, increasing concentrations of the compounds led to a consistent decrease in aromatase gene expression in the gonads (Alberti *et al.*, 2005). In view of these studies, the present study, and the recent review by Hutchinson *et al.* (2006), it is clear that DMSO has the potential of modulating the endocrine system in addition to other effects. Therefore, the use of DMSO as carrier vehicle in both *in vitro* and *in vivo* fish endocrine disruption studies should be reevaluated.

In summary, studies on endocrine disruption, particularly with regard to steroidogenesis, have focused mainly on reproductive steroids, but very little is known about the effects and mechanisms of xenoestrogens on neurosteroids. Recently, it was described that the brain is not only a target but also a steroid-producing area and that steroid concentration in plasma and brain fluctuate independently (Sierra, 2004). The effect of endocrine disruptors on steroidogenic pathways might have a more serious consequence for the organism than end points like egg yolk and eggshell protein inductions (Arukwe and Goksøyr, 2003). The relevance of these findings in terms of physiological, endocrine, reproductive, and ecotoxicological consequences will depend on factors such as bioavailability, biotransformation, and environmental concentration of EE2 and TBT, in addition to their interaction with other xenoestrogens. In this regard, it should be noted that the data in this report are based on nominal EE2 and TBT concentrations, and that the concentrations used might not necessarily represent the concentration of EE2 and TBT available in the experimental tanks. Regardless, in view of the present study, the fact that the concentration of EE2 reported in effluents and surface waters from Europe range between 0.5 and 7 ng/l (Desbrow *et al.*, 1998; Larsson *et al.*, 1999) and concentrations of up to 50 ng/l have been reported (Aherne and Briggs, 1989), and also the U.S. study survey of 139 streams showing that several rivers had concentrations > 5 ng/l with an extreme EE2 concentration up to 273 ng/l reported at some riverine sites (Kolpin *et al.*, 2002), pharmaceuticals in the environment represent serious health concern both to humans and wildlife.

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REFERENCES

Aherne, G. W., and Briggs, R. (1989). The relevance of the presence of certain synthetic steroids in the aquatic environment. *J. Pharm. Pharmacol.* **41,** 735–736.

Alberti, M., Kausch, U., Haindl, S., and Seifert, M. (2005). Gene expression analysis for exposure to estrogenic substances. *Acta Hydrochim. Hydrobiol.* **33,** 38–44.

Ankley, G. T., Kahl, M. D., Jensen, K. M., Hornung, M. W., Korte, J. J., Makynen, E. A., and Leino, R. L. (2002). Evaluation of the aromatase inhibitor fadrozole in a short-term reproduction assay with the fathead minnow (*Pimephales promelas*). *Toxicol. Sci.* **67,** 121–130.

Arukwe, A. (2005). Modulation of brain steroidogenesis by affecting transcriptional changes of StAR protein and cholesterol side chain cleavage (P450scc) in juvenile Atlantic salmon (Salmo salar) is a novel aspect of nonylphenol toxicity. *Environ. Sci. Technol.* **39,** 9791–9798.

Arukwe, A., and Goksøyr, A. (1998). Xenobiotics, xenoestrogens and reproduction disturbances in fish. *Sarsia* **83,** 225–241.

Arukwe, A., and Goksøyr, A. (2003). Eggshell and egg yolk proteins in fish: Hepatic proteins for the next generation: Oogenetic, population, and evolutionary implications of endocrine disruption. *Comp. Hepatol.* **2,** 4.

Arukwe, A., Kullman, S. W., and Hinton, D. E. (2001). Differential biomarker gene and protein expressions in nonylphenol and estradiol-17beta treated juvenile rainbow trout (*Oncorhynchus mykiss*). *Comp. Biochem. Physiol. C Comp. Pharmacol. Toxicol.* **129,** 1–10.

Babler, S. J. M. (1970). The occurrence of a penis-like outgrowth behind the right tentacle in spent female of *Nucella lapillus* (L.). *Proc. Malacol. Soc. Lond.* **39,** 231–233.

Borghi, V., and Porte, C. (2002). Organotin pollution in deep-sea fish from the northwestern Mediterranean. *Environ. Sci. Technol.* **36,** 4224–4228.

Bradford, M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **72,** 248–254.

Bryan, G. W., Gibbs, P. E., Hummerstone, L. G., and Hurt, G. R. (1986). The decline of the gastropod *Nucella lapillus* around south-west England: Evidence for the effects of tributyltin from antifouling paints. *J. Mar. Biol. Assoc. U.K.* **66,** 611–640.

Callard, G. V., Tchoudakova, A. V., Kishida, M., and Wood, E. (2001). Differential tissue distribution, developmental programming, estrogen regulation and promoter characteristics of cyp19 genes in teleost fish. *J. Steroid Biochem. Mol. Biol.* **79,** 305–314.

Colborn, T., vom Saal, F. S., and Soto, A. M. (1993). Developmental effects of endocrine-disrupting chemicals in wildlife and humans. *Environ. Health Perspect.* **101,** 378–384.

Depledge, M. H., and Billinghurst, Z. (1999). Ecological significance of endocrine disruption in marine invertebrates. *Mar. Pollut. Bull.* **39,** 32–38.

Desbrow, C., Routledge, E. J., Brighty, G., Sumpter, J. P., and Waldock, M. J. (1998). Identification of estrogenic chemicals in STW effluent. 1. Chemical fractionation and in vitro biological screening. *Environ. Sci. Technol.* **32,** 1559–1565.

Donohoe, R. M., and Curtis, L. R. (1996). Estrogenic activity of chlordecone, o,p′-DDT and o,p′-DDE in juvenile rainbow trout: Induction of vitellogenesis and interaction with hepatic estrogen binding sites. *Aquat. Toxicol.* **36,** 31–52.

Feral, C., LeGal, S. (1983). *Proceedings of International Mini-Symposium on Molluscan Endocrinology.* (J. Lever and H. H. Boer, Eds.), pp. 173–175. North Holland, Amsterdam.

Flouriot, G., Pakdel, F., Ducouret, B., Ledrean, Y., and Valotaire, Y. (1997). Differential regulation of two genes implicated in fish reproduction: Vitellogenin and estrogen receptor genes. *Mol. Reprod. Dev.* **48,** 317–323.

Gelinas, D., Pitoc, G. A., and Callard, G. V. (1998). Isolation of a goldfish brain cytochrome P450 aromatase cDNA: mRNA expression during the seasonal cycle and after steroid treatment. *Mol. Cell. Endocrinol.* **138,** 81–93.

Gibbs, P. E., and Bryan, G. W. (1994). Biomonitoring of tributyltin (TBT) pollution using the imposex response of Neogastropod molluscs. In *Biomonitoring of Coastal Waters and Estuaries* (K. J. M. Kramer, Ed.), pp. 205–222. CRC Press, Inc., London.

Gonzalez, A., and Piferrer, F. (2002). Characterization of aromatase activity in the sea bass: Effects of temperature and different catalytic properties of brain and ovarian homogenates and microsomes. *J. Exp. Zool.* **293,** 500–510.

Gonzalez, A., and Piferrer, F. (2003). Aromatase activity in the European sea bass (*Dicentrarchus labrax* L.) brain. Distribution and changes in relation to age, sex, and the annual reproductive cycle. *Gen. Comp. Endocrinol.* **132,** 223–230.

Haubruge, E., Petit, F., and Gage, M. J. (2000). Reduced sperm counts in guppies (*Poecilia reticulata*) following exposure to low levels of tributyltin and bisphenol A. *Proc. Biol. Sci.* **267,** 2333–2337.

Hori, S. H., Kodama, T., and Tanahashi, K. (1979). Induction of vitellogenin synthesis in goldfish by massive doses of androgens. *Gen. Comp. Endocrinol.* **37,** 306–320.

Horiguchi, T., Shiraishi, H., Shimizu, M., and Morita, M. (1994). Imposex and organotin compounds in *Thais clavigera* and *T. bronni* in Japan. *J. Mar. Biol. Assoc. U.K.* **74,** 651–669.

Hornung, M. W., Jensen, K. M., Korte, J. J., Kahl, M. D., Durhan, E. J., Denny, J. S., Henry, T. R., and Ankley, G. T. (2004). Mechanistic basis for estrogenic effects in fathead minnow (*Pimephales promelas*) following exposure to the androgen 17α-methyltestosterone: Conversion of 17α-methyltestosterone to 17α-methylestradiol. *Aquat. Toxicol.* **66,** 15–23.

Hutchinson, T. H., Shillabeer, N., Winter, M. J., and Pickford, D. B. (2006). Acute and chronic effects of carrier solvents in aquatic organisms: A critical review. *Aquat. Toxicol.* **76,** 69–92.

Jobling, S., Beresford, N., Nolan, M., Rodgers-Gray, T., Brighty, G. C., Sumpter, J. P., and Tyler, C. R. (2002). Altered sexual maturation and gamete production in wild roach (*Rutilus rutilus*) living in rivers that receive treated sewage effluents. *Biol. Reprod.* **66,** 272–281.

Kazeto, Y., Goto-Kazeto, R., Place, A. R., and Trant, J. M. (2003). Aromatase expression in zebrafish and channel catfish brains: Changes in transcript abundance associated with the reproductive cycle and exposure to endocrine disrupting chemicals. *Fish Physiol. Biochem.* **28,** 29–32.

Kazeto, Y., Ijiri, S., Place, A. R., Zohar, Y., and Trant, J. M. (2001). The 5′-flanking regions of CYP19A1 and CYP19A2 in zebrafish. *Biochem. Biophys. Res. Commun.* **288,** 503–508.

Kazeto, Y., Place, A. R., and Trant, J. M. (2004). Effects of endocrine disrupting chemicals on the expression of CYP19 genes in zebrafish (Danio rerio) juveniles. *Aquat. Toxicol.* **69,** 25–34.

Kim, B. H., Takemura, A., Kim, S. J., and Lee, Y. D. (2003). Vitellogenin synthesis via androgens in primary cultures of tilapia hepatocytes. *Gen. Comp. Endocrinol.* **132,** 248–255.

Kishida, M., and Callard, G. V. (2001). Distinct cytochrome P450 aromatase isoforms in zebrafish (*Danio rerio*) brain and ovary are differentially programmed and estrogen regulated during early development. *Endocrinology* **142,** 740–750.

Kishida, M., McLellan, M., Miranda, J. A., and Callard, G. V. (2001). Estrogen and xenoestrogens upregulate the brain aromatase isoform (P450aromB) and perturb markers of early development in zebrafish (*Danio rerio*). *Comp. Biochem. Physiol. B Biochem. Mol. Biol.* **129,** 261–268.

Kolpin, D. W., Furlong, E. T., Meyer, M. T., Thurman, E. M., Zaugg, S. D., Barber, L. B., and Buxton, H. T. (2002). Pharmaceuticals, hormones, and other organic wastewater contaminants in U.S. streams, 1999–2000: A national reconnaissance. *Environ. Sci. Technol.* **36,** 1202–1211.

Kwak, H. I., Bae, M. O., Lee, M. H., Lee, Y. S., Lee, B. J., Kang, K. S., Chae, C. H., Sung, H. J., Shin, J. S., Kim, J. H., *et al.* (2001). Effects of nonylphenol, bisphenol A, and their mixture on the viviparous swordtail fish (*Xiphophorus helleri*). *Environ. Toxicol. Chem.* **20,** 787–795.

Lange, R., Hutchinson, T. H., Croudace, C. P., Siegmund, F., Schweinfurth, H., Hampe, P., Panter, G. H., and Sumpter, J. P. (2001). Effects of the synthetic estrogen 17 alpha-ethinylestradiol on the life-cycle of the fathead minnow (*Pimephales promelas*). *Environ. Toxicol. Chem.* **20,** 1216–1227.

Larsson, D. G. J., Adolfsson-Erici, M., Parkkonen, J., Pettersson, M., Berg, A. H., Olsson, P. E., and Förlin, L. (1999). Ethinyloestradiol—An undesired fish contraceptive. *Aquat. Toxicol.* **45,** 91–97.

Lee, Y. H., Lee, F. Y., Yueh, W. S., Tacon, P., Du, J. L., Chang, C. N., Jeng, S. R., Tanaka, H., and Chang, C. F. (2000). Profiles of gonadal development, sex steroids, aromatase activity, and gonadotropin II in the controlled sex change of protandrous black porgy, *Acanthopagrus schlegeli Bleeker. Gen. Comp. Endocrinol.* **119,** 111–120.

Matthiessen, P., and Gibbs, P. E. (1998). Critical appraisal of the evidence for tributyltin-mediated endocrine disruption in mollusks. *Environ. Toxicol. Chem.* **17,** 37–43.

McAllister, B. G., and Kime, D. E. (2003). Early life exposure to environmental levels of the aromatase inhibitor tributyltin causes masculinisation and

irreversible sperm damage in zebrafish (*Danio rerio*). *Aquat. Toxicol.* **65,** 309–316.

Meucci, V., and Arukwe, A. (2005). Detection of vitellogenin and zona radiata protein expressions in surface mucus of immature juvenile Atlantic salmon (*Salmo salar*) exposed to waterborne nonylphenol. *Aquat. Toxicol.* **73,** 1–10.

Meucci, V., and Arukwe, A. (2006). Transcriptional modulation of brain and hepatic estrogen receptor and P450arom isotypes in juvenile immature Atlantic salmon (Salmo salar) after waterborne exposure to the xenoestrogen, 4-nonylphenol. *Aquat. Toxicol.* doi:10.1016/j.aquatox.2005.11.008.

Nakayama, K., Oshima, Y., Yamaguchi, T., Tsuruda, Y., Kang, I. J., Kobayashi, M., Imada, N., and Honjo, T. (2004). Fertilization success and sexual behavior in male medaka, *Oryzias latipes*, exposed to tributyltin. *Chemosphere* **55,** 1331–1337.

Nash, J. P., Kime, D. E., Van der Ven, L. T., Wester, P. W., Brion, F., Maack, G., Stahlschmidt-Allner, P., and Tyler, C. R. (2004). Long-term exposure to environmental concentrations of the pharmaceutical ethynylestradiol causes reproductive failure in fish. *Environ. Health Perspect.* **112,** 1725–1733.

Ohno, S., Nakajima, Y., and Nakajin, S. (2005). Triphenyltin and Tributyltin inhibit pig testicular 17β-hydroxysteroid dehydrogenase activity and suppress testicular testosterone biosynthesis. *Steroids* **70,** 645–651.

Pasmanik, M., Schlinger, B. A., and Callard, G. V. (1988). In vivo steroid regulation of aromatase and 5α-reductase in goldfish brain and pituitary. *Gen. Comp. Endocrinol.* **71,** 175–182.

Pawlowski, S., Sauer, A., Shears, J. A., Tyler, C. R., and Braunbeck, T. (2004). Androgenic and estrogenic effects of the synthetic androgen 17α-methyltestosterone on sexual development and reproductive performance in the fathead minnow (*Pimephales promelas*) determined using the gonadal recrudescence assay. *Aquat. Toxicol.* **68,** 277–291.

Pelissero, C., Bennetau, B., Babin, P., Le Menn, F., and Dunogues, J. (1991). The estrogenic activity of certain phytoestrogens in the Siberian sturgeon *Acipenser baeri*. *J. Steroid Biochem. Mol. Biol.* **38,** 293–299.

Segner, H., Navas, J. M., Schafers, C., and Wenzel, A. (2003). Potencies of estrogenic compounds in *in vitro* screening assays and in life cycle tests with zebrafish *in vivo*. *Ecotoxicol. Environ. Saf.* **54,** 315–322.

Sierra, A. (2004). Neurosteroids: The StAR protein in the brain. *J. Neuroendocrinol.* **16,** 787–793.

Simpson, E. R., Mahendroo, M. S., Means, G. D., Kilgore, M. W., Hinshelwood, M. M., Graham-Lorence, S., Amarneh, B., Ito, Y., Fisher, C. R., Michael, M. D., *et al.* (1994). Aromatase cytochrome P450, the enzyme responsible for estrogen biosynthesis. *Endocr. Rev.* **15,** 342–355.

Specker, J. L., and Sullivan, C. V. (1994). Vitellogenesis in fishes: Status and perspectives. In *Perspectives in Comparative Endocrinology* (K. G. Davey, R. E. Peter, and S. S. Tobe, Eds.), pp. 304–315. National Research Council of Canada, Ottawa, Ontario.

Spooner, N., Gibbs, P. E., Bryan, G. W., and Goad, L. J. (1991). The effect of tributyltin upon steroid titers in the female dogwhelk, *Nucella lapillus*, and the development of imposex. *Mar. Environ. Res.* **32,** 37–49.

Tchoudakova, A., Kishida, M., Wood, E., and Callard, G. V. (2001). Promoter characteristics of two cyp19 genes differentially expressed in the brain and ovary of teleost fish. *J. Steroid Biochem. Mol. Biol.* **78,** 427–439.

Thompson, E. A., Jr., Siiteri, P. K. (1974). The involvement of human placental microsomal cytochrome P450 in aromatization. *J. Biol. Chem.* **429,** 5373–5378.

Trant, J. M., Gavasso, S., Ackers, J., Chung, B. C., and Place, A. R. (2001). Developmental expression of cytochrome P450 aromatase genes (CYP19a and CYP19b) in zebrafish fry (*Danio rerio*). *J. Exp. Zool.* **290,** 475–483.

Tsai, C. L., Wang, L. H., Chang, C. F., and Kao, C. C. (2000). Effects of gonadal steroids on brain serotonergic and aromatase activity during the critical period of sexual differentiation in tilapia, *Oreochromis mossambicus*. *J. Neuroendocrinol.* **12,** 894–898.

Yadetie, F., Arukwe, A., Goksoyr, A., and Male, R. (1999). Induction of hepatic estrogen receptor in juvenile Atlantic salmon in vivo by the environmental estrogen, 4-nonylphenol. *Sci. Total Environ.* **233,** 201–210.

Yamazaki, T., Shimodaira, M., Kuwahara, H., Wakatsuki, H., Horiuchi, H., Matsuda, H., and Kominami, S. (2005). Tributyltin disturbs bovine adrenal steroidogenesis by two modes of action. *Steroids* **70,** 913–921.

Zerulla, M., Lange, R., Steger-Hartmann, T., Panter, G., Hutchinson, T., and Dietrich, D. R. (2002). Morphological sex reversal upon short-term exposure to endocrine modulators in juvenile fathead minnow (*Pimephales promelas*). *Toxicol. Lett.* **131,** 51–63.