Disruption of the Steroid Metabolome in Fish Caused by Exposure to the Environmental Estrogen 17α-Ethinylestradiol

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Exposure to environmental estrogens such as 17α-ethinylestradiol (EE2) has been associated with feminization and a decline in fertility of male fish. To investigate the effect of estrogen exposure on steroid homeostasis, we exposed roach (Rutilus rutilus) to EE2 (1—29 ng/L) for 18 days and analyzed steroid profiles in bile and plasma using targeted analyses and in liver and gonadal tissues using mass spectrometry metabolite profiling techniques (metabolomics). Exposure to EE2 resulted in a concentration dependent reduction of estrogens and androgens in bile and plasma of both male and female fish. At 10 ngEE2/L, significant reductions in concentrations of hydroxyprogesterone, androstenedione, 11-hydroxycorticosterone, and 11-ketotestosterone were detected in the testes metabolome, indicating disruption of steroid biosynthesis upstream of androgen metabolism. Estrogen exposure also resulted in increased biosynthesis of cortisol and cortisones in testes and ovaries, respectively, but did not alter glucocorticoid concentrations in the liver or plasma. This first report on the effect of EE2 exposure on the steroid metabolome in fish tissues suggests that both sex steroid and glucocorticoid pathways are one of the primary targets of estrogen exposure in fish gonads and provides further insights into the mode of action of this endocrine disrupting chemical.

Introduction

Fish exposed to wastewater effluents can bioaccumulate a complex mixture of estrogenic chemicals which comprise a variety of synthetic and naturally produced steroidal estrogens present in human waste as well as other estrogen receptor-active compounds such as alkylphenols which arise from industrial usage (1). Short-term exposure to estrogenic chemicals can induce feminization responses in male and female fish, which include increased expression of the estrogen-dependent genes vitellogenin and vitelline envelope proteins (2). The most potent estrogen present in wastewaters is the birth control pharmaceutical 17α-ethinylestradiol (EE2) (3), and long-term exposure of newly hatched roach (Rutilus rutilus, a fish susceptible to estrogenic pollution in the wild) to 4 ng/L EE2 results in complete sex reversal of all males and an all female population (4). Estrogenic chemicals with similar modes of action can act in an additive manner to induce feminization responses in male fish which include biosynthesis of vitellogenin as well as suppression of secondary sexual features (3, 5). There is also a strong correlation between the predicted exposures to mixtures of steroidal estrogens and the incidence of feminized intersex fish in roach populations in U.K. rivers (6). Intersex fish have reduced fecundity and are characterized by the appearance of oocytes within the testes and/or the presence of an ovarian cavity in males (7). The effect of estrogen exposure on female fish is less well documented but has been shown to result in an increase in the oocyte atresia and a decrease in egg production (8, 9). Together, the effects of estrogen exposure on reproductive physiology, behavior, and sexual selection may pose a threat to the long-term sustainability of fish populations in wastewater impacted waters (7).

The impact of long-term exposures to complex mixtures of environmental estrogens and other endocrine disrupting chemicals (EDCs) contaminants has raised concerns for the health of both humans and wildlife (10). The advent of genomic approaches to (eco)toxicological investigations should enable an understanding of the multiple mechanisms of action of EDCs which, in turn, could aid risk assessment strategies by the development of biomarker systems to identify contaminants with similar modes of action and biomarkers to monitor chemical exposure (11, 12). Ecotoxicogenomic investigations on the modes of action of environmental estrogens in fish have mainly concentrated on transcriptomic approaches; exposure of the fathead minnow (Pimephales promelas) to 17β-estradiol (E2) resulted in a feminization of ‘male’ steroidogenic enzyme expression profiles as well as changes in expression of other genes involved in growth, development, thyroid, and interrenal function (13). In further work, E2 was also shown to inhibit genes involved in androgen synthesis in the testes and to increase expression of aromatase genes involved in estrogen synthesis (14). In teleosts, like mammals, steroidogenesis is controlled via feedback mechanisms involving the brain-pituitary-gonadal axis. Depending on the stage of the life cycle, estrogens are known to have either positive and negative feedback effects on teleost gonadotrophins (GTH I and II), which in turn control the expression of genes regulating steroidogenesis in the gonads (15). The disruption of expression of genes involved in steroidogenesis is in broad agreement with studies which have reported reduced plasma concentrations of androgens as a result of EE2 exposure (16, 17). However, molecular responses to estrogens are complex, and the overall result of alterations in expression of a variety of steroidogenic enzymes on steroid metabolism in individual tissues and blood plasma are difficult to predict.

Metabolite profiling techniques (metabolomics) enables a semiquantitative approach to obtain information on the downstream final products and is increasingly being used in ecotoxicological investigations (18). Metabolomics has been used to investigate the response of the fathead minnow to EE2 exposure, and using NMR spectroscopy, changes in liver metabolites associated with lipid, energy, and protein metabolism were detected (19, 20). Similarly in rainbow trout, NMR metabolomics of plasma revealed increases in concentrations of phospholipids and vitellogenin and decreases in levels of cholesterol and alanine in fish exposed to 10 ng/L of EE2 (21). However, the effect of estrogen exposure on the steroid metabolome in fish has not been reported, primarily due to the lack of sensitive methods to profile nanomolar concentrations of these analytes. Recently, ultraperformance liquid chromatography-time-of-flight mass spectrometry
(UPLC-TOFMS) methods have been developed to profile the steroid metabolome in animal tissues, allowing an investigation on the effect of estrogen exposure a range of sex steroids and glucocorticoids (22).

The aim of this study was to determine the effect of EE2 exposure on the steroid metabolome of sexually mature male and female roach. An 18 day exposure period was used as studies with other fish species have revealed that exposure to estrogens for a 10–21 day period resulted in reduced expression of genes involved in steroidogenesis (14, 23) and decreased concentrations of androgens in plasma (24, 25). We used a combination of a targeted analysis of biofluids and metabolomic analyses of tissues to determine the effect of exposure to EE2 (1–29 ng/L) on steroid concentrations in the fish. A major route of excretion of sex steroids in fish is via the bile (26), and gas chromatography-tandem mass spectrometry (GC-MSMS) analyses used for targeted analyses of selected androgens and estrogens in bile as this is a highly selective technique for quantitation of known analytes in a complex matrix. From these data, fish samples from two of the EE2 treatments (1 and 10 ngEE2/L) were selected to further study the effect of EE2 exposure on changes in the steroid metabolome in extracts of gonad and liver tissues. Nontargeted metabolomic analyses were undertaken using a semiquantitative profiling technique (UPLC-TOFMS) to detect a range of steroids in the tissue extracts. Following the results of the metabolomic analyses, targeted analyses of both sex steroids and corticoids were undertaken in blood plasma using sensitive immunoassay techniques which allowed for detection of pg levels of analytes in the samples.

Materials and Methods

Materials. See the Supporting Information for details of chemicals.

Fish Exposure. Sexually mature roach, mean ± s.e. length and weight 11.4 ± 0.2 cm, 28.2 ± 1.3 g, > 10.4 ± 0.1 cm, 20.2 ± 0.9 g, were obtained from Jon Wall Fisheries (Melton Mowbray, Leicestershire, U.K.) in late November 2005. After one week of acclimation, the roach were randomly distributed among 5 treatment tanks in flow-through conditions (see the Supporting Information Table S1 for details). Stock solutions of EE2 in water were prepared (3, 10, 24, 68 µg/L) and delivered by peristaltic pump at 20 mL/h to a mixer chamber to which 40 L/h of charcoal filtered water was supplied. This gave nominal EE2 concentrations of 0, 1, 5, 12, and 34 ng/L. Replicate tank water samples were taken on 1, 7, and 14 days of exposure and analyzed for EE2 concentrations according to ref 27. Measured (GC-MS) concentrations (mean ± s.e. n = 8 for each concentration) over the exposure period were < LOD (<0.6), 1.1 ± 0.1, 3.7 ± 0.2, 9.8 ± 0.3, 28.5 ± 0.5 ngEE2/L, respectively, i.e. 0, 1, 4, 10, and 29 ngEE2/L. Each day all fish were fed to satiation with commercial fish food until day 16 of the exposure. After 18 days of exposure, fish from all the tanks were anaesthetised by immersion in MS222, and a heparinized blood sample was taken from the caudal vein. Gonads, liver, bile, and plasma were sampled from each fish and stored at −70 °C until analysis. Gender, gonadometric index (GSI, gonad mass as % of total body mass), and hepatometric index (HSI, liver mass as % of total body mass) were recorded for each individual.

Targeted Analyses of Sex Steroids in Bile. Conjugated steroids in bile samples (30–100 µL bile) were hydrolyzed with β-glucuronidase and sulfatase enzymes as described in ref 26. Deuterated steroids (10 ng, Table S2) were added as internal standards to each bile sample, which was extracted by solid phase extraction (SPE) cartridges. Samples were dried and derivatized by trimethylsilylation with a mixture of MSTFA:mercaptoethanol:NH4I prior to tandem GC-MSMS analyses (see the Supporting Information for SPE and GC-MSMS details).

Proiling of the Tissue Metabolomes. The steroid metabolome in roach liver and gonad tissues was analyzed according to ref 22 (see the Supporting Information for details of chemical and multivariate analyses). Briefly, metabolites were extracted from tissues by ultrasonication in methanol. Free and conjugated steroids in tissue extracts were separated on Strata-X-AW SPE, and the free steroid fraction was further purified using NH2 SPE. SPE fractions were separated using UPLC-TOFMS profiling in both +ESI and -ESI modes. Spectral peaks were deconvoluted and aligned, and the data were analyzed by partial least-squares-discriminant analysis (PLS-DA) to find class separating differences in the data sets. The performance of the PLS-DA models was described by the explained variation (R2Y) and the predictive ability (Q2Y) parameters of the models. Loading variables influencing treatment classification were determined using orthogonal projections partial least-squares to latent structures (OPLS) to select data that was only due to class separation. The significance of discriminatory variables (metabolites) between control and any one EE2 treatment was determined by Dunnet’s post hoc test (p < 0.05) after ANOVA of each variable and also from an estimate of the false discovery rate (FDR). The identity of discriminatory metabolites was determined from their accurate mass composition, their isotopic fit, and from comparison of the retention time (RT) with authentic standards. Structures of androgens and glucocorticoids in samples were also confirmed by GC-MS methodology (22).

Targeted Analyses of Steroids in Blood Plasma. Steroids in blood plasma (50 µL) were extracted 3 times with 4 volumes of dichloromethane, the extracts were combined, and solvent was removed under vacuum. The sample residue was redissolved in enzyme immunoassay buffer (1 mL) and analyzed for free steroids using the appropriate enzyme immunoassay kit (Cayman Chemical Co, Ann Arbor, MI, U.S.A.). The performance of the extraction method and the assays for each analyte was checked using additional plasma samples spiked with steroid standards.

Statistical Analyses. The correlation between the concentrations of analytes in the bile and EE2 uptake was determined using Pearson’s correlation coefficient. A regression analysis was used to investigate the relationship between EE2 concentration and values of GSI, HSI, or plasma concentrations of steroids. Differences in analytic concentrations between male and female fish in the same exposure tank were investigated using the students t-test. Non-normally distributed data (determined by the Kolmogorov–Smirnov test) were log-transformed prior to statistical analysis. Statistical differences were considered significant at p < 0.05.

Results and Discussion

Gonadosomatic and Hepatosomatic Indices. Exposure to concentrations of 1–29 ngEE2/L was significantly related to a decrease in GSI values for both male (p < 0.05) and female fish (p < 0.001) according to regression analyses (Table S1). This observation was consistent with reports of gonadal recrudescence in fish exposed to estrogens e.g. ref 16. Regression analysis revealed a significant increase in HSI of only female roach (p < 0.01) with EE2 concentration, which may reflect increased synthesis of estrogen-dependent vitellogenin in the liver (5). A 37% increase in male HSI was observed in all EE2-treated groups compared with the control group, but this was not statistically significant for the number of fish used per group (n = 8).

Effect of EE2 Exposure on Steroid Concentrations in Roach Bile. EE2 concentrations (analyzed as both free and conjugated forms) in bile increased in female and male fish as the concentration increased (Table 1). The mean bio-
concentration values of EE2 in bile of roach at the end of the 18 day exposure period were calculated from a comparison of the measured levels in water compared with bile. Bioconcentration of EE2 were between 63,000–136,000-fold for

<table>
<thead>
<tr>
<th>TABLE 1. Effect of EE2 Exposure on the Concentrations of Sex Steroids in Roach Bile*</th>
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<tbody>
<tr>
<td>sex steroid</td>
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<tr>
<td>--------------------</td>
</tr>
<tr>
<td>EE2</td>
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<tr>
<td></td>
</tr>
<tr>
<td>estrone</td>
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<tr>
<td></td>
</tr>
<tr>
<td>17β-estradiol</td>
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<td></td>
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<tr>
<td>testosterone</td>
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<td></td>
</tr>
<tr>
<td>11-ketotestosterone</td>
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* (p ≤ 0.05).  † (p ≤ 0.01).  ‡ (p ≤ 0.001).  § (p ≤ 0.0001).  * not determined.  * Concentration of steroid analytes in bile given as mean ng/mL ± SD. Nine fish per tank for all treatments, except for ♀ 4 ng EE2/L treatment which had 10 fish, and ♂ 1 ng/L treatment 7 fish, and ♂ 4 ng/L 4 fish.  † Pearson’s correlation coefficient (r) and significance value (p one tailed) of the relationship between the uptake of EE2 and the decrease in concentrations of each sex steroid in the bile of either male or female roach.

FIGURE 1. Partial least-squares-discriminant analyses (PLS-DA) scores plots of changes in the metabolome of gonads and liver of roach exposed to EE2. SPE fractions containing free steroids were analyzed in +ESI mode by UPLC-TOFMS. Data sets from control (black) roach were compared with those exposed to 1 ngEE2/L (red) and 10 ngEE2/L (blue). The % of Y variation modeled in the first three latent variables, t1, t2, t3, is shown on the relevant axis, and the number of latent variables included in each model was determined from internal cross-validation. One outlier has been removed from each of the testes and ovaries 10 ngEE2/L groups and the female liver control group.

of the measured levels in water compared with bile. Bioconcentration of EE2 were between 63,000–136,000-fold for
females and 59,000–97,000-fold for males, and a students t-test analysis of fish within each tank revealed that there were no significant gender differences in EE2 bioconcentration. Similar bioconcentration values of 10^4–10^5 for the uptake of estrogens into fish bile were determined for roach held in a WwTW effluent for 10 days (I) and for rainbow trout caged below a WwTW effluent for 2 weeks (28).

Targeted analysis of sex steroids (total of free and conjugated forms) in the bile revealed that exposure to EE2 resulted in a concentration dependent reduction of both estrogen and androgen concentrations in both sexes (Table 1). The concentrations of EE2 in the bile were significantly correlated with a decrease in concentrations of estrone, E2, testosterone, and, in males, 11-ketotestosterone (11-KT). These results indicate that the observed reductions in sex steroid concentrations in the bile were a result of EE2 exposure rather than due to confounding variables and indicated that EE2 uptake disrupted sex steroid homeostasis in both male and female roach. Two EE2 concentrations were chosen for a detailed study of the steroid metabolome in roach tissues; these were 10 ng EE2/L, which is a concentration 2.5-fold higher than that known to cause intersexuality in roach (29), and 1 ng/L, which is a concentration more relevant to environmental levels of EE2 in U.K. final wastewater effluents which range between <1 and 4 ng/L (30).

**Effect of EE2 Exposure on Steroid Metabolome of Gonads.** Liver and gonad tissue samples from roach exposed 0, 1, and 10 ng EE2/L were extracted and fractionated by SPE. UPLC-TOFMS analysis of SPE fractions of tissue extracts resulted in data sets with 1158–3600 RT x m/z signals. The only EE2 metabolite that was detected in any fraction was EE2 glucuronide which was detected in the conjugate fraction in +ESI mode, and this was removed from the data set before multivariate analyses. PCA models of all data sets gave poor predictive ability (Q^2 < 0.2); however, models of the free steroid fractions (+ESI mode) did show some separation between treatment groups (Figure S1). PLS-DA models with a high predictive ability (where Q^2 Y value > 0.5) were obtained from +ESI analysis of the free steroid fraction of testes (Q^2 Y 0.71), ovaries (Q^2 Y 0.60), male liver (Q^2 Y 0.82), and female liver (Q^2 Y 0.85) (Figure 1). Validation of the models revealed 93–100% accuracy of classification of the control and 10 ng EE2/L exposure data sets and 80–100% accuracy between the control and 1 ng EE2/L data sets. PLS-DA of the free steroid fraction from testes extracts analyzed in -ESI mode also resulted in a predictive model (Q^2 Y 0.52) with 76% of the Y variation modeled in the first 2 latent variables. PLS-DA models of the data sets from the conjugate steroid fraction from gonads and liver extracts gave Q^2 Y values of < 0.5 and were not analyzed further.

Data sets from the free steroid fractions were analyzed by OPLS, and ‘S’ scatter plots of loading variables were used to detect metabolite signals that differed between the control and either the 1 ng EE2/L or 10 ng EE2/L treatments (for examples see Figures S2 and S3). Many of the marker metabolites whose concentrations changed in response to EE2 treatment were identified as steroids, and the significance of the difference between their relative concentrations in the different treatments was estimated using p values and an estimate of the FDR for each metabolite. Some of the steroidal markers detected in the testes extracts were present as multiple signals as either adducts or fragments or were detected as markers in the data sets obtained from both +ESI and -ESI modes. The detection of several m/z signals for
TABLE 3. Effect of EE2 Exposure on Concentrations of Steroids in Roach Plasmand

<table>
<thead>
<tr>
<th>steroid (concentration units in plasma)</th>
<th>gender</th>
<th>EE2 concentration ng/L</th>
<th>LOD</th>
<th>regression coefficient, r²</th>
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<tbody>
<tr>
<td></td>
<td></td>
<td>control 1</td>
<td>4</td>
<td>10</td>
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<tr>
<td>estrone (pg/mL)</td>
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<td></td>
<td></td>
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<tr>
<td>d</td>
<td>328 ± 231</td>
<td>241 ± 430</td>
<td>10 ± 2</td>
<td>&lt;LOD</td>
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<td>171 ± 150</td>
<td>965 ± 582</td>
<td>43 ± 30</td>
<td>&lt;LOD</td>
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<td>estradiol (pg/mL)</td>
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<tr>
<td>d</td>
<td>525 ± 380</td>
<td>276 ± 275</td>
<td>76 ± 8</td>
<td>&lt;LOD</td>
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<tr>
<td>testosteron (pg/mL)</td>
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<tr>
<td>d</td>
<td>525 ± 380</td>
<td>378 ± 380</td>
<td>66 ± 28</td>
<td>53 ± 14</td>
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<tr>
<td>11-ketosteron (pg/mL)</td>
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<tr>
<td>d</td>
<td>1961 ± 3051</td>
<td>83 ± 84</td>
<td>15 ± 13</td>
<td>9 ± 8</td>
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<tr>
<td>cortison (ng/mL)</td>
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<td></td>
<td></td>
<td></td>
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<tr>
<td>d</td>
<td>232 ± 180</td>
<td>290 ± 263</td>
<td>359 ± 251</td>
<td>128 ± 201</td>
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<tr>
<td>cortisonen (ng/mL)</td>
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<tr>
<td>d</td>
<td>3237 ± 2161</td>
<td>2270 ± 2222</td>
<td>8091 ± 9947</td>
<td>15655 ± 2319</td>
</tr>
</tbody>
</table>

* Linear regression analysis of EE2 concentration against log concentration of steroid in the bile.

a p ≤ 0.01. b p ≤ 0.001. c p ≤ 0.0001, ns = not significant. d Concentration of free steroid analytes in plasma given as mean ± SD. Measurements of 6–9 fish for all treatments, except for d 4 EE2 ng/L which comprised 5 measurements.

Each metabolite also decreased the likelihood of detection of false positive (type I errors) during multivariate analyses. Three androgens, androstenedione and its metabolites 11-ketone and 11-KT, were identified in testes extracts confirming previous reports that these are the most abundant androgen in fish testes (31). Exposure to 10 ngEE2/L resulted in a significant reduction in androgen signals in roach testes (Table 2). Concentrations of androstenedione and 11-ketone (detected in +ESI mode) were reduced by 7- and 233-fold, respectively. Hydroxyandrostenedione was detected in testes extracts in +ESI mode as both the [M+H]⁺ and [M+Na]⁺ ions, and in -ESI mode as the [M-H]⁻ ions, and concentrations of these ions were reduced in the EE2 treatment by 11-, 48-, and 149-fold, respectively. The observed differences in reduction of the different hydroxyandrostenedione species in +ESI mode could be due to changes in the relative abundance of the [M+H]⁺ and [M+Na]⁺ ions which may depend on the Na⁺ concentration in each sample. The decrease in androgen concentrations indicated that steroid biosynthetic pathways were disrupted upstream of androstenedione, and this was confirmed by a significant reduction in the concentration of 17-hydroxyprogesterone (identified from the mass, isotopic composition and retention time of an authentic standard) in response to 10 ngEE2/L exposure. The observed decrease in concentrations of 17-hydroxyprogesterone, androstenedione and 11-oxygenated androgens in roach testes are consistent with transcriptomic studies showing that EE2 (10 ng/L) inhibited expression of cytochrome P450 17 (CYP 17), 11β-hydroxysteroid dehydrogenase (11β-HSD), and 17β-hydroxysteroid dehydrogenase (17β-HSD) genes in testes of fathead minnow (Pimephales promelas) (14). Both EE2 and E2 (20 mg/kg) also inhibited cytochrome P450 11-hydroxylases (CYP 11B) in trout testes (23, 32). Suppression of androgen biosynthesis by EE2 may occur due to the negative feedback via the pituitary follicle stimulating hormone (GTH I) (15). However, some in vitro studies also suggest that estrogens can have a direct effect on expression of genes involved in steroidogenesis in fish (32).

Estrogens were not detected by metabolomic analyses of testes extracts possibly due to their low concentrations in this tissue. Neither androgens nor estrogens were detected in ovary extracts of control fish due to strong ion suppression effects of this matrix (22). Cortisol was detected in the testes extracts in both +ESI mode as the [M+H]⁺ ion and [M+Na]⁺ adduct, and in -ESI mode as the [M-H]⁻ ion, the formate adduct and the [M-H-CH₃O]⁻ fragment. Cortisol concentrations in the testes were significantly increased by exposure to 10 ngEE2/L, and, depending on the m/z signal, levels increased by 6–12-fold. Two other glucocorticoids, with the same empirical formula and isotope fit, but different RTs to cortisol, were detected in testes extracts in -ESI mode and were named as cortisol-type 1 and type II. Their identity is currently unknown but they are likely to be metabolites of either corticosterone (e.g., hydroxycorticosterone) or cortisone (e.g., dihydrocortisone). Concentrations of the cortisol-type II metabolite significantly decreased with 10 ngEE2/L exposure by 4–13-fold. Although cortisol and cortisone were both detected in testes and ovary extracts (22), cortisone was the only glucocorticoid detected in ovary extracts whose concentrations changed as a result of EE2 exposure. Levels of cortisone were significantly increased by 7-fold at 10 ngEE2/L (Table 2). Box-plots of the effect of EE2 treatment on the relative concentrations of androgens and glucocorticoids in roach gonads are given in Figure S4.

It is possible that glucocorticoid biosynthesis is dependent on different gene products (e.g., 21-hydroxylases) and isozymes (e.g., 11β-hydroxylases) to those involved in androgen metabolism, and these may not be downregulated by EE2 treatment. In this case, a reduction in androgen biosynthesis may result in more substrate availability (e.g., pregnenolone or progesterone) and a shift toward glucocorticoid metabolism (Figure S5). Similarly a reduction in glucocorticoid catabolism and excretion could also account for EE2-induced increased levels of cortisol and cortisone in the gonads. It is not clear why EE2 exposure increased cortisol concentrations in testes, whereas levels of its oxidized metabolite, cortisone, increased in ovaries. The relative concentrations cortisol and cortisone are determined by 11β-HSD enzymes which could be regulated differently in male and female roach.

No changes in steroid concentrations were detected from the metabolomic analyses of the liver tissues, despite the detection of cortisol in liver extracts from both male and female roach (22). An additional finding from the metabolomic profiling of liver or gonad extracts was that concentrations of metabolites other than steroids were significantly altered in fish in the EE2-exposed groups. The concentrations of these metabolites were either significantly decreased or increased in the 10 ngEE2/L group or in both the 1 and 10 ngEE2/L groups compared with the control group (Table S3). There was not enough material for further chemical analysis of these metabolites, and their identities remain to be elucidated. It is unclear whether changes in these metabolites were linked to EE2 exposure or were a result of other differences in the fish between the tank groups.

**Effect of EE2 Exposure on Plasma Concentrations of Steroids.** In order to investigate whether the EE2-induced...
changes observed in the steroidal metabole of roach gonads were reflected in the blood, the plasma concentrations of free steroids in roach were analyzed by enzyme immunoassay. The concentrations of free sex steroids measured in plasma of control roach were in good agreement with previously reported studies for this species (33) (Table 3).

There was a significant relationship between EE2 concentration and a reduction in levels of testosterone, 11-KT, E2 and estrone concentrations in the plasma of both sexes indicating that androgen biosynthesis was a likely limiting factor in estrogen production in females too. EE2 exposure results in a significant increase in expression of CYP 19 A and B genes in fish testes and ovaries, and these encode for aromatase enzymes responsible for estrogen biosynthesis (13, 14). In our study, the observed reduction in concentrations of precursor androgens in the testes and in the plasma of both sexes of roach indicates that upregulation of aromatase activity may not necessarily result in increased synthesis of estrogens.

At 1 ngEE2/L, 11-KT concentrations were reduced by 2.6-fold in the testes, although this was not significant in terms of the FDR (Table 2). Additionally at 1 ngEE2/L, 11-KT concentrations were reduced in bile and plasma of males by 20% and 47%, respectively, indicating that environmentally relevant concentrations of EE2 can reduce 11-KT concentrations in roach. Similarly, reductions in plasma 11-KT levels in male muumichog and zebrafish have been observed after exposure to 1 and 2 ngEE2/L correspondingly (24, 25). Reduction in steroid biosynthesis in the gonads may have significant consequences not only for normal gonadal development during the seasonal cycle of sexually maturing adult fish but also for reproductive behavior of fish where reductions in plasma 11-KT concentrations have been associated with disruption of sexual selection and decreased reproductive success of exposed males (24, 25).

The observed EE2-induced changes in gonadal concentrations of glucocorticoids were not reflected in the plasma. No significant changes in cortisol or corticosterone (another major glucocorticoid in fish blood) concentrations were detected in plasma as a result of EE2 treatment (Table 3), and these results were consistent with metabolic analyses of roach liver extracts which did not detect any changes in cortisol concentrations in the EE2-treated groups. In fish glucocorticoids are synthesized in the interrenal tissue, but there is increasing evidence that they can also be produced in both ovaries and testes (34). Therefore plasma concentrations of glucocorticoids represent their total synthesis from interrenal tissue and gonads and may not necessarily reflect levels in an individual tissue (35). In addition, the plasma concentrations of glucocorticoids were very variable, and concentrations in control fish reflected the range reported for undisturbed roach or those exposed to brief handling stress (36). Although, in this study, the roach were sampled in a random manner, the variable levels of cortisol in the plasma may reflect handling stress which could have masked some treatment effects. There is also the possibility that there was cross reactivity in the immunoassay with other cortisol type metabolites, and this may have confounded the estimation of true cortisol levels in the plasma.

Cortisol has been shown to play an important part in gonad development and can interfere with estrogen signaling in adult females and at high concentrations can inhibit spermatogenesis (34). Increased cortisol or cortisone concentrations in the gonads, together with a decrease in androgen biosynthesis may account for some of the changes in testes and ovarian histopathology observed with estrogen exposure, such as feminization, increased oocyte atresia and decreased GSI (7, 9). It is possible that shorter or longer time periods of exposure to estrogens may have different effects on steroidalogenesis due to both the positive and negative feedback mechanisms of steroids on the brain-pituitary gonadal axis in fish. Further metabolomic studies are needed to identify other metabolite pathways and end points that are targets of estrogen exposure in fish in order to improve our understanding of the mode of action of environmental estrogens and to result in sensitive biomarkers of exposure to these endocrine disrupting chemicals.

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Supporting Information Available
Additional information on methodology and multivariate analyses as noted in the text. This material is available free of charge via the Internet at http://pubs.acs.org.

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