Published in final edited form as:

Aquat Toxicol. 2014 November ; 156: 148–160. doi:10.1016/j.aquatox.2014.08.008.

Dietary exposure of 17-alpha ethinylestradiol modulates physiological endpoints and gene signaling pathways in female largemouth bass (*Micropterus salmoides*)

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Abstract

17alpha-ethinylestradiol (EE2), used for birth control in humans, is a potent estrogen that is found in wastewater at low concentrations (ng/L). EE2 has the ability to interfere with the endocrine system of fish, affecting reproduction which can result in population level effects. The objective of this study was to determine if dietary exposure to EE2 would alter gene expression patterns and key pathways in the liver and ovary and whether these could be associated with reproductive endpoints in female largemouth bass during egg development. Female LMB received 70 ng EE2/g feed (feed administered at 1% of body weight) for 60 days. EE2 dietary exposure significantly reduced plasma vitellogenin concentrations by 70%. Hepatosomatic and gonadosomatic indices were also decreased with EE2 feeding by 38.5% and 40%, respectively. Transcriptomic profiling revealed that there were more changes in steady state mRNA levels in the liver compared to the ovary. Genes associated with reproduction were differentially expressed such as vitellogenin in the liver and aromatase in the gonad. In addition, a set of genes related with oxidative stress (e.g. glutathione reductase and glutathione peroxidase) were identified as altered in the liver and genes associated with the immune system (e.g. complement component 1, and macrophage-inducible C-type lectin) were altered in the gonad. In a follow-up study with 0.2 ng EE2/g feed for 60 days, similar phenotypic and gene expression changes were observed that support these findings with the higher concentrations. This study provides new insights into how dietary exposure to EE2 interferes with endocrine signaling pathways in female LMB during a critical period of reproductive oogenesis.

Keywords

17alpha-ethinylestradiol; largemouth bass; transcriptomic approach

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1. Introduction

17 α -ethinylestradiol (EE2) is a synthetic estrogen and one of the main components used in oral contraceptives as well as the most widely studied endocrine disruptor worldwide. It enters the environment mainly through wastewater discharges. To date, a large number of studies have reported its widespread presence in aquatic systems with reported detection levels typically at low concentrations (<1 ng/L) in surface waters (review by Aris et al. 2014; Caldwell et al. 2008; Wise et al. 2010).

The effects of EE2 have been summarized for a number of different fish species. What is clear from these studies is that exposure of fish to EE2 (1) causes disruption of sexual development; (2) results in abnormal development of early life stages, thereby altering the sex ratio of fish; (3) alters gonadal differentiation and development in adult fishes; (4) disrupts sex steroid receptor signaling; and (5) in some extreme cases, decreases reproductive output (Aris et al. 2014; Garcia-Reyero et al. 2009; Kidd et al. 2007; Santos et al. 2007; Schäfers et al. 2007). Conclusive work by Kidd et al. (2007), who studied a contaminated lake at a level of 5 ng EE2/L over three years, indicated that fathead minnow populations in the lake declined, confirming that the effects of EE2 can be detected at the population level.

In addition to these reproductive effects, the toxic potential of EE2 in several exposed organisms is also recognized (Aris et al. 2014; Kato et al. 2004; Micael et al. 2007; Yamamoto et al. 2006). A concentration of 10 ng EE2/L over 60 days post hatch in zebrafish caused liver and kidney lesions (Weber et al. 2004). In addition, it is well known that E2 can also activate cellular processes through activation of the membrane ER, in particular triggering Ca⁺⁺-dependent proteases and protein kinases (Kelly and Levin 2001; Zadrán et al. 2009). It is clear that exogenous estrogens can exert effects on multiple levels of biological organization and can interact with other physiological processes. There are studies that suggest an association with the immune system. EE2 has been reported to modulate the inflammatory response and alter genes involved in this response in several species of fish including gilthead seabream and carp (Cabas et al. 2011; Cabas et al. 2012; Liarte et al. 2011; Moens et al. 2007).

EE2 has an octanol-water-partitioning coefficient (Kow) of 4.15 (Lai et al. 2000), suggesting that a significant amount of the chemical may be adsorbed on suspended particulate matter (e.g., algae, bacteria, detritus) and/or on sediments (Lai et al. 2002). This implies that another route of exposure for aquatic organisms is through the diet, as fish may eat particulate matter with their food. There are only a few studies that expose fish (medaka, mosquitofish, gilthead seabream and tilapia) to EE2 in the diet (Angus et al. 2005; Cabas et al. 2011; Chikae et al. 2003; Shved et al. 2007) and these studies suggest very similar effects to those observed with aqueous exposures including increases of plasma levels of VTG in males, altered sexual development and maturation, inhibition of pituitary hormones and effects related with the immune system.

In this study, female largemouth bass (*Micropterus salmoides*, LMB) in late stage of reproduction (vitellogenesis/egg maturation) were used to examine the effects of a

subchronic dietary (60 day) exposure to 0.2 ng EE2/g and 70 ng EE2/g. We evaluated gene expression patterns in both the liver and ovary and associated these responses to changes in physiological endpoints observed during the critical late stage period in their reproductive cycle. The liver was investigated in this study because it is an important reproductive organ, synthesizing vitellogenin and other proteins that are required for maturing oocytes (Arukwe and Goksoyr 2003; Celius et al. 2000). This is also the main organ for the detoxification of contaminants including estrogenic compounds (Yamamoto et al. 2006). These two primary liver functions may be affected directly by long-term treatments with EE2. The ovary is also a target of endocrine disrupting chemicals (EDCs) since it is involved in oogenesis and in hormone biosynthesis; synthesizing reproductive sex steroids that feedback to the hypothalamus and pituitary to modulate reproduction (Craig et al. 2011; Van den Belt et al. 2003).

We hypothesized that a dietary exposure to EE2 would produce changes in physiological endpoints associated with key genes and biochemical pathways involved in reproduction and development. We used a transcriptomics approach to investigate a wide range of molecular responses in the liver and ovary of female LMB for the higher EE2 concentration and qRT-PCR for select genes at the lower EE2 concentration. We linked these changes with altered biological endpoints caused by exposure to EE2. Transcriptomics approaches have been used to identify specific modes of action associated with biological responses to chemical exposures (Garcia-Reyero et al. 2014; Levi et al. 2009; Miller et al. 2012). Identifying genes and pathways that may result in specific phenotypic alterations in organisms provides valuable insight into the mechanism of action of EE2. Moreover, most studies investigating the effects of EE2 at the molecular level have been conducted in male teleost fish and with waterborne exposures, and there is a lack of data for females. Therefore, in the present study, we investigated the effects of feeding EE2 to female LMB during the critical late stage period of reproductive development.

2. Materials and methods

2.1 Chemicals and reagents

EE2 standard was obtained from Sigma Chemical (St. Louis, Mo, USA); 17 α -ethinylestradiol-d4 standard (chemical purity 98%) was purchased from CDN Isotopes (Quebec, Canada). HPLC-grade methanol, methylene chloride, water and ethyl acetate were purchased from Fisher Scientific (Pittsburgh, PA, USA), as were Optima-grade acetone, acetonitrile and hexane, ACS-grade formic acid, and certified n-butyl chloride. Pyridine was obtained from Acros (Fair Lawn, NJ, USA). N,O-bis(trimethylsilyl)fluoroacetamide (BSTFA) + 1% trimethylchlorosilane (TMCS) was purchased from Pierce (Rockford, IL, USA).

2.2 Fish and dietary exposures to EE2

Adult LMB were purchased from American Sport Fish Hatchery (Montgomery, AL, USA). Fish were acclimated in aerated 150 gallon fiberglass tanks under constant conditions for a minimum of 3 months prior to exposure within the Aquatic Toxicology Laboratory at the University of Florida. LMB are not sexually dimorphic and for this reason, we included

twice as many fish as needed. Sex was determined at the time of sacrifice and confirmed by histology. At the beginning of the experiment, randomly selected LMB were placed into separate tanks for control and treatment with 11–15 fish per tank, for a week before exposure. Flow rates of water were verified daily. The average temperature throughout the experiment was $22.60 \pm \text{SD } 1.41$ °C. All procedures were in accordance with a protocol approved by the University of Florida IACUC. This study assessed the effects of EE2 on females only.

To prepare the diet, a stock solution of 1 mg/ml EE2 (Sigma Chemical Company, St. Louis, MO, USA) was made in 95% ethanol. 490 µl of this solution was mixed with 70 ml menhaden oil (JanDell Fishing Products, Boyd, TX, USA) and then mixed with 7 kg of floating trout pellets (Silvercup, Murray, UT, USA) in a sealed container within a cement mixer, to make a final concentration of 70 ng EE2/g food. In a second experiment, we adjusted the final concentration to 0.2 ng EE2/g food. Control feed was also mixed with menhaden oil. Fish were fed with the EE2 diet once per day for 60 days and bass normally consumed 1% of their average body weight. Food was weighed prior to administration and all that was provided at the time of feed was consumed. The concentration of EE2 in the food was not measured, but the concentrations of EE2 in fish plasma for the 70 ng EE2/g feed group were measured to determine the actual plasma levels in fish.

After 60 days of dietary exposure to EE2, fish were euthanized. Blood was collected from the caudal vein for plasma vitellogenin (VTG), plasma 17β estradiol (E2) and testosterone (T), as well as plasma EE2 measurements. Liver and gonads were dissected, weighed, and then immediately flash frozen in liquid nitrogen. Samples were then stored at -80 °C until the RNA was isolated. A sample of gonad tissue was preserved in 10% buffered formalin for histology and *ex vivo* studies. Only samples from female liver and ovary ($n=6-10$) were used for microarrays. We also checked Vtg levels in male plasma and by qRT-PCR to verify the exposures.

2.3 Histology of LMB ovary

Histological analyses of LMB ovaries were conducted as described previously (Martyniuk et al. 2009a). Briefly, fixed ovarian LMB tissues were embedded in plastic and cut into 4 µm sections and stained with hematoxylin and eosin following standard histological techniques. The LMB ovaries were classified by stage using six ovarian growth categories and atresia as reported previously (Dominguez et al. 2012; Martyniuk et al. 2009a). The six categories considered are category I, primary growth phase/perinucleolar (PGpn); category II, primary growth phase/cortical alveoli (PGca); category III, early vitellogenesis (eVtg); category IV, late vitellogenesis (lVtg); category V, early oocyte maturation (eOM); category VI, late oocyte maturation (lOM), and atresia (AT).

2.4 Ex vivo ovarian cultures

Ex vivo ovarian tissue cultures were performed from EE2-exposed LMB (70 ng EE2/g fish group) to examine gonadotropin (hCG) responsiveness. Ovarian tissues were extracted from control and treated individuals ($n = 4/\text{group}$) and immediately minced into ~25 mg pieces in L-15 medium (phenol red, +L-glutamine, +1% ABAM), and transferred to individual wells

in 24 well plates. Following a 4 h equilibration at room temperature on a rocking platform in the dark, the media was aspirated from wells and fresh medium alone or medium supplemented with 1 U/mL human chorionic gonadotropin was added. Tissues were incubated for an additional 20 hours in the dark on a rocking platform under ambient conditions, then subsequently tissues were snap-frozen in liquid N₂ and stored at -80 °C until processed for RNA.

2.5 GSI, HSI, VTG, and Plasma steroid levels T, E2

Gonadosomatic index (GSI) and the hepatosomatic index (HSI) were calculated for all individuals as gonad weight/absolute body weight x 100; liver weight/total body weight x 100, respectively. Plasma concentrations of VTG using the monoclonal antibody Mab 3G2 were determined by ELISA, as previously described (Denslow et al. 1999; Sepúlveda et al. 2002). Vitellogenin standards ranging from 0.005 µg/ml to 1.0 µg/ml along with positive interassay control were loaded onto an ELISA plate. All assays were performed in triplicate and reported as the mean of three measurements. The coefficient of variation and correlation for the assay were < 10% and >95%, respectively for all samples analyzed. E2 and T measurements were determined in LMB plasma using an ELISA as described by (Martyniuk et al. 2009b).

2.6 Analysis of plasma EE2 levels

In order to determine EE2 concentrations in LMB, plasma samples from the 70 ng EE2/g food group female LMB, as well as EE2 standards prepared in control plasma, were extracted and derivatized for analysis by gas chromatography–mass spectroscopy (GCMS). Control plasma standards were spiked at six known EE2 concentrations before extraction. Deuterated EE2 (10 µg) was added as an internal standard before sample processing to control for extraction efficiency. The method of Li et al. (2005) was modified for the extraction of EE2. Briefly, plasma samples (0.2–0.4 ml in volume) were extracted with 4 ml of n-butyl chloride. After centrifugation for 10 min at 2000 rpm, the upper n-butyl chloride layer was transferred to clean borosilicate tubes and dried under nitrogen at 35 °C. Derivatization of the EE2 extracts was carried out by the addition of 50 µl each of pyridine and N,O-bis(trimethylsilyl)fluoroacetamide (BSTFA) + 1% trimethylchlorosilane (TMCS). After brief vortexing, the mixtures were heated to 60 °C for 30 min, and then dried under nitrogen at 35 °C. Finally, the samples were reconstituted in ethyl acetate for GCMS analysis.

Analysis was conducted using an HP-6890 series gas chromatograph equipped with a ZB-5MS column (Phenomenex Torrance, CA., USA) and an HP-5973 mass selective detector in selected-ion mode. Temperatures for the GC inlet and MS transfer line were 260 °C and 280 °C, respectively. The initial GC oven temperature of 150 °C was maintained for 2 min, followed by temperature ramping of 10°/min until the final temperature of 300 °C was achieved. Monitored ions for EE2 were 425 and 440 (M⁺), while ions 429 and 444 (M⁺) were used to identify EE2-d4 (Li et al. 2005).

2.7. Extraction of total RNA and RNA quality control

Total RNA was extracted from LMB livers and ovaries using RNA STAT-60 reagent (Tel-Test Inc., Friendswood, TX, USA) and DNase-treated with Turbo DNA-free (Ambion Austin, TX, USA). RNA quantity was measured using the NanoDrop ND-1000 (Nanodrop Technologies, Wilmington, DE, USA) and RNA quality was evaluated using the Agilent 2100 BioAnalyzer with the RNA 6000 Nanochip. RNA integrity values (RIN) were > 8.0 for all samples used in the analysis.

2.8. Quantitative real-time PCR (qRT-PCR)

Total RNA was DNase treated using the TURBO DNA-free kit (Ambion, Austin, TX, USA) and cDNA was synthesized using Super-Script[™] II Reverse Transcriptase (Invitrogen). In order to validate the results of the microarray analyses, mRNA levels of the following genes were measured by qRT-PCR: vitellogenin (*VTG*), estrogen receptor alpha (*ESR1*), estrogen receptor beta b (*ESR2*), glutathione S-transferase alpha like (*GST*) and glutathione peroxidase (*GPX*). *ESR1* was measured in both tissues while *ESR2*, *VTG*, *GST* and *GPX* were measured in the liver. Because human homologs are used in this manuscript, all gene names are capitalized. All primers used for these transcripts are provided in Table SI-1. Primers for *VTG*, *ESR1*, *ESR2*, and *GST* and *GPX* were published previously (Martyniuk et al. 2010a; Sabo-Attwood et al. 2004). Absolute quantification was used in order to calculate the number of copies for each gene. Standard curves using cloned plasmids for each gene were generated using seven points in a serial dilution from 1×10^2 through 1×10^9 copies with efficiencies ranging from 93 % to 104 %. Detailed methods for cloning, sequencing, and primer optimization for LMB genes have been previously described (Martyniuk et al. 2010a; Sabo-Attwood et al. 2004). Standards and experimental samples were run in duplicate, along with a “no reverse transcriptase (-RT)” control and a “no template control (NTC)”. Melting curves for each gene indicated a single product was formed. Ribosomal RNA *18S* rRNA was used as an internal reference and it was determined to be an acceptable and stable reference in this experiment (Table SI-2). *18S* rRNA levels were not affected by exposure to EE2 in both tissues studied. In addition, earlier work performed in our laboratory with LMB using *18S* rRNA as a normalizer gene has demonstrated that it does not significantly vary during this specific reproductive time period (Sabo-Attwood et al. 2004). Copy numbers for each gene were normalized to the copy number of ribosomal *18S* rRNA within each individual sample.

2.9. Microarray and Pathway Analysis

Gene expression patterns of the liver and ovarian samples for fish exposed to 70 ng EE2/g feed were investigated using a custom-designed LMB $8 \times 15K$ oligonucleotide microarray manufactured by Agilent (Palo Alto, CA, USA). Four biological replicates for control and EE2-treated liver and ovary were used for microarray analysis. Microarray hybridizations were conducted according to the Agilent One-Color Microarray-Based Gene Expression Analysis protocol using Cyanine 3 (Cy3) (Agilent, Palo Alto, CA, USA). Microarrays were kept in the dark until scanning using an Agilent G2505B microarray scanner. Data extraction was performed using Agilent Feature Extraction software (v 9.5). Raw expression data were imported into JMP Genomics v5 (SAS, Cary, NC, USA). Data were log2-

transformed and all control and non-uniform spots were removed prior to the final analysis. Raw intensity data for each microarray was normalized by median centering before performing One-Way ANOVA ($p < 0.05$; fold change greater than ± 1.5). The data was checked for a false discovery rate (FDR) using the method of Benjamin and Hochberg et al. (1995). For pathway analysis, a false discovery rate was not applied. All microarrays were submitted to NCBI's Gene Expression Omnibus (GEO) database (GSE28143).

Pathway Studio V9 (Elsevier, Inc., Rockville, MD, USA) operating with the ResNet 9.0 database was used to obtain further insight into pathways affected by dietary exposure to EE2. Enrichment analysis in Pathway Studio V9 was performed by gene set enrichment analysis (GSEA) and sub-network enrichment analysis (SNEA) algorithms using the Mann-Whitney test with an alpha level of $p < 0.05$ (Kotelnikova et al. 2012; Sivachenko et al. 2007; Sivachenko et al. 2005; Subramanian et al. 2005). Recently these approaches have been applied to studies conducted in LMB (Doyle et al. 2013; Martyniuk et al. 2013). GSEA is an analytical method used to evaluate microarray data at the level of gene sets. These gene sets are defined based on prior biological knowledge of gene ontology and curated pathways. The Ariadne's Curated Reference Pathway Collection included cell process and metabolic pathways and gene ontology categories for biological processes and molecular functions.

2.10 Statistical Analyses

Differences in GSI, HSI, plasma VTG and plasma steroids (T, E2), plasma EE2 and gene expression data among treatment groups were detected using One-Way Analysis of Variance (ANOVA). When data were not normally distributed, the Kruskal–Wallis non-parametric test followed by the Mann-Whitney U-test was used to determine whether there were significant differences ($p < 0.05$) in mRNA levels between control and treatment groups. All analyses were performed using Prism 6 (GraphPad Software Inc., San Diego CA).

3. Results

3.1 EE2 plasma analysis

EE2 was detectable in the blood of treated fish after 2 months exposure to 70 ng EE2/g feed. The average of the measured concentrations of EE2 in blood plasma was 260 ng/ml (0.8 μ M) whereas EE2 was not detectable in the control samples (Fig. 1). We used four of the fish for our microarray analysis with plasma EE2 values spanning the full range. Plasma concentrations in fish treated with lower EE2 was not attempted, as these concentrations would likely be below the limit of detection.

3.2 Effects of dietary EE2 on female LMB reproduction: body weight, GSI, LSI and plasma VTG

In fish treated with the 70 ng EE2/g feed, there were no significant differences detected in body weight (d.f. = 14; $t = 1.62$; $p = 0.12$). However, there was a significant reduction of ~40% in GSI (d.f. = 14; $t = 2.3$; $p = 0.037$) between control fish and the dietary EE2 group. Moreover, in this EE2 group, both HSI (d.f. = 14; $t = 4.19$; $P < 0.001$) and VTG plasma levels (d.f. = 14; $H = 5.00$; $p = 0.0047$) were significantly decreased by 38.5 % and 70.2 %, respectively compared to controls (Fig. 2).

In the follow up experiment, fish were treated with a super low concentration of EE2, 0.2 ng EE2/g feed. Again there were no significant differences in body weight, GSI or plasma VTG; however, there was a statistically significant increase in HSI for females only. This data is summarized in Table 1.

3.3 Ovarian Histology: Gonad regression

Histology revealed that the ovaries from fish treated with the higher concentration of EE2 showed a higher number of atretic follicles than control fish (Fig. 3) and contained oocytes in earlier stages of development (primary growth). At the initiation of the experiment, all fish were presumed to be in the late stages of vitellogenesis, based on the water temperature and month (March) (Martyniuk et al. 2013). These data suggest that the long term feeding of EE2 may cause a shutdown of steroidogenesis and oocyte growth in the ovary. This is supported by the decreases in GSI and *ex vivo* production of E2 (described below). Fish treated with the lower concentration of EE2 (0.2 ng EE2/g/day) showed no differences in gonadal histology compared to controls.

3.4 Sex steroid and Ex vivo ovarian cultures

There were no significant differences detected in plasma E2 (d.f. = 6; H = 5.0; p = 0.49) and T (d.f. = 6; H = 5.0; p = 0.49) levels between control fish and the 70 ng EE2/g feed dietary EE2 group (Figure 3). However, concentrations of E2 in media from an ovarian tissue culture were significantly reduced in the treated group compared to control (d.f. = 6; H = 11.5; p = 0.009), but not for T (d.f. = 6; H = 1.9; p = 0.62) (Fig. 4).

3.5 Gene expression changes in liver and ovary by dietary EE2

Microarray results for the fish treated with 70 ng EE2/g feed showed that 1897 transcripts were significantly affected in the liver (973 increased and 924 reduced) and 738 genes in the ovary (522 increased and 216 reduced) by dietary exposure to EE2. Using a false discovery rate (FDR) of 5%, the number of transcripts altered in the liver and in the gonad was 199 and 21, respectively. As expected, gene expression profiles were specific for individual tissues.

In both tissues, genes related to the reproductive pathway were affected, but the altered genes associated with this pathway were specific to each tissue. Hepatic altered genes involved in reproduction included vitellogenin (*VTG*), encoding the egg yolk precursor protein, and nothepsin (*nots*), encoding a cathepsin like protease. Within the gonad, gene expression included transcripts such as cytochrome P450, family 19 (*CYP19A*) and insulin-like growth factor 1 (*IGF-1*). Also, identified in both tissues were significant changes in expression levels of estrogen receptor 1 (*ESR1*). In addition, a set of genes related with oxidative stress were significantly altered in the liver such as glutathione reductase, glutathione peroxidase 1a and caspase 3. In the gonad, genes associated with the immune system such as complement component 1, myostatin and macrophage-inducible C-type lectin, among others were over-represented. All gene expression data for significantly expressed transcripts are shown in Table SI-3, without FDR correction.

3.6 Pathway analysis

Subnetwork Enrichment Analysis (SNEA) and Gene Ontology (GO) analysis to determine pathways that are altered by dietary EE2 in the liver and the ovary

1) Regulated pathways that are similar for both the liver and ovary: SNEA showed that many of the sets of genes were altered in both the liver and the ovary which are involved in reproduction and development, both processes which are known to be regulated by estrogens. These included vitellogenesis, female gonad development, follicle-stimulating hormone secretion and sperm entry. In addition, other sets of altered genes were associated with hormone biosynthesis (*e.g.* low density lipoprotein (LDL) oxidation and hormone metabolism). The common GO terms identified for the full set of regulated genes included “Response to estradiol stimulus” and “Hormone activity,” matching the SNEA selected pathways. In both tissues, there were also common cell processes and GO terms that are indirectly associated with estrogens. These included kidney filtration, fibrinolysis, mesenchymal to epithelial transition, among others (see Table 2). For biological processes, these included, positive regulation of tyrosine phosphorylation of Stat3 protein, activation of JUN kinase activity, inositol or phosphatidylinositol kinase activity, drug metabolic process, synaptic vesicle exocytosis, branching morphogenesis of a tube, negative regulation of DNA binding, among others. Molecular functions included hormone activity and Hsp90 protein binding (Table 3).

2) Tissue-specific pathways: As one would expect, there were pathways that were specific for each of the tissues. Some of these were also related to reproduction, for example, in the liver, we identified sex maturation, fertilization and reproduction; and in the ovary, we identified ovary function, oocyte development, and reproductive behavior.

Additional processes and GO terms that were specific to the liver included xenobiotic metabolic process, negative regulation of DNA replication, ribosome biogenesis, RNA splicing, sphingolipid biosynthetic process, I-kappa B phosphorylation, glutathione peroxidase activity, SMAD binding, microfilament motor activity, and calcium-dependent phospholipid binding, among others (Table S1–4).

In the ovary, specific altered processes included androgen metabolic processes, chromatin modification, cerebral cortex radially oriented cell migration, neurotransmitter binding, motor activity and GABA-A receptor binding, among others. The complete list of SNEA results for liver and ovary are presented in (Table SI-5).

Gene Set Enrichment Analysis (GSEA)—GSEA showed additional pathways influenced by dietary EE2. As above, some pathways were common to both tissues and some were specific. The pathway that was most likely changed in the liver by the exposure was ROS metabolism (p-value = 0.002). This pathway was changed overall by 1.28-fold with 16 different elements within the pathway changed. A representative component of the pathway involving glutathione peroxidase is seen in Fig. 5. The most likely changed pathway for the ovary was “Classical complement pathway” (p-value = 0.00002) with an overall fold change of 1.6 and eleven different elements. A representative pathway involving

the classical complement pathway is seen in Fig. 6. Thus we are focusing our discussion on these two pathways. This information is also included in detail in Table SI-4.

3.7 qRT-PCR analysis

There is a favorable concordance between microarray and qRT-PCR. Genes implicated in oxidative stress such as *GST* and *GPX* were investigated due to their presence in GO terms and many of the sub-networks. The qRT-PCR results showed that hepatic *GST* mRNA expression was significantly increased by the EE2 exposure compared to controls (d.f. = 6; $H = 0.00$; $p = 0.029$) while *GPX* mRNA levels were significantly decreased (d.f. = 6; $H = 0.00$; $p = 0.029$), indicating that there may be an oxidative response in the liver (Fig. 7A and Fig. 7B) with the EE2 treatment. Both genes were significantly affected in the microarray. However, the *GPX* probe on the array did not permit its grouping into a particular class. In contrast, qRT-PCR analysis showed no significant changes in mRNA levels for *ESR1* and *ESR2* in the liver and no significant changes for *ESR1* in the gonad for the high treatment (Fig 7C). We observed that there was a high variability in the expression for *ESR1*, *ESR2*, in the control fish which may be related to their cyclic seasonal regulation (Sabo-Attwood et al. 2004). This may have resulted in low power for qRT-PCR to detect changes in *ESR1* and *ESR2* mRNA since qRT-PCR involves geometric amplification for quantification and a small variability at the start of the process results in a large variance. Microarray analysis, on the other hand, involves linear amplification of the starting RNA, thereby more closely resembling the actual concentration in tissues. *VTG* trended towards reduction but this was not significant for *VTG* in agreement with the *VTGB* probe on the arrays (Fig 7D; Table SI-3). We did not test other *VTG* genes by qRT-PCR.

In the follow up study with 0.2 ng EE2/g feed, the same transcripts were measured by a focused qRT-PCR approach (Fig. 8). There were significant decreases in *VTG* mRNA and *ESR1*, matching the trend seen for the higher concentration of EE2. We also saw a trend for increased expression for *GST* and *GPX*.

Discussion

4.1. Reproductive effects in female LMB after EE2 feeding

EE2 is a potent estrogen that has been detected at low concentration (0.2 – 5 ng/L) in aquatic systems (review by Aris et al. 2014; Caldwell et al. 2008). Most studies performed with EE2 have been in aqueous exposures and only a few studies have focused on dietary exposures (Angus et al. 2005; Cabas et al. 2011; Shved et al. 2007). However, both routes of exposure can lead to adverse effect on development and reproduction (Angus et al. 2005; Balch et al. 2004; Cabas et al. 2011; Länge 2001; Nash et al. 2004; Parrott and Blunt 2005; Shved et al. 2007).

In this study, we performed two feeding experiments with LMB; the first one was to a relatively high concentration of EE2 (70 ng EE2/g feed) and the second to a much lower concentration (0.2 ng EE2/g feed) mimicking what the bass would see in the environment. The second experiment was performed to confirm the findings of the first. For both studies, we observed that there was a negative effect of EE2 exposure on several reproductive

parameters when administered at later stages of reproduction. GSI of fish treated with the 70 ng EE2/g feed was significantly decreased and showed a decreasing trend for the 0.2 ng EE2/g feed exposure. These results correspond well with two previous studies that showed that dietary EE2 (1 – 200 µg EE2/g) resulted in a significant decrease in GSI in gilthead seabream and mosquitofish, respectively (Angus et al. 2005; Cabas et al. 2011).

Other physiological endpoints such as the reduction in plasma VTG were similar in the two treatments, significantly different for the higher concentration of EE2, and trending towards diminution in the lower concentration fish. This is counter to what is found in the early stages of reproduction where VTG levels are increased in females (Doyle et al. 2013; Hoffmann et al. 2006; Van den Belt et al. 2003).

Interestingly, the HSI was significantly increased in the fish treated with the lower concentration, suggesting increased hepatic activity, but significantly decreased in the fish treated with the higher concentration, possibly suggesting diminished activity of liver in this group, for example this may be related to the reduction in VTG output as well as albumin and other lipoproteins that are known to be regulated by estrogen. This result is consistent with studies with long term use of dietary EE2 exposures in female rats, which resulted in liver reduction (Kato et al. 2004). Clearly EE2 changes expression of many genes in all vertebrates.

We also observed alterations in the histology of the ovaries for the high treatment, with evidence of increased atresia in the ovaries of fish treated with 70 ng EE2/g feed. This agrees with other studies in fish that have shown increase of ovarian follicle atresia after EE2 exposure (Kidd et al. 2007; Länge 2001; Van den Belt et al. 2003). There was no observable change in ovary stages or in GSI of fish treated with the 0.2 ng EE/g exposure. This lower EE2 treatment is in the low environmental range.

We did not detect significant decreases in plasma levels of E2 and T in fish treated with the higher EE2 amount and this may be due to the high individual variability in these endpoints. However, E2 in the media of oocyte cultures was significantly decreased in LMB fed 70 ng EE2/g feed but not T, suggesting a direct effect on aromatase activity. Interestingly, the mRNA for aromatase (*CYP19A*) was up-regulated in the ovary, which would go along with a compensatory change for that mRNA due to the inactivity of protein (Ankley et al. 2002; Villeneuve et al. 2009). At first look, the up regulation of *CYP19* in the gonad seemed backward since ex-vivo cultures showed depression of E2 synthesis but not of T synthesis. However, it is possible that somehow the EE2 interferes with the activity of the enzyme and the homeostatic response to this interference is to up-regulate the expression of the mRNA in order to make more protein. This type of response has already been documented for fish, in elegant studies performed with fadrozole, an inhibitor of aromatase activity (Villeneuve et al. 2009).

In addition, EE2 treatment may impair steroidogenesis, in general, via a negative feedback inhibition of the hypothalamus, especially for the high EE2 treatment (Van den Belt et al. 2003). This has been observed in other studies too. For example, in a zebrafish study with

aqueous exposure to EE2 (3.5 – 25 ng/L), reduction of GSI and concomitant changes in ovarian histopathology were attributed to a negative feedback effect on the HPG axis.

For chemical analysis of surface waters, it is typical to remove particulate matter (including algae, bacteria, detritus) before sorbing chemicals to hydrophobic matrices, and thus these methods only analyze the freely dissolved portion of EE2 (Chen et al. 2008; LaFleur and Schug 2011; Noppe et al. 2005; Wang et al. 2012; Zuehlke et al. 2004). These methods may underestimate the amount of EE2 in the environment. The aqueous solubility and Kow of EE2 suggests that the chemical might be adsorbed on suspended particulate matter and/or to sediments (review by Aris et al. 2014; Lai et al. 2002; Pojana et al. 2007) and can be assimilated in fish through the food chain (Al-Ansari et al. 2010; Aris et al. 2014; Lai et al. 2002). Our results quantifying EE2 levels in plasma of the fish treated with the higher level of EE2 showed that it is present at relatively high levels after 60 days and bioaccumulates rather than being quickly metabolized. All our fish were equally exposed to the dietary EE2, but plasma levels varied either because the fish consumed different amounts of EE2-laced food or metabolized it differently. Nevertheless, the gene expression patterns associated with different plasma levels of EE2 did not vary, suggesting that for gene expression even the lowest value already caused maximal effect.

Hierarchical clustering of the gene expression data for the high treatment group showed no correlation to the level of EE2 in the plasma of the fish, suggesting that tissue concentrations were probably similar despite plasma levels being somewhat different among the fish. The average value detected of EE2 in the plasma (260 ng/ml) in this study is higher than the range (60 to 90 ng/ml) reported by Skillman et al. (2006) for rainbow trout from water exposure at 125 ng EE2/L for 61 d, indicating that the first diet contributed a very high dose of EE2 to the fish and that it was not quickly excreted or metabolized. Studies by others suggest that EE2 is slowly metabolized and bioaccumulates in fish tissue (Al-Ansari et al. 2010; Aris et al. 2014; Lai et al. 2002; Larsson et al. 1999; Tyler et al. 1998). Nevertheless, the low EE2 treatment showed some of the same changes in gene expression as were seen with the high treatment, suggesting that these changes are environmentally relevant.

4.2 Molecular pathways affected by EE2 (high dose) in the liver and ovary

4.2.1. EE2 in the feed affects reproduction at multiple levels of biological organization—Microarray analysis suggested that there were major molecular themes affected in the liver and ovary. One theme was related to a direct negative effect on reproduction in both tissues. Other major themes are related with stress and chronic toxicity, as well as the immune system. Global bioinformatics methods confirm that there were key processes impacted by EE2 that are related to endocrine disruption. For example, cell processes that included follicle-stimulating hormone secretion, vitellogenesis and female gonad development were identified as altered in both tissues with SNEA (Table 1), and this indicates that dietary EE2 exposure may compromise LMB reproduction. This is in agreement with other studies that emphasize that reproduction is the main target by EE2 (Caldwell et al. 2008; Fenske et al. 2005; Nash et al. 2004).

The most straight forward interpretation of the data is that in addition to direct effects from EE2 on the liver and ovary, the high dose of EE2 also initiated negative feedback on the

HPG axis and that some of the phenotypic changes (decreased E2 production *ex vivo* in ovaries as well as diminished GSI) we saw were due to that mechanism of action. Feedback inhibition of the HPG axis by high levels of estrogens has been documented in a number of studies (Harding et al. 2013; Hoffmann et al. 2006; Hogan et al. 2010; Kato et al. 2004; Van den Belt et al. 2003).

4.2.2. EE2 affects pathways associated with a stress response—GSEA was used to identify known pathways or gene ontologies that were significantly affected by EE2 exposure. This bioinformatic analysis suggested that the oxidative stress response in liver was increased by the treatment. There are several studies that suggest that E2 can directly affect mitochondrial function and produce reactive oxygen species (ROS) which may also come into play (reviewed by Felty and Roy 2005). Our results suggest that the high dose of EE2 produced an oxidative stress response at the level of the transcriptome. This is supported by the observation of the altered glutathione peroxidase activity pathway in the liver of female LMB (Fig. 5), which is related to ROS production and activation of the oxidative stress and antioxidant systems. This is in agreement with the significant changes observed for hepatic *GST* mRNA levels which were up regulated in the experiment with the high dose, but not at the low dose. This may be associated with a compensatory response of the fish to protect against lipid peroxidation and ROS production by the high dose of EE2. *GPX* expression has been used as an indicator of the antioxidant status of organisms and serves as a biomarker of oxidative stress in response to pollutants (Valavanidis et al. 2006). It is well known that increases in ROS production leads to apoptosis and activation of signaling pathways that elicit changes in gene expression, particularly transcription factors (Apel and Hirt 2004; Klaunig et al. 2001). EE2-induced oxidative stress has been widely reported in several organisms, and can be associated with adverse effects in reproduction (Aris et al. 2014; Brown et al. 2004; Nadzialek and Kestemont 2010).

4.2.3. EE2 alters pathways associated with the immune system—Our results suggest that the high concentration of EE2 might be altering the immune system in the gonad such as the inflammatory response, T-cell response, and humoral immune response (Fig. 6). Cabas et al. (2011) demonstrated that dietary exposures with EE2 (range 5–200 µg EE2/g feed) for 28 days induced immune responses that included inflammation and infiltration of acidophilic granulocytes and B lymphocytes in the testis of fish, which resulted in disruption of spermatogenesis. Endocrine disrupting chemicals, such as EE2 can produce more than one effect on several physiological systems that go beyond the reproductive system, including for example the immune response (Ahmed 2000; Jin et al. 2010). In this study, GSEA suggested that in the ovary, pathways associated with the immune system such as the classical complement pathway and the lectin-induced complement pathway were significantly affected by EE2. The classical complement and the lectin-induced complement pathways are critical for activation of the complement system during innate immunity. Both of these pathways play a vital role of defense against infections (Brown et al. 2002; Celik et al. 2001).

Estrogens such as E2 and EE2 have been shown by others to alter genes involved in the inflammatory and humoral immune responses in other fish. In our study, C1R, C1S, C3, C5,

C7 and C9, were altered and these have been associated with the inflammatory and humoral immune responses in fish. Similar hepatic transcriptomics results were observed for carp exposed to a variety of estrogenic chemicals (E2, EE2, nonylphenol and bisphenol A) in the water (Moens et al. 2007) and for flounder exposed to a high dose of E2 by injection (10 mg/Kg) (Williams et al. 2007). Moreover, a dietary exposure of rainbow trout to E2 (20 ug E2/g dry food) for 14 days altered the expression of a wide number of genes in the liver involved in GO categories associated with immune functions like immune response, humoral immune response, complement activation, among others (Casanova-Nakayama et al. 2011).

A review by Milla et al. (2011) suggested that there are extensive interactions between sex-steroid pathways and the immune system, and a number of genes that encode for immune proteins are also co-expressed with those that encode for sex-steroid receptors (lectins lysozyme, hepcidin and complement component 3). In addition, other studies have shown that EE2 modulated gonadal genes related to cytokines, chemokines and adhesion molecules (Cabas et al. 2011; Cabas et al. 2012). Lastly, E2 has been shown to regulate phagocyte responses through macrophage activation in the gonad of seabream (Liarte et al. 2011).

In summary, in our study sub-chronic exposure of LMB to EE2 altered pathways related with ROS in the liver and with the immune system in the gonad, responses that are consistent with previous transcriptomics studies.

Conclusions

Reproductive endpoints were in general depressed with feeding EE2 to female LMB, suggesting feedback inhibition of the HPG axis. There were two common molecular themes that were affected by EE2 in the liver and gonad that were identified using a transcriptomics approach. One theme was represented by changes in pathways that were associated with reproductive impairment in both the liver and the ovary. This reduction in reproductive potential follows the adverse outcome pathway for plasma VTG reduction in female fish, first proposed by Ankley et al. (2009). A second theme suggests chronic toxicity by EE2 that is manifested by increased oxidative stress in the liver (as exemplified by the changes in the glutathione peroxidase activity pathway and ROS) and in the ovary, by inflammation and atresia. These later gene changes are anchored by the morphological changes seen in the ovary via histopathology. Taken together, this study provides new insights into how dietary exposure to EE2 interferes with the endocrine signaling pathways in female LMB during a critical period of reproductive maturation.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This research was funded by the Superfund Research Program from the National Institute of Environmental Health Sciences, RO1 ES015449, awarded to NDD and DSB and to the postdoctoral fellowship (124367) from CONACYT awarded to RCCD.

Abbreviations

GPX4	glutathione peroxidase 4 (phospholipid hydroperoxidase)
GPX3	glutathione peroxidase 3 (plasma)
ALOX5	arachidonate 5-lipoxygenase
TXN	thioredoxin
HDL	high-density lipoprotein
CASP3	caspase 3, apoptosis-related cysteine peptidase
CAT	catalase
MGST3	microsomal glutathione S-transferase 3
GPX1	glutathione peroxidase 1
BAX	BCL2-associated X protein
IL6	interleukin 6 (interferon, beta 2)
GPX2	glutathione peroxidase 2 (gastrointestinal)
MAPK14	mitogen-activated protein kinase 14
AKT1	v-akt murine thymoma viral oncogene homolog 1
PRDX6	peroxiredoxin 6
GSTK1	glutathione S-transferase kappa 1
GSTT1	glutathione S-transferase theta 1
GPX7	glutathione peroxidase 7
C1R	Complement Component 1, R Subcomponent
C1S	Complement Component 1, S Subcomponent
C2	Complement Component 2
C4B	Complement Component 4B
C3	Complement Component 3
C5	Complement Component 5
C6	Complement Component 6
C7	Complement Component 7
C8A	Complement Component 8, Alpha Polypeptide
C9	Complement Component 9
C3AR1	Complement Component 3a Receptor 1
GPR77	G protein-coupled receptor 77, C5AR1, Complement Component 5a Receptor 1
IgM	Immunoglobulin Heavy Constant Mu

IgG3	Immunoglobulin Heavy Constant Gamma 3 (G3m Marker)
Ig1	Indeterminate gametophyte 1
Ca2+	calcium ion

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Highlights

Dietary exposure of female bass to EE2 decreased vitellogenin mRNA and protein

Dietary EE2 reduced GSI and ex vivo synthesis of E2 and increased ovarian atresia

EE2 altered expression of genes for oxidative stress and the innate immune response

Figure 1.

Plasma concentrations of EE2 (scatter plot) with median values (horizontal black line within scatter plot) are plotted. Analyses were performed using Prism 6.0.

Figure 2.

Morphometric and physiological endpoints in female LMB exposed to 70 ng EE2/g feed. (A) Body Weight, (B) Gonadosomatic index (GSI), (C) Liver Somatic index (LSI) and (D) plasma vitellogenin (Vtg). Box plots indicate the upper and lower percentile (10–90th) and the median values (horizontal black line within box). Whiskers represent the highest and lowest points. Different letters indicate significant differences among groups. Analyses were performed using Prism 6.0.

Figure 3.

Histological micrographs of the female LMB ovary after sub-chronic feeding (60 days) with control and EE2-laced diets. Ovaries from control females (A) were characterized as late vitellogenesis/maturation with some evidence of atresia. The ovaries from the dietary EE2 treatment (B) showed more atretic follicles than the control fish. The scale bar corresponds to 500 µm. Abbreviations are as follows: Primary growth perinuclear (PGpn), Primary growth cortical alveoli (PGca), atresia (AT), germinal vesicle (GV), lipoprotein yolk (LY).

Figure 4.

Plasma hormone concentrations and *ex vivo* production of sex steroids from minced ovarian tissues of fish exposed to 70 ng EE2/g feed. Sex steroid plasma levels (A) 17 β -estradiol (E2) and (B) testosterone (T). *Ex vivo* production of (C) E2 and (D) T by ovarian tissue from adult female LMB from either control or EE2 fed groups (n=4). Box plots indicate the upper and lower percentile (10–90th) and the median values (horizontal black line within box). Whiskers represent the highest and lowest points. Different letters indicate significant differences among groups.

Figure 5.

Glutathione peroxidase activity pathway which was significantly altered in the liver of female LMB, obtained from GSEA, featuring GO terms. Blue color indicates down-regulation, and red indicates up regulation of gene expression; color intensity indicates the degree of response, grey indicates the potential processes likely affected, and purple represents potential diseases associated with gene changes. The abbreviations of the genes are listed in the abbreviations list.

Figure 6. Classical Complement Pathway affected in the ovary of female LMB (by gene set enrichment analysis (GSEA)).

Figure 7.

qRT-PCR validation of genes involved in stress for the 70 ng EE2/g feed treatment. (A) Glutathione-S-Transferase (*GST*); (B) Glutathione Peroxidase (*GPX*); Vitellogenin (*VTG*) and Estrogen receptor 1 (*ESR1*). Horizontal line in box plots is the median, the boundaries of the box represent the 10th and 90th percentiles and the minimum and maximum data points are represented by the whiskers. Differences among groups ($p < 0.05$) are denoted with different letters. Data were not normally distributed (Wilcoxon rank sum test) and a non-parametric Mann-Whitney test was used to determine if there were significant differences ($p < 0.05$) between control and treatment.

Figure 8. qRT-PCR of genes in the liver for the 0.2 ng EE2/g feed treatment. (A) *VTG*; (B) *ESR1*; (C) *GST*; and (D) *GPX*. Horizontal line in box plots is the median, the boundaries of the box represent the 10th and 90th percentiles and the minimum and maximum data points are represented by the whiskers. Differences among groups ($p < 0.05$) are denoted with different letters.

Table 1

Morphometric and physiological endpoints in female LMB treated with the 0.2 ng EE2/g feed.

Biological endpoints **	ng EE2/g			
	Cntrl-M Mean(±SE)	0.2 EE2 -M Mean(±SE)	Cntrl-F Mean(±SE)	0.2 EE2-F Mean(±SE)
Body Weight	332.0 ± 38	252.0 ± 20	282 ± 24	269 ± 35
GSI	0.61 ± 0.4	0.47 ± 0.02	2.3 ± 0.25	1.8 ± 0.38
HSI	0.78 ± 0.05	0.91 ± 0.11	0.83 ± 0.06	1.2 ± 0.11 *
Plasma VTG (mg/ml)	0.52 ± 1	0.56 ± 0.20	2.6 ± 0.34	2.1 ± 0.53

* Significantly affected

** Males (M), n= 9 for controls and 9 for treatment; Females (F), n=6 for controls and 7 for treatment.

Common Sub-networks significantly affected in both the liver and ovary of female LMB exposed to dietary EE2.

Table 2

Theme	Common Cell Processes Gene Set Seed	Liver		Ovary	
		Median fold change	p-value	Median fold change	p-value
Reproduction	vitellogenesis	1.31	0.0003	1.33	0.0065
	Female gonad development	-1.05	0.0090	1.22	0.0258
	follicle-stimulating hormone secretion	1.12	0.0252	1.41	0.0162
Hormone Biosynthesis	sperm entry	1.17	0.0497	1.28	0.0059
	low density lipoprotein (LDL) oxidation	1.44	0.0329	1.20	0.0091
	hormone metabolism	-1.29	0.0335	1.33	0.0121
Blood	menarche	1.14	0.0114	1.57	0.0003
	blood vessel contraction	1.17	0.0098	1.11	0.0140
	hemolysis	1.26	0.0372	1.14	0.0028
Other	kidney filtration	1.36	0.0272	1.19	0.0211
	fibrinolysis	1.13	0.0313	1.14	0.0142
	mesenchymal to epithelial transition	1.12	0.0380	1.21	0.0229
	blastocyst development	-1.28	0.0438	1.33	0.0007
	histone acetylation	1.07	0.0452	1.29	0.0206

Table 3

Common Gene Ontology processes over-represented in the liver and ovary of dietary exposure to EE2 in female LMB (p-value <0.05).

Gene Ontology		Liver		Ovary	
Gene Set Category	Common GO Terms	Median Fold Change	p-value	Median Fold Change	p-value
Biological Process	Response to estradiol stimulus	-1.02	0.0055	1.17	0.0005
Molecular Function	Hormone activity	-1.19	0.0133	1.19	0.0005
Molecular Function	Oxidoreductase activity, acting on paired donors, with incorporation or reduction of molecular oxygen	1.47	0.0013	1.34	0.0121
Biological Process	Microtubule-based movement	-1.30	0.0026	-1.09	0.0409
Biological Process	Inositol or phosphatidylinositol kinase activity	1.21	0.0029	1.18	0.0249
Molecular Function	Monooxygenase activity	1.21	0.0029	1.18	0.0249
Biological Process	Positive regulation of tyrosine phosphorylation of Stat3 protein	1.95	0.0032	1.41	0.0131
Biological Process	Synapse organization	1.30	0.0071	1.27	0.0307
Biological Process	Cell growth	1.04	0.0150	1.16	0.0059
Biological Process	Drug metabolic process	1.56	0.0168	1.23	0.0208
Biological Process	Negative regulation of DNA binding	1.24	0.0195	1.11	0.0337
Biological Process	Induction of apoptosis by oxidative stress	-1.34	0.0247	-1.29	0.0326
Biological Process	Response to vitamin D	-1.19	0.0259	1.22	0.0165
Biological Process	Branching morphogenesis of a tube	1.36	0.0275	1.18	0.0073
Biological Process	Synaptic vesicle exocytosis	1.46	0.0299	1.30	0.0038
Biological Process	Sertoli cell proliferation	-1.46	0.0313	1.29	0.0283
Biological Process	Dibenzo-p-dioxin metabolic process	1.52	0.0321	1.23	0.0139
Biological Process	Phosphatidylinositol phosphorylation	1.16	0.0394	1.23	0.0163
Biological Process	Activation of JUN kinase activity	1.35	0.0411	1.33	0.0173
Molecular Function	Hsp90 protein binding	1.25	0.0412	-1.28	0.0341