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Short-term treatment of adult male zebrafish (*Danio Rerio*) with 17α-ethinyl estradiol affects the transcription of genes involved in development and male sex differentiation

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ABSTRACT

The synthetic estrogen 17α-ethinyl estradiol (EE_2) disturbs reproduction and causes gonadal malformation in fish. Effects on the transcription of genes involved in gonad development and function that could serve as sensitive biomarkers of reproductive effects in the field is, however, not well known. We have studied mRNA expression in testes and liver of adult zebrafish (*Danio rerio*) males treated with 0, 5 or 25 ng/L $EE_2$for 14 days. qPCR analysis showed that the mRNA expression of four genes linked to zebrafish male sex determination and differentiation, *Anti-Mullerian Hormone*, *Double sex and mab-related protein*, *Sry-related HMG box-9a* and *Nuclear receptor subfamily 5 group number 1b* were significantly decreased by 25 ng/L, but not 5 ng/L EE_2 compared with the levels in untreated fish. The decreased transcription was correlated with a previously shown spawning failure in these males (Reyhanian et al., 2011. Aquat Toxicol 105, 41–48), suggesting that decreased mRNA expression of genes regulating male sexual function could be involved in the functional sterility. The mRNA level of *Cytochrome P-45019a*, involved in female reproductive development, was unaffected by hormone treatment. The transcription of the female-specific *Vitellogenin* was significantly induced in testes. While testicular *Androgen Receptor* and the *Estrogen Receptor-alpha* mRNA levels were unchanged, *Estrogen receptor-beta* was significantly decreased by 25 ng/L EE_2. Hepatic *Estrogen Receptor-alpha* mRNA was significantly increased by both exposure concentrations, while *Estrogen Receptor-beta* transcription was unaltered. The decreased transcription of male-predominant genes supports a demasculinization of testes by EE_2 and might reflect reproductive disturbances in the environment.

1. Introduction

Endocrine disrupting chemicals (EDC), precipitated in the aquatic environment from municipal sewage, industry and agricultural sources, represent a health concern for fish and other water-living organisms (Vos et al., 2000; Segner et al., 2003). The synthetic estrogen 17α-ethinyl estradiol (EE_2), commonly used in contraceptive pills and hormone replacement therapy, is frequently found in effluents from sewage treatment plants (STP) and surface waters in concentrations from less than 1 ng/L up to the 200-300 ng/L range (Kolpin et al., 2002; Hannah et al., 2009; Laurenson et al., 2014).

The reproductive organs have been regarded as the main targets for estrogenic EDC, causing alterations in reproductive capability and physiology. Affected end points in adult zebrafish (*Danio rerio*) include morphological changes in gonads (Van den Belt et al., 2002, 2003), reduction of fecundity and fertilization success (Santos et al., 2007), retardation of germ cell maturation (Silva et al., 2012), reduced gonadosomatic index (Van den Belt et al., 2004; Versonnen and Janssen, 2004), as well as expression of the egg yolk precursor protein Vitellogenin (Vtg) in male liver (Tyler et al., 1996; Cheek et al., 2001; Henry et al., 2009; Chen et al., 2010). Studies on developing embryos and larvae demonstrate high sensitivity to EE_2 exposure (Van den Belt et al., 2002; Hill and Janz, 2003; Weber et al., 2003; Cardinali et al., 2004; Maack and Segner, 2004; Nash et al., 2004). While effects on adults are essentially reversible (Van den Belt et al., 2002; Maack and

Abbreviations: AMH, Anti-Mullerian Hormone; AR, Androgen Receptor; Cyp19a, Cytochrome p-45019a; Dmrt1, double sex and mab-related protein; Dmy, master sex determination gene of the Y chromosome; EDC, endocrine disrupting chemical; ER-α, Estrogen Receptor alpha; ER-β, Estrogen Receptor beta; E_2, estrogen; EE_2, 17α-ethinyl estradiol; NR5a1b, Nuclear Receptor subfamily 5 group number 1b; qPCR, quantitative real time polymerase chain reaction; Sox9a, Sry-related HMG box-9; SRY, sex determining region Y; STP, sewage treatment plant; Vtg, Vitellogenin; Vtg2, Vitellogenin splice variant 2; 18s RNA, 18s small ribosomal unit RNA.

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Segner, 2004), exposure during development often leads to irreversible effects on reproductive parameters such as gonad morphology, fertility and fecundity (Van den Belt et al., 2003; Fenske et al., 2005). Other treatment effects, such as decreased body weight, arrested gonadal development and elevated Vtg levels may be partly recovered from, yet the reversibility depends on timing, duration and concentration of EE_2 exposure (Van den Belt et al., 2002; Maack and Segner, 2004; Schäfers et al., 2007; Larsen et al., 2008; Xu et al., 2008a).

EE_2 is capable of altering zebrafish gonad development and sex differentiation, and even cause sex reversal of males (Örn et al., 2003; Weber et al., 2003; Brion et al., 2004). In zebrafish all embryos develop an immature ovary-like gonad prior to sex differentiation. The immature gonad undergoes a transition stage where it starts to develop into either testis or ovary (Orban et al., 2009). The genetic mechanism behind gonadal differentiation and sex determination in zebrafish is unknown. No sex chromosome or male/female-inducing master gene has been found. However, several candidate genes have been proposed, which have been linked to either zebrafish sex determination or differentiation (von Hofsten et al., 2005; Siegfried, 2010). *Dmrt1* (*double sex and mab-3 related gene 1*), *Sox9a* (*Sry-related HMG box-9*), *AMH* (*Anti-Mullerian Hormone*) and *NR5a1b* (*nuclear receptor subfamily 5, group a, number 1b*) are male-predominant genes involved in the development and maintenance of the male sex. *Cyp19a* (*Cytochrome P450 19a*) encodes the enzyme aromatase, which catalyzes the aromatization of androgens to estrogens and is a key gene in ovarian differentiation in teleosts. As no sex chromosome has been found, it seems less likely that sex is determined by a single gene, rather the collective gene dosage and temporal expression patterns seem to determine the gonadal sex (von Hofsten and Olsson, 2005).

To date, there is a lack of biomarkers indicating exposure to estrogen-like chemicals, especially demonstrating exposure of target organs like the gonads. The hepatic egg-yolk precursor protein Vtg, one of the few biomarkers (Kausch et al., 2008; Henry et al., 2009), is induced in the liver in response to estrogens or xenoestrogen in adult males and juveniles (Van den Belt et al., 2002; Matozzo et al., 2008). The majority of studies on EE_2 exposure in zebrafish have included Vtg quantifications of either plasma protein or hepatic mRNA (Schäfers et al., 2007; Jin et al., 2008; Kausch et al., 2008; Soares et al., 2009; Lange et al., 2012). Very few studies concern differences in expressions of genes related to fertility and gonad function that could serve as biomarkers more related to observed damage in reproductive organs. It is vital to find biomarkers that could identify effects of exposure during several development stages, reflecting continuous environmental exposure of aquatic populations. Genes regulating gonadal differentiation as well as maintenance of reproductive functions are good candidates for this purpose, as effects of developmental exposure could be expected to be persistent.

In search for candidate biomarkers related to fertilization and reproduction, we have studied mRNA expression of genes involved in sex differentiation, maintenance of gonadal function and hormone regulation in testes of adult zebrafish males exposed to 0, 5 or 25 ng/L EE_2 for 14 days. A previous study of these males has shown reduced fertility and effects on non-reproductive behavior in response to the EE_2 treatment (Reyhanian et al., 2011). Here we follow up the findings of reduced fertility with studies of transcription of genes related to gonadal function. We found a significant downregulation of transcription of male-predominant genes, suggesting that the hormone treatment might cause a demasculinization of the testes.

2. Materials and methods

2.1. Animals and treatments

As reported in a previous study (Reyhanian et al., 2011), adult male zebrafish (AB strain) from the Karolinska Institute Zebrafish Core Facility, Huddinge, Sweden, were kept in tap water (25–27 °C, pH 7.8, conductivity 20.7 mSi) with 12 h light/12 h dark cycles, and fed twice daily with Sera dry flakes (Vipan, Germany) and *Artemia nauplii* (Artemia International LCC, USA). Groups of 30 males each were exposed for two weeks to 0, 5 and 25 ng/L EE_2 (Sigma Aldrich, Germany) in separate 30 × 50 × 38 cm glass tanks in a flow-through system. The flow rate was set to 200 mL/min corresponding to a steady state of 16 L in each tank. Stock solutions were kept in dark bottles at 4 °C. From the stock solutions, fresh solutions in dark bottles were made every other day containing EE_2 at concentrations 2 and 10 μg/L EE_2 in 0.4% Acetone. These solutions were mixed with pre-heated tap water via peristaltic pumping through silicon tubing. The pump flow rates were adjusted to final nominal concentrations of 0, 5 and 25 ng/L EE_2 in 10 ppm acetone (400 fold dilution). No physical signs of negative health effects were observed, and all fish survived the two-week treatment period.

2.2. Determination of EE_2 in water samples

Water samples (100 mL) were collected once per week from each treatment tank during the exposure and stored in darkness at −20 °C. 50 mL of the water samples were extracted on 100 mg Strata X-33 μ Polymeric Reversed Phase cartridges according to the method described by Volkova (Volkova et al., 2012) except that preconditioning of the cartridge was performed only with MeOH. The water samples were analyzed for EE_2 using an LC system (Dionex ultimate 3000, Thermo Scientific, San Jose, CA, USA) coupled to a triple quadruple mass spectrometer (TSQ Vantage, Thermo Scientific). The quantification range of the method was 1–100 ng/L of EE_2 in aquaria water. EE_2-d_4 was used as the internal standard.

2.3. Tissue handling

Of the 30 fish per treatment group used for behavior studies (Reyhanian et al., 2011), 15 fish from each group were sacrificed at 14 days directly after the behavior tests. Organs were isolated for the analysis of gene expression. Fish were sacrificed by anaesthetization in 0.5‰ 2-phenoxyethanol (Sigma-Aldrich, Sweden) followed by decapitation. Testes (15/group) and livers (5/group) were dissected and immediately frozen at −80 °C in RNA later (Sigma-Aldrich) to be used for the current gene expression study by qPCR.

All experiments and fish husbandry were performed according to the Swedish Animal Care legislation, approved by the Southern Stockholm Animal Research Ethics Committee (Stockholms Södra Djurförsöksetiska Nämnd, Dnr S130-09).

2.4. RNA isolation and reverse transcription

Zebrafish gonads and livers were homogenized in 0.8 mL TriReagent (Sigma-Aldrich, Germany). RNA isolation was performed according to the manufacturer's protocol. Total RNA was quantified using NanoDrop ND-1000 Spectrophotometer and the quality was verified by absorbance ratio 260/280 nm. cDNA was obtained from 0.5 μg RNA/sample using Quantitec Reverse Transcription Kit (Qiagen, Germany).

2.5. Reverse transcription

The Reverse Transcriptase-PCR was carried out by using HotStarTaq Plus Master Mix Kit (Qiagen, Germany), with the PCR reactions containing the following: 10 μL Mastermix, 3 μL forward primer, 3 μL reverse primer, 1 μL cDNA template (350 ng RNA). The cycling parameters were set according to manufacturer's recommendations.

2.6. Quantitative Real-Time PCR

The transcription of the different zebrafish genes was analyzed by quantitative real-time PCR (qPCR) using Bio-Rad iCycler, MyIQ single-