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Reversibility of endocrine disruption in zebrafish (*Danio rerio*) after discontinued exposure to the estrogen 17α-ethinylestradiol

Lisa Baumann [a,b,*], Susanne Knörr [b], Susanne Keiter [b], Kristina Rehberger [b], Sina Volz [b], Viktoria Schiller [c], Martina Fenske [c], Henrik Holbech [d], Helmut Segner [a], Thomas Braunbeck [b]

[a] Centre for Fish and Wildlife Health, Vetsuisse Faculty, University of Bern, PO Box 8466, CH-3001 Bern, Switzerland
[b] Aquatic Ecology and Toxicology Section, Centre for Organismal Studies, University of Heidelberg, Im Neuenheimer Feld 230, D-69120 Heidelberg, Germany
[c] Fraunhofer Institute for Molecular Biology and Applied Ecology, Forckenbeckstr. 6, D-52074 Aachen, Germany
[d] Department of Biology, University of Southern Denmark, Campusvej 55, DK-5230 Odense M, Denmark

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ABSTRACT

The aim of the present study was to investigate the persistence of the feminizing effects of discontinued 17α-ethinylestradiol (EE2) exposure on zebrafish (*Danio rerio*). An exposure scenario covering the sensitive phase of sexual differentiation, as well as final gonad maturation was chosen to examine the estrogenic effects on sexual development of zebrafish. Two exposure scenarios were compared: continuous exposure to environmentally relevant concentrations (0.1–10 ng/L EE2) up to 100 days post-hatch (dph) and developmental exposure up to 60 dph, followed by 40 days of depuration in clean water. The persistence of effects was investigated at different biological organization levels from mRNA to population-relevant endpoints to cover a broad range of important parameters. EE2 had a strong feminizing and inhibiting effect on the sexual development of zebrafish. Brain aromatase (cyp19b) mRNA expression showed no clear response, but vitellogenin levels were significantly elevated, gonad maturation and body growth were inhibited in both genders, and sex ratios were skewed towards females and undifferentiated individuals. To a large extent, all of these effects were reversed after 40 days of recovery, leading to the conclusion that exposure to the estrogen EE2 results in very strong, but reversible underdevelopment and feminization of zebrafish. The present study is the first to show this reversibility at different levels of organization, which gives better insight into the mechanistic basis of estrogenic effects in zebrafish.

Introduction

17α-Ethinylestradiol (EE2) is one of the most potent xenoestrogens in the aquatic environment. It can regularly be measured in surface waters, mostly due to human excretion, and typically occurs at concentrations in the lower ng/L range (Johnson et al., 2013; Wise et al., 2011). It is a major constituent in contraceptive pills, which are consumed by over 100 million women worldwide (Owen and Jobling, 2012). The synthetic estrogen is very potent, with a higher binding affinity to estrogen receptors than natural 17β-estradiol (Blair et al., 2000). Very low concentrations (ng/L) are sufficient to induce vitellogenin (VTG) production in male fish (Andersen et al., 2003; Holbech et al., 2001; Rose et al., 2002), to modify sexual behavior of fish (Coe et al., 2010; Filby et al., 2012; Reyhanian et al., 2011), to disrupt reproductive capacities of fish (Fenske et al., 2005; Länge et al., 2001; Nash et al., 2004; Pawlowski et al., 2004; Schäfers et al., 2007) and eventually to adversely impact recruitment of fish populations (Kidd et al., 2007; Lange et al., 2011).

For investigations of the impact of EDCs on reproductive capabilities of fish, the zebrafish (*Danio rerio*) has proven to be a valuable model (Scholz and Klüver, 2009; Segner, 2009; Van der Ven et al., 2003) and is a recommended species to be used in the risk assessment of potential EDCs (OECD, 2009a,b, 2011). Due to its fast development, zebrafish can be used for full life cycle and trans-generation studies. It shows a protogynous sexual development, which is very sensitive to exposure to EDCs (Maack and Segner, 2004; Segner, 2009). Altered sexual development in zebrafish is easily inducible with different EDCs such as estrogens (Hill and Janz, 2003; Maack and Segner, 2003), androgens (Holbech et al., 2006; Örn et al., 2006) or aromatase inhibitors (Fenske and Segner, 2004; Kinnberg et al., 2007; Thorpe et al., 2011). Nevertheless, feminization of zebrafish can easily be misinterpreted as sex reversal, if a substance only inhibits and/or retards sexual differentiation, which results in an arrest of genetic males in the juvenile "all-female" stage (Fenske et al., 2005).

* Corresponding author at: Centre for Fish and Wildlife Health, Vetsuisse Faculty, University of Bern, PO Box 8466, CH-3001 Bern, Switzerland.
E-mail addresses: lisa.baumann@vetsuisse.unibe.ch (L. Baumann), susanne.knoerr@gmx.de (S. Knörr), susanne.keiter@cos.uni-heidelberg.de (S. Keiter), k.rehberger@stud.uni-heidelberg.de (K. Rehberger), s.volz@stud.uni-heidelberg.de (S. Volz), schiller@molbiotech.rwth-aachen.de (V. Schiller), martina.fenske@ime.fraunhofer.de (M. Fenske), hol@biology.sdu.dk (H. Holbech), helmut.segner@vetsuisse.unibe.ch (H. Segner), braunbeck@uni-hd.de (T. Braunbeck).

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The present study has been designed to provide insight into the mechanisms underlying the phenotypic observations of developmental arrest or transient feminization of zebrafish. Therefore, an exposure scenario with recovery phase was chosen. Since exposure of wildlife to EDCs is mostly not continuous, but intermittent, realistic exposure scenarios have become more popular over the last years. Several publications described the ability of zebrafish to recover from estrogen exposure (Hill and Janz, 2003; Larsen et al., 2009; Nash et al., 2004; Schäfers et al., 2007), whereas other authors have documented exposure to androgens as more persistent (Larsen and Baatrup, 2010; Morthorst et al., 2010). However, since most studies focused on the description of apical endpoints such as gonad histology and reproduction, information about the mechanisms leading to these phenotypes is still scarce. E.g., changes at the level of mRNA expression of relevant markers have not been investigated with respect to their reversibility. This is even more important, since vitellogenin (VTG), and recently also aromatase (cyp19b), are used as biomarkers for the detection of endocrine disruptive effects (OECD, 2010a,b). Discontinued exposure to a potent estrogen gives the opportunity to compare sub-apical key parameters of sexual differentiation (aromatase and VTG expression) with apical endpoints (gonad differentiation and reproductive output) and, thus, to understand the mechanisms underlying the transient nature and reversibility of feminization by estrogenic EDCs. This approach will further our insight into the mechanisms of endocrine disruption in zebrafish.

Materials and methods

Test substance. 17α-Ethinylestradiol (EE2; CAS-No.: 57-63-6) was obtained from Sigma-Aldrich (Deisenhofen, Germany). The following test concentrations were used for the exposure of zebrafish in the 100 d assay: 0, 0.1, 1, 3 and 10 ng/L, dissolved in dimethylsulfoxide (DMSO; maximum concentration in the test solutions ≤ 0.01%). Fresh stock solutions were prepared every second day in light-proof glass reservoirs from which they were added to the exposure tanks via peristaltic pumps (Minipuls 3, Gilson, Wiesbaden, Germany).

Exposure and sampling. The exposure of zebrafish (*D. rerio*, West aquarium strain) to the different chemicals started at latest 1 h post-fertilization and ended at 100 days post-hatch (dph). Fish were held in aerated 12 L flow-through glass tanks at 26–27 °C and a dark–light cycle of 10/14 h. Water temperature and flow-through rate (complete water exchange every 8 h) were controlled twice daily. Hardness (200–280 mg/L), conductivity (600–750 μS), pH (8.0–8.2) and oxygen saturation (90–95%) were tested at least twice weekly. Feces and food leftovers were removed daily. From days 4 to 14, larvae were fed with powdered dry food (Staubfutter, Sera Micron, Heinsberg, Germany); from day 14, larvae were fed with granular flake food (TetraMin™, Tetra-Werke, Melle, Germany) and newly hatched nauplii of *Artemia* spec. (Great Salt Lake *Artemia* Cysts, Sanders Brine Shrimp Company, Ogden, USA).

The exposure to EE2 was carried out until 60 dph, followed by a recovery-period in clear water of 40 days in one of the two replicates. Additionally, control groups with continuous and without any exposure over the whole duration of the experiment (100 days) were analyzed. Each treatment was run in two replicates. 100 fertilized eggs per replicate were used at the beginning. After 30 and 60 days, 30 individuals from each tank were randomly removed for analyses. In case of slight differences in the number of individuals per tank, some additional fish were removed to avoid density-dependent effects. Fish were euthanized with a saturated solution of tricaine (ethyl-*m*-aminobenzoate, Sigma-Aldrich). Length and wet weight of each individual were documented. Head and tail (for ELISA) or only the head (for qPCR) were cut off with a razor blade immediately behind the operculum and behind the anal fin, respectively (as described in OECD TG 234, 2011), weighed and frozen in liquid nitrogen for subsequent quantification of VTG protein concentrations or cyp19b mRNA expressions. Half of the sampled fish were used for VTG analyses, the other half for cyp19b analyses. Trunks of all 30 fish were placed in embedding cassettes (Histosette, Neolab, Heidelberg, Germany) and fixed in modified Davidson's fixative (Romeis and Böck, 2001) for subsequent histological analyses.

Histology. Samples were incubated in modified Davidson's fixative (Romeis and Böck, 2001) at 4 °C for at least 24 h before embedding into paraffin by means of a tissue processor (TP 1020, Leica Microsystems, Nussloch, Germany). Embedding into blocks was performed with a heated paraffin embedding module (EG 1140H, Leica Microsystems, Nussloch, Germany) with trunks orientated ventrally to the cutting surface. Sections of the gonads with a thickness of 4–5 μm were cut with a microtome (HN 40, Reichert-Jung, Heidelberg, Germany), mounted on glass slides (Langenbrinck, Langenseibold, Germany) and then stained with hematoxylin–eosin (Romeis and Böck, 2001) the next day. Light microscopical evaluation of the gonad tissue sections was performed according to the OECD Histopathology Guidance Document (OECD, 2010a,b). Each fish was identified either as female, male or intersex individual or was recorded as undifferentiated. Additionally, the maturation stages of the gonads were categorized as relative proportions of various gametogenic cell types into a numerical staging system (ovary: stages 0–5, testis: stages 0–4) according to the OECD Histopathology Guidance Document (OECD, 2010a,b).

Maturity index. As an enhancement to the regular gonad staging system in the OECD Histopathology Guidance Document (OECD, 2010a,b), each stage of maturity was given a fixed maturity value, increasing with the maturity of the fish (stage 0 corresponds to value 1; stage 1 corresponds to value 2; etc.). The sum of these values, from all individuals of each replicate, was divided by the respective number of fish. The sex-specific mean values for each treatment were calculated from the replicates and termed the maturity indices for females or males (Baumann et al., 2013). Completely undifferentiated or intersex individuals were not included into this assessment, since they could not be classified as female or male.

ELISA. The measurement of the VTG concentration in head and tail homogenates of zebrafish was performed as described by Holbech et al. (2006). In short, the frozen tissues were homogenized with a plastic pistil in 1.5 mL centrifuge tubes and mixed with 10 times the weight of homogenization buffer (50 mM Tris–HCl, pH 7.4: 1% protease inhibitor cocktail, Sigma-Aldrich). The homogenate was centrifuged for 30 min at 25,000 ×*g* at 4 °C (Multifuge 1S-R, Heraeus, Hanau, Germany) where after the supernatant was collected and stored at −80 °C. The VTG concentration in the supernatant was measured by a direct non-competitive sandwich ELISA based on polyclonal affinity purified antibodies against zebrafish lipovitellin developed by Holbech et al. (2001).

qPCR. Total RNA isolation of zebrafish heads was performed with TriReagent (guanidine thiocyanate and phenol, Sigma-Aldrich) according to the manufacturer's instructions. The tissue was homogenized with the aid of a Tissue Lyser II (Qiagen, Hilden, Germany) for 3 min at a frequency of 18 beats per second. RNA concentration and purity were measured with the NanoVue™ Plus Spectrophotometer (General Electric, Fairfield, USA). Subsequent cDNA synthesis was performed with the following reagents per reaction with 1 μg RNA: 1 μg hexanucleotide random-primer-mix (Roth, Karlsruhe, Germany), 2.5 μL M-MLV RT reaction buffer (Sigma-Aldrich), 1.25 μL deoxynucleotide (dNTP) Mix (Sigma Aldrich), 1 μL RiboLock RNase inhibitor (40 U; Fisher Scientific, Schwerte, Germany), 1 μL M-MLV reverse transcriptase (Sigma-Aldrich), and 4.25 μL RNase-free water. The qPCR reaction with the cDNA was performed using the StepOne™ real-time PCR System (Applied Biosystems, Foster City, USA) and the Fast SYBR Green master mix (Applied Biosystems, Foster City, USA). Expressions