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Developmental exposure of zebrafish (*Danio rerio*) to 17α-ethinylestradiol affects non-reproductive behavior and fertility as adults, and increases anxiety in unexposed progeny

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ABSTRACT

Exposure to estrogenic endocrine disruptors (EDCs) during development affects fertility, reproductive and non-reproductive behavior in mammals and fish. These effects can also be transferred to coming generations. In fish, the effects of developmental EDC exposure on non-reproductive behavior are less well studied. Here, we analyze the effects of 17α-ethinylestradiol (EE_2) on anxiety, shoaling behavior and fertility in zebrafish after developmental treatment and remediation in clean water until adulthood. Zebrafish embryos were exposed from day 1 to day 80 post fertilization to actual concentrations of 1.2 and 1.6 ng/L EE_2. After remediation for 82 days non-reproductive behavior and fertilization success were analyzed in both sexes. Males and females from the 1.2 ng/L group, as well as control males and females, were bred, and behavior of the untreated F1 offspring was tested as adults.

Developmental treatment with 1.2 and 1.6 ng/L EE_2 significantly increased anxiety in the novel tank test and increased shoaling intensity in both sexes. Fertilization success was significantly reduced by EE_2 in both sexes when mated with untreated fish of opposite sex. Progeny of fish treated with 1.2 ng/L EE_2 showed increased anxiety in the novel tank test and increased light avoidance in the scototaxis test compared to control offspring.

In conclusion, developmental exposure of zebrafish to low doses of EE_2 resulted in persistent changes in behavior and fertility. The behavior of unexposed progeny was affected by their parents' exposure, which might suggest transgenerational effects.

Introduction

Endocrine disrupting chemicals (EDCs) have been shown to disrupt the function of both vertebrate and several invertebrate hormone systems (Guillette and Gunderson, 2001; Waring and Harris, 2005). Exposure during narrow windows of development can lead to irreversible changes in both the morphology and function of affected organs (McLachlan, 2001). The reproductive organs and brain have long been regarded as the main targets for estrogenic EDCs. Human and rodent data alike shows impaired fertility and reproduction, malformations and cancers of the reproductive organs as a result of developmental EDC exposure (McLachlan, 2001; Newbold et al., 2006). In fish, the effects of estrogenic EDCs on the reproductive tract are well established. Findings include abnormal gonad structure and differentiation, intersexuality, sex reversal and decreased fertility (Arukwe, 2001; Fenske et al., 2005; Weber et al., 2003). A significant amount of studies has examined the effects of EDCs on reproductive behaviors in fish (for review see Söffker et al., 2012). Disturbed reproductive behavior has been observed in the three-spined stickleback, goldfish, guppy and zebrafish (Bayley et al., 1999; Bjerselius et al., 2001; EspmarkWibe et al., 2002a; Larsen et al., 2009; Shenoy, 2014). While the reproductive organs are able to recover from the effects of EDC exposure after remediation in clean water (Baumann et al., 2014; Larsen et al., 2009; Maack and Segner, 2004; Weber et al., 2003), the effects on fertilization success and reproductive behavior appear to be more persistent (Hill and Janz, 2003; Larsen et al., 2009; Schäfers et al., 2007; Van den Belt et al., 2003).

Disrupting the hormonal balance at an early stage of development can not only disturb gonad development but also interfere with the development of brain regions involved in adult endocrine and behavioral responses (McEwen, 1987). Brain development is tightly regulated and guided not only by transcription factors but also by endogenous hormones such as gonadal steroids (Fernandez-Galaz et al., 1997). EDCs have been shown to affect non-reproductive behavior. Prenatal EE_2 increases fear, anxiety and social neophobia in adult rats (Dugard

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et al., 2001). Developmental exposure to bisphenol A in mice results in altered social behavior, increased anxiety, altered spatial recognition and impaired memory (Ryan and Vandenbergh, 2006; Wolstenholme et al., 2011; Xu et al., 2010). In humans, prenatal exposure to phthalates affects aggression, attention and depression (Engel et al., 2010). Exposure to diethylstilbestrol during development has been associated with an increased frequency of depression (O'Reilly et al., 2010). Children of women exposed to polychlorinated biphenyls exhibit alterations in distractibility, verbal skills, learning and memory (Zala and Penn, 2004).

While reproductive behaviors are well studied in fish, data on non-reproductive behavior as a result of EDC exposure is relatively scarce. Adult EDC exposure is shown to alter risky behavior, schooling behavior and bottom dwelling (Bell, 2004; Dzieweczynski et al., 2014; EspmarkWibe et al., 2002b; Xia et al., 2010). Short term adult exposure to 17α-ethinylestradiol (EE_2) increases anxiogenic behavior in adult guppy and zebrafish males, and intensifies shoaling behavior in zebrafish (Hallgren et al., 2011; Reyhanian et al., 2011). Aggressive behavior has been shown to be affected by EE_2 exposure in several fish species (Colman et al., 2009; Filby et al., 2012; Majewski et al., 2002) and was modified by aromatase inhibitors in the African cichlid fish (Huffman et al., 2013). Studies on effects of developmental EDC exposure on non-reproductive behavior are few in fish, but developmental EE_2 exposure in guppies increases the stress response as adults (Volkova et al., accepted for publication), and developmental bisphenol A exposure causes learning deficits in adult zebrafish (Saili et al., 2012).

This study investigates the effects of developmental exposure to low doses of the potent EDC EE_2, on three non-reproductive behaviors in zebrafish. EE_2 is the main component of most contraceptive pills, and is released into the environment through waste water at concentrations ranging from below detectable levels up to 200–300 ng/L (Hannah et al., 2009; Kolpin et al., 2002; Laurenson et al., 2014; Ternes et al., 1999). The doses currently released into the environment have been shown to be harmful to aquatic animals (Aris et al., 2014), with a predicted no-effect-concentration for aquatic organisms at 0.1 ng/L (Caldwell et al., 2012). EE_2 is persistent, widespread (Aris et al., 2014) and has a strong binding affinity to the estrogen receptor (Denny et al., 2005). It has been detected in German drinking water (Kuch and Ballschmiter, 2001) and is, together with other estrogenic compounds, not only a growing public health concern (Mompelat et al., 2009; Vulliet and Cren-Olivé, 2011) but also a great ecological risk (Bull and Vogt, 1979). We studied the effects of EE_2 on anxiety and shoaling behavior, parameters of high ecological significance in wild fish populations, likely affecting fitness by influencing food foraging, reactions to predators and opportunities to reproduce.

We hypothesized that zebrafish (*Danio rerio*) larvae exposed to low doses of EE_2 for 80 days post fertilization followed by 82 days in clean water would show organizational effects on anxiety and shoaling intensity in the exposed F0 generation and unexposed F1 generation, and on fertilization success in the exposed F0 generation.

Materials and methods

Animals and treatments

Animals were kept in a 12/12 hour light/dark cycle at 25–27 °C, pH 7.0. Fertilized zebrafish (*Danio rerio*) embryos of the wild type strain AB were obtained from the Karolinska Institute Zebrafish Core Facility, Huddinge, Sweden. 17α-Ethinylestradiol (Sigma Aldrich, USA) was dissolved in acetone and stock solutions were mixed with pre-heated fish maintenance system water to final nominal concentrations of 0, 3 and 10 ng EE_2/L. The final concentration of acetone was 5 ppm in control and EE_2 solutions. All solutions were kept in dark glass bottles.

Fertilized eggs from 8 different parental pairs were collected and kept separately throughout the experiment. The fertilized eggs from each parental pair were divided into three lots and assigned to treatment groups of 0, 3 and 10 ng EE_2/L respectively. The animals were treated for 80 days (0–80 dpf). During the first 6 weeks, fish larvae were raised in 1 L glass tanks (maximally 50 eggs per tank) with partial solution exchange of 60% every second day. Fish larvae were fed *Paramecia* daily. Artemia (Artemia International LCC, USA) was added to the diet twice a day from week 5. Sera Dry Flakes (Vipan, Germany) were added to the diet twice daily from week 6. After 6 weeks, offspring from the parental pairs of each treatment dose were placed in separate net cages and transferred to 20 L tanks in a flow-through system with a flow rate of 280 mL/h, resulting in 1/3 exchange of the total volume per day. Premixed EE_2 or control solutions were peristaltically pumped through silicon tubing, with fresh solutions added every second day. After 80 days the treatment was stopped and fish families were transferred to 2 L tanks and kept in clean water under normal zebrafish maintenance conditions until adulthood, resulting in an 82 day remediation period before behavior and fertility testing. The sexes were separated based on secondary sexual characteristics after 40 days of remediation in clean water (4 months of age) and re-checked weekly. The sexes were then kept separated for the rest of the experiment.

In order to produce an F1 generation, males and females treated with the nominal concentration of 3 ng/L were mated with fish of opposite sex from the corresponding treatment group of a different family to avoid sibling mating. The progeny was raised in clean water according to standard breeding procedures. The same procedure was used to produce a control F1 generation.

Dissection and sex verification

At experimental termination fish were sacrificed by anaesthetization in 0.5‰ 2-phenoxyethanol (Sigma-Aldrich) followed by immediate decapitation. Fish were dissected and gonads examined under a microscope. Livers were removed and stored at −80 °C in RNA later (Sigma-Aldrich) to be used in qPCR analysis of Vtg mRNA expression. All experiments and handling of the animals was performed according to the Swedish Animal Care legislation, and approved by the Southern Stockholm Animal Research Ethics Committee (Dnr S130-09).

EE_2 concentrations

Water samples were collected at three different occasions during the exposure and stored in darkness at −20 °C. Analyses was performed according to the method previously described (ReyhanianCaspillo et al., 2014). Briefly, 100 mL water samples were extracted on 100 mg Strata X-33 μ Polymeric Reversed Phase cartridges, reconditioned with MeOH. EE_2 content was analyzed using Dionex Ultimate 3000 LC system (Thermo Scientific, San Jose, CA, USA), coupled to a triple quadruple mass spectrometer (TSQ Vantage, Thermo Scientific, San Jose, CA, USA). The quantification range of the method was 0.5–100 ng/L of EE_2, with EE_2-d4 as the internal standard.

Behavior studies

Behavior was analyzed in a combination of two previously described behavior tests: the novel tank test (Egan et al., 2009) and shoaling test (Moretz et al., 2007). The novel tank test, reflecting stress responses in an unfamiliar environment is well-defined by means of robust responses to anxiogenic and anxiolytic drugs (Stewart et al., 2011). Increased bottom-dwelling in the novel tank indicates higher stress. The shoaling test, detecting group cohesion as social interaction, boldness/wariness of the fish and possibly also stress, is less well-defined. Shoaling is, however, extremely ecologically significant in fish. Higher intensity of shoaling could indicate higher stress. The tests were performed one after the other in the same test episode (Reyhanian et al., 2011). The test tank (20 × 20 × 40 cm) was filled with 15 L preheated pure tap water. At the right end, a transparent Plexiglas screen