Aquatic Toxicology 105 (2011) 41–48

Contents lists available at ScienceDirect

Aquatic Toxicology

journal homepage: www.elsevier.com/locate/aquatox

17α-Ethinyl estradiol affects anxiety and shoaling behavior in adult male zebra fish (*Danio rerio*)

Nasim Reyhanian[a,b], Kristina Volkova[a,b], Stefan Hallgren[a], Tomas Bollner[a], Per-Erik Olsson[b], Håkan Olsén[a], Inger Porsch Hällström[a,*]

[a] School of Life Sciences, Södertörn University, SE-141 86 Huddinge, Sweden
[b] Örebro Life Science Center, School of Science and Technology, Örebro University, SE-701 82 Örebro, Sweden

ARTICLE INFO

Article history:
Received 4 March 2011
Received in revised form 4 May 2011
Accepted 10 May 2011

Keywords:
Endocrine disruption
Behavior
Zebra fish
Anxiety
Shoaling
Ethinyl estradiol

ABSTRACT

Ethinyl estradiol is a potent endocrine disrupting compound in fish and ubiquitously present in the aquatic environment. In this study, we exposed adult zebra fish (*Danio rerio*) males to 0, 5 or 25 ng Ethinyl estradiol/L for 14 days and analyzed the effects on non-reproductive behavior. Effects of treatment of the exposed males was shown by vitellogenin induction, while brain aromatase (CYP 19B) activity was not significantly altered. Both concentrations of Ethinyl estradiol significantly altered the behavior in the Novel tank test, where anxiety is determined as the tendency to stay at the bottom when introduced into an unfamiliar environment. The effects were, however, opposite for the two concentrations. Fish that were exposed to 5 ng/L had longer latency before upswim, fewer transitions to the upper half and shorter total time spent in the upper half compared with control fish, while 25 ng Ethinyl estradiol treatment resulted in shorter latency and more and longer visits to the upper half. The swimming activity of 25, but not 5 ng-exposed fish were slightly but significantly reduced, and these fish tended to spend a lot of time at the surface. We also studied the shoaling behavior as the tendency to leave a shoal of littermates trapped behind a Plexiglas barrier at one end of the test tank. The fish treated with Ethinyl estradiol had significantly longer latency before leaving shoal mates and left the shoal fewer times. Further, the fish exposed to 5 ng/L also spent significantly less time away from shoal than control fish. Fertilization frequency was higher in males exposed to 5 ng/L Ethinyl estradiol when compared with control males, while no spawning was observed after treatment with 25 ng/L. The testes from both treatment groups contained a normal distribution of spermatogenesis stages, and no abnormality in testis morphology could be observed.

In conclusion, we have observed effects on two behaviors not related to reproduction in zebra fish males after treatment with Ethinyl estradiol, adding to the ecological consequences of contamination of aquatic environments with estrogenic substances.

1. Introduction

Endocrine disrupting chemicals (EDC) are interfering with the function of the hormone system of all vertebrates and several invertebrate species (Basrur, 2006; Guillette and Gunderson, 2001; Rasier et al., 2006; Waring and Harris, 2005; Vos et al., 2000). Primarily, the reproductive organs have been regarded as the main targets for EDC with estrogenic effects in vertebrates. Effects on fertility and reproduction, as well as reproductive tract malformations and cancers have been identified in rodents and humans (Crews and McLachlan, 2006; O'Donnell et al., 2001; Rasier et al., 2006). Developing embryos and newborn are very sensitive, and exposure during narrow windows of development often leads to irreversible effects on the level of tissue organization (Li et al., 2003; McLachlan, 2001). It has also been shown that the effects can be transmitted to future generations without further exposure, probably by epigenetic mechanisms (Anway et al., 2005; Anway et al., 2008; Crews et al., 2007; Newbold et al., 2006).

Many targets of the endocrine system are in the brain. Parameters such as reproductive behavior and mate choice, regulated by sex-differentiated imprinting of the hypothalamic regions by sex steroids, are shown to be affected by EDC (Gore, 2008; Gore and Patisaul, 2010). Furthermore, estrogen is involved in many aspects of the development of the neuroendocrine system influencing brain structure as well as behavior (Dickerson and Gore, 2007; Gore, 2008). Behavior variables shown to be affected by EDC in wildlife and/or experimental animals include aggression, anxiety,

Abbreviations: EDC, Endocrine disrupting chemicals; EE_2, 17α-Ethinyl estradiol.

* Corresponding author. Tel.: +46 8 608 47 02; fax: +46 8 608 45 10.
E-mail address: inger.porsch-hallstrom@sh.se (I.P. Hällström).

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doi:10.1016/j.aquatox.2011.05.009

play behavior, attention, learning and memory (Xu et al., 2010; Zala and Penn, 2004). Human foetal exposure to the estrogen analogue diethylstilbestrol has been associated with increased frequency of depression (O'Reilly et al., 2010), and polychlorinated biphenyls have been shown to affect distractibility, verbal skills, learning and memory in children of exposed women (Zala and Penn, 2004). Prenatal phtalate exposure was found to reduce masculine play in boys (Swan et al., 2010) and was associated with poorer scores on clinical scales for i.e. aggression, conduct and attention problems and depression (Engel et al., 2010). EDC have been associated with symptoms similar to attention deficit hyperactivity disorders (ADHD) as well as Parkinson's-like symptoms (Gore and Patisaul, 2010; Jones and Miller, 2008).

A wide variety of EDC are contaminating aquatic environments (Kolpin et al., 2002; Loos et al., 2009), and evidence from field studies suggests relationship between exposure to environmental EDC and developmental and reproductive alterations in fish. Effects of estrogenic chemicals on reproductive variables are well established in many fish species, including abnormal gonad structure and differentiation, intersexuality and sex reversal, decreased fertility and fecundity and expression of egg yolk protein in males (Ankley et al., 2009; Arukwe, 2001; Baatrup and Junge, 2001; Bayley et al., 2002; Fenske et al., 2005; Fenske and Segner, 2004; Hill and Janz, 2003; Kidd et al., 2007; Lin and Janz, 2006; Pottinger et al., 1996; Santos et al., 2007; Van den Belt et al., 2002; Weber et al., 2003). Disturbed reproductive behavior have been observed in three-spined stickleback (*Gasterosteus aculeatus)*, goldfish (*Carassius auratus)*, guppy *(Poecilia reticulata)* and zebra fish (Bayley et al., 1999; Bjerselius et al., 2001; Espmark Wibe et al., 2002a; Larsen et al., 2009).

Compared with reproductive endpoints, effects of EDC on non-reproductive behavior is practically unstudied in fish. Only a few reports on EDC effects in different fish species exist, showing decreased aggression, affected shoaling and increased anxiety and risky behavior in response to EDC (Bell, 2004; Colman et al., 2009; Espmark Wibe et al., 2002b; Majewski et al., 2002; Ward et al., 2006; Xia et al., 2010).

In this study, we have analyzed the effects of 17α-Ethinyl estradiol (EE_2), widely used for oral contraception and a ubiquitous contaminant in sewage water, on two previously described non-reproductive behaviors in adult zebra fish (Egan et al., 2009; Moretz et al., 2007). The Novel Tank diving test is well characterized, and studies the anxiety response when a fish is introduced into an unknown environment. In this test, increased anxiety has been observed in zebra fish treated with drugs that act anxiogenically in mammals, whereas decreased anxiety is described in zebra fish treated with anxiolytic substances (Bencan et al., 2009; Levin et al., 2007; Sackerman et al., 2010). The Shoaling test is less well defined, but proposed to detect group attachment as a social interaction, boldness/wariness of the fish, and might possibly also be related to stress (Moretz et al., 2007). Also, some reproductive parameters were included in the study, to compare the sensitivity of reproductive and behavior endpoints.

2. Materials and methods

2.1. Animals and treatments

Adult male zebra fish (*Danio rerio*) of the wild type strain AB were obtained from the Karolinska Institute Zebra fish Core Facility, Huddinge, Fish were kept in tap water (25–27 °C, pH 7, 8, conductivity 20.7 mSi) with 12/12 h light/dark cycles, and fed twice daily with Artemia nauplii (Artemia International LCC, USA) and Sera Dry Flakes (Vipan, Germany). Groups with 30 males each were treated for 2 weeks with 17α-Ethinyl estradiol (Sigma–Aldrich, USA) in separate 30 × 50 × 38 cm glass tanks in a flow-through system. Water depth in the tanks was set to 11 cm, corresponding to a steady-state of 16 L in each tank. The flow rate was 200 mL/min. EE_2 was dissolved in acetone and stock solutions (0, 1.2 and 4.0 g/L in 4% acetone) were mixed with pre-heated tap water with peristaltic pumping through silicon tubing. The pump flow rates were adjusted to give final nominal concentrations of 0, 5 and 25 ng EE_2/L in10 ppm acetone. All fish survived the treatment period, with no signs of negative health effects.

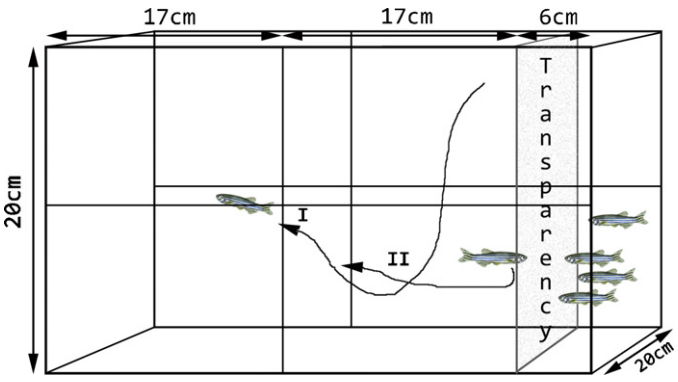

Fig. 1. Non-scaled design of behavior tests. The aquarium used for both tests is divided by a horizontal midline and a vertical midline. Male peers are trapped behind the Plexiglas™ transparency which is covered by a black sheet (not shown) during the Novel tank test. The test fish is released and latency to first horizontal midline crossing, total number of midline transitions and time in the surface half is video-recorded (I). The black sheet is removed from the transparency and the shoaling test is recorded as latency to first vertical midline crossing, total number of midline transitions and time in the opposite half (II).

All experiments and handling of the animals were performed according to the Swedish Animal Care legislation, and approved by the Southern Stockholm Animal Research Ethics Committee (Stockholm's Södra Djurförsöksetiska Nämnd, Dnr S130-09).

2.2. Behavior studies

The fish were studied in a combination of two previously described behavior tests, the Novel Tank diving test first described by Egan (Egan et al., 2009), and the shoaling test (Moretz et al., 2007). The tests were performed essentially as described, but modified to be performed one after the other during the same test episode. The glass test tank (20 × 20 × 40 cm, Fig. 1) was filled with 15 L pre-warmed tap water (the same water as used for fish maintenance, without previous contact with fish). In the right end, a vertical zone was created by a transparent Plexiglas screen 6 cm from the short end of the tank, trapping a shoal of 4 males of the same AB strain that had not been treated with EE_2. Visual contact was prevented by a black plastic sheet covering the Plexiglas screen. A horizontal and a vertical midline divided the rest of the tank into top/bottom and right/left halves, respectively.

The tests were started by introducing a single fish into the tank by gentle netting (max 3 s above water). As soon as the fish had entered the bottom half, video recording was started. Three tanks were run in parallel, and all experiments were performed between 9.00 and 13.00 a.m. Two fish in the 25 ng/L group were excluded due to technical problems with the filming. After a 5 min observation period of the behavior in the Novel Tank assay, the black screen was removed, visualizing the presence of the shoal group. When the test fish had observed the shoal it started to interact by swimming together with the shoal on the other side of the Plexiglas screen. Most fishes rapidly made contact, but a few took longer time. Fishes not approaching the group within 5 min after screen displacement were excluded (one each in 0 and 5 ng/L, 2 in 25 ng/L). The behavior in the presence of the shoal was recorded for 5 min after contact was made.