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Disruption of zebrafish (*Danio rerio*) embryonic development after full life-cycle parental exposure to low levels of ethinylestradiol

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ABSTRACT

Exposure of fish to the synthetic estrogen ethinylestradiol (EE2) has been shown to induce a large set of deleterious effects. In addition to the negative impact of EE2 in reproductive endpoints, concern has recently increased on the potential effects of EE2 in fish embryonic development. Therefore, the present study aimed at examining the effects of EE2 on the full embryonic development of zebrafish in order to identify the actual phases where EE2 disrupts this process. Hence, zebrafish were exposed to environmentally relevant low levels of EE2, 0.5, 1 and 2 ng/L (actual concentrations of 0.19, 0.24 and 1 ng/L, respectively) from egg up to eight months of age (F_1), and the survival as well as the occurrence of abnormalities in their offsprings (F_2), per stage of embryonic development, was investigated. A thorough evaluation of reproductive endpoints and transcription of *vtg1* gene in the parental generation (F_1) at adulthood, was performed. No significant differences could be observed for the two lowest EE2 treatments, in comparison with controls, whereas *vtg1* transcripts were significantly elevated (40-fold) in the 2 ng/L EE2 treatment. In contrast to the findings in the F_1 generation,a significant concentration-dependent increase in egg mortality between 8 and 24 hours post-fertilization (hpf) was observed for all EE2 treatments, when compared with controls. The screening of egg and embryo development showed a significant increase in the percentage of abnormalities at 8 hpf for the highest EE2 concentration, a fact that might explain the increased embryo mortality at the 24 hpf time-point observation. Taken together, these findings indicate that the two lowest tested EE2 concentations impact late gastrulation and/or early organogenesis, whereas exposure to 2 ng/L EE2 also disrupts development in the blastula phase. After early organogenesis has been completed (24 hpf), no further mortality was observed. These results show that increased embryo mortality occurs at EE2 levels below those inducing reproductive impairment and *vtg1* gene induction in the male parental generation, thus suggesting that EE2 may impact some fish populations at levels below those inducing an increase in *vtg1* transcripts. Hence, these findings have important implications for environmental risk assessment, strongly supporting the inclusion of embryonic development studies in the screening of endocrine disruption in wild fish populations.

1. Introduction

The presence, in the aquatic ecosystems, of chemicals able to interfere with the normal endocrine function of animals, generally named endocrine disrupting chemicals (EDCs), is a mat-

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ter of great concern (Ashby et al., 1997; Mills and Chichester, 2005; Iguchi et al., 2006; Sanderson, 2006). Many EDCs modulate sex steroid signalling and the hypothalamic–pituitary–gonad axis (Ankley and Johnson, 2004; Segner et al., 2006), interfering with both development and reproduction (Guillette, 1995; Bigsby et al., 1999). Estrogenic chemicals (ECs) are among the most widely studied EDCs, mostly because field studies have shown fish feminization in many aquatic ecosystems (Sumpter, 2005). Fish can be exposed to EDCs through a variety of sources, but waste waters are the primary source of ECs such as the active ingredient of contraceptive pills, 17α-ethynilestradiol (EE2) (Folmar et al., 2000; Metcalfe et al., 2001; Kolpin et al., 2002).

Environmental EE2 concentrations in water are highly variable, from non-detectable to a maximum concentration of 830 ng/L in USA rivers (Kolpin et al., 2002). In Europe, the majority of the measured concentrations are below 5 ng/L (Desbrow et al., 1998; Belfroid et al., 1999; Ternes et al., 1999; Johnson et al., 2000; Svenson et al., 2002). EE2 has the ability to bioconcentrate in fish tissue (650-fold in whole body tissues), and is not only more effective in eliciting estrogenic responses but also more stable than the natural estrogen 17β-estradiol (Länge et al., 2001; Lai et al., 2002; Thorpe et al., 2003). Under laboratory conditions, EE2 has been reported to cause a wide variety of negative effects in fish reproduction, such as a bias in the sex ratio toward females, decreased fertility and fecundity, and vitellogenin induction in males (Nash et al., 2004). In the model teleost, zebrafish (*Danio rerio*), several studies involving EE2 exposure have been performed. Only few, however, cover the full life-cycle, and have tested the impact on reproductive parameters. Segner et al. (2003) tested EE2 exposure from fertilization to adult stage and observed a decline in the egg number per female and the fertilization success at concentrations above 1.67 ng/L; Fenske et al. (2005) found an inhibition on egg production when fish were exposed to 3 ng EE2/L from 0 to 180 days post-fertilization (dpf). More recently, Schäfers et al. (2007) observed a decrease in the number of eggs and also a decline on the number of fertilized eggs of zebrafish exposed to concentrations of 1.1 ng/L, from 0 to 177 dpf; Larsen et al. (2008) observed a decline in the number of fertilized eggs at 0.05 and 0.5 ng EE2/L, from 1 to 124 dpf.

In addition to the negative effects of EE2 in reproductive endpoints, it has recently been pointed out that embryo development may also be a target of EE2 (Länge et al., 2001; Nash et al., 2004; Brown et al., 2007). The effects of EE2 on fish embryo survival have been reported after chronic parental EE2 exposure, thus raising considerable concern as it occurs at environmentally relevant EE2 concentrations. While these early studies have determined embryo mortality at specific time-points over the embryonic period, none has investigated in detail the effects of EE2 on the full embryonic development. Hence, in order to understand the mechanism(s) by which EE2 disrupts embryonic development, it is essential to identify the actual phases where EE2 acts.

Therefore, the main aim of the present study was to examine the concentration-dependent effects of parental zebrafish full life-cycle exposure to low levels of EE2 on the different stages of the offspring embryonic development. In order to achieve this, zebrafish were exposed to low levels of EE2 (nominal concentrations of 0.5, 1 and 2 ng/L), from egg up to eight months of age, and the survival and the occurrence of abnormalities in their offspring, per stage of embryonic development, evaluated. The results of embryo mortality patterns were integrated with the data of the reproductive parameters and vitellogenin gene induction in the parental generation and discussed in relation to the potential population-level impact of EE2 for wildlife fish populations.

2. Materials and methods

2.1. Species selection

Zebrafish (*Danio rerio*) are recommended as test species in a number of ecotoxicological test protocols (Oberemm, 2000). This species size, robustness, short life-cycle and the fact that it can be induced to breed all year round under laboratory conditions, are advantages for its use as bioassay organism. Furthermore, particularly important for the present study, zebrafish eggs are translucent and non-sticky thus allowing an easy screening of embryonic development under a stereo-microscope.

2.2. Parental animals (F_0)

Adult wild-type zebrafish, obtained from local suppliers in Singapore, were used as breeding stocks. The stock was kept at a water temperature of 28 ± 1 °C and under a photoperiod of 14:10 h (light:dark), in 250 L aquaria with dechlorinated and aerated water in a recirculation system with both mechanical and biological filters. The fish were fed *ad libitum* twice a day with a commercial fish diet Tetramin (Tetra, Melle, Germany) supplemented with live brine shrimp (*Artemia* spp.).

2.3. Egg production of F_0 generation

In the afternoon before breeding, two groups of 4–6 males and 10–12 females were independently housed in cages with a net bottom cover with glass marbles within a 30 L aquarium under the same water and photoperiod conditions as the stock and fed with live brine shrimp. At the following day, breeding fish were removed 1.5 h after the beginning of the light period and the eggs were collected and cleaned. Fertilized eggs were randomly allocated to experimental aquaria.

2.4. Exposure of F_1 generation

Exposures were performed using a flow through system with slight modifications from the experimental setup described in Santos et al. (2006). Before entering the system, the water was heated and charcoal-filtered. The water flow (50 mL/min) was maintained by a peristaltic pump (ISM 144, ISMATEC) whereas EE2 and DMSO solutions (0.018 mL/min flow) were administered by a second peristaltic pump (205U Watson-Marlow). The water and the solutions were mixed together, in a mixing chamber, before entering, by gravity, in the continuously aerated aquaria. The utilization of peristaltic pumps to control water and contaminant fluxes increases the accuracy of dosage over time, which was confirmed by weekly measurements. Throughout the experimental period, zebrafish were under a 14:10 h (light:dark) photoperiod and water physical–chemical parameters were measured weekly with the exception of temperature that was checked on a daily basis (28 ± 1 °C of temperature; pH 7.7 ± 0.2; 6 ± 1 mg/L of dissolved oxygen; 376 μS/cm of conductivity; 0.08 ± 0.06 mg/L of ammonium and 0.01 ± 0.01 mg/L of nitrite).

Five exposure conditions, in duplicate, were set up: an experimental control, a solvent control (DMSO) and three EE2 concentrations (nominal concentration: 0.5; 1 and 2 ng/L).

17α-Ethynilestradiol (EE2 98%, Sigma) (stock solution: 1 mg/mL) was diluted in dimethylsulfoxide (DMSO 99.5%, Sigma). From this solution, aliquots of the working solutions were prepared and kept at −20 °C until use. Working EE2 solutions were diluted in MilliQ water and renewed three times a week; all solutions were prepared in order to have a final DMSO volume of 0.000002%.

In order to test EE2 effects when zebrafish reached their maximal fecundity, the study lasted for 8 months, with continuous exposure