Aquatic Toxicology 95 (2009) 330–338

Contents lists available at ScienceDirect

Aquatic Toxicology

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

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

J. Soares[a,d,1], A.M. Coimbra[a,g,h,1], M.A. Reis-Henriques[a,d], N.M. Monteiro[b,f], M.N. Vieira[b,e], J.M.A. Oliveira[i], P. Guedes-Dias[i], A. Fontaínhas-Fernandes[g,h], S. Silva Parra[h], A.P. Carvalho[e], L. Filipe C. Castro[c], M.M. Santos[a,*]

[a] *CIMAR/CIIMAR, Interdisciplinary Centre of Marine and Environmental Research, Laboratory of Environmental Toxicology, University of Porto, Rua dos Bragas 289, 4050-123 Porto, Portugal*
[b] *CIMAR/CIIMAR – Interdisciplinary Centre of Marine and Environmental Research, Laboratory of Ecology, University of Porto, Rua dos Bragas 289, 4050-123 Porto, Portugal*
[c] *CIMAR/CIIMAR, Interdisciplinary Centre of Marine and Environmental Research, Laboratory of Cellular, Molecular and Analytical Studies, University of Porto, Rua dos Bragas 289, 4050-123 Porto, Portugal*
[d] *ICBAS – Institute of Biomedical Sciences Abel Salazar, University of Porto, Largo Professor Abel Salazar, 2, 4099-003 Porto, Portugal*
[e] *FCUP – Dept of Zoology and Anthropology, Faculty of Sciences, University of Porto, Portugal*
[f] *CEBIMED, FCS-UFP – Faculty of Health Sciences, University Fernando Pessoa-Oporto, Portugal*
[g] *CITAB – Centro de Investigação e de Tecnologias Agroambientais e Biológicas – Quinta de Prados, Apartado 1013, 5001-801 Vila Real, Portugal*
[h] *DeBA-ECVA-UTAD – Departamento de Biologia e Ambiente, Escola de Ciências da Vida e Ambiente, Universidade de Trás-os-Montes e Alto Douro, Quinta de Prados, Apartado 1013, 5001-801 Vila Real, Portugal*
[i] *REQUIMTE, Serviço de Farmacologia, Faculdade de Farmácia da Universidade do Porto, Portugal*

ARTICLE INFO

Article history:
Received 27 May 2009
Received in revised form 25 July 2009
Accepted 29 July 2009

Keywords:
Zebrafish
Ethinylestradiol
Embryonic development
Endocrine disruptor
Estrogenic chemical
Fish
Vitellogenin
Environmental risk assessment

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-

* Corresponding author. Tel.: +351 223401812; fax: +351 223390608.
E-mail address: santos@ciimar.up.pt (M.M. Santos).
[1] These authors contributed equally to the study.

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doi:10.1016/j.aquatox.2009.07.021

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

of the F_1 generation (Westerfield, 2000). Before the beginning of the exposure, the aquaria were equilibrated during 15 days. The experiment was initiated by randomly allocating 450 eggs ≈2 h post-fertilization (hpf) in 5 L aquaria, placed within each 30 L aquarium. At 20 dpf, zebrafish were allocated to 30 L aquaria and their number adjusted to fit 100 juveniles per aquarium. The number of zebrafish in each aquarium was again reduced to 70 at 40 dpf, and later to 30 at 60 dpf, a density that was maintained up to the end of the experiment (8 months). Feeding was initiated at 6 dpf with two meals of Tetramin supplemented with one brine shrimp meal per day based on a slight modification of Carvalho et al. (2006); at 9 dpf this was changed to two brine shrimp meals and one Tetramin meal per day, which was maintained up to the end of exposure.

Mortality was assessed by daily recordings during the entire exposure period, and dead eggs/individuals removed. At the end of the experiment, the remaining zebrafish were immobilized in ice-cold water, immediately decapitated, and their weight and length recorded for body condition factor calculation (K = weight × length^{-3} × 100). Liver was removed and stored in RNAlater (Sigma) for molecular biology determinations. Gonads were also excised and weighed for gonadosomatic index calculation (GSI = gonad weight as percentage of total weight), and observed under a stereo-microscope for sex determination, complemented with histology.

2.5. EE2 concentration in water

In order to determine actual EE2 concentrations, water samples were collected twice during the exposure of F_1 generation (at 40 and 210 dpf) and pre-treated according to the "Sample Pre-treatment Protocol for Female Steroid Hormones" (Japan Envirochemicals). Briefly, steroids were extracted in a solid phase C18 column (Sep-Pak Plus C18 cartridges, Waters Corporation), 80% methanol eluted, concentrated under nitrogen gas flow and frozen at −20 °C until analysis. EE2 concentration was determined using the "Jec ELISA kit" (Japan Envirochemicals) according to the manufacturer's protocol. Concentrations of EE2 were found to be below the detection limit in the reference aquaria and to be 1.00 ± 0.12, 0.24 ± 0.02 and 0.19 ± 0.02 ng/L, respectively, for the 2, 1 and 0.5 ng/L nominal concentrations. The extraction procedure rendered a recovery of over 90%, and therefore no correction was performed to the obtained EE2 concentrations.

2.6. Reproductive capability of F_1 generation

Reproductive capability studies were performed after 8 mpf (months post-fertilization) in all experimental groups. Reproductive success was evaluated as fecundity (number of eggs per female per day) and % of viable eggs per female per day after 1–1.5 hpf. For reproductive studies, the 30 L aquaria were divided in two compartments and female and male zebrafish were randomly distributed in cages, with a net bottom covered with glass marbles, in a manner that matched the sex ratio of each treatment (4 replicates per treatment) (Fig. 1). Subsequently, during three consecutive days, 1–1.5 h after the beginning of the light period, eggs were collected, counted and the percentage of viable eggs recorded. The sex ratio per replicate was confirmed at the end of the reproductive trial.

2.7. Histology and stereological analysis of F_1 generation

In order to perform a histological sex determination, approximately 50 fish per treatment (30 fish for the 0.5 ng/L EE2 treatment) were collected at the end of exposure (8 months). Animals were fixed in Bouin solution (Panreac) for 48 h, paraffin-embedded, sectioned into 5 μm sections and mounted on slides. Slides were stained with hematoxylin–eosin and mounted with Entellan®. Identification of gonad cell populations was conducted according to Weber et al. (2003). Briefly, ovarian follicles were staged as: oogonia (Oo – small in size and eosinophilic ooplasm with relatively large nucleus); previtellogenic follicles (PreV – small in size with basophilic ooplasm, large nucleus with visible chromatin and single somatic cell layer); vitellogenic follicles (Vit – basophilic ooplasm that is enlarging relative to nucleus and has one or more layers of somatic cells) and preovulatory follicles (PreO – distinct zona radiata, multiple differentiated somatic cell layers and vacuolated ooplasm). Testicular populations were identified as follows: spermatogonia (Sg – eosinophilic cytoplasm with relatively large nucleus); spermatocytes (Sc – thread-like or condensed chromatin, with relatively smaller cytoplasm) and spermatids or mature sperm (M – tightly packaged nuclear material lacking surrounding cytoplasm).

To determine the relative volume density (VV) of spermatogonia, spermatocytes, spermatids and spermatozoa in testes as well as oogonia, previtellogenic follicles, vitellogenic follicles and preovulatory follicles in ovaries, from 8 months zebrafish (n = 6), a stereological approach was designed based on point counting (Freere and Weibel, 1967), using a microscope (Olympus IX 51, Japan) equipped with a CCD camera (Olympus U CMDA3, Color View soft imaging system). A detailed description of the method can be found in Monteiro et al. (2009).

2.8. Sperm quality parameters in F_1 males

Functional imaging of the spermatozoids was performed with a system composed of an inverted microscope (Eclipse TE300, Nikon, Tokyo, Japan) equipped with a 40× air objective and a CCD camera (C6790; Hamamatsu Photonics, Hamamatsu, Japan). Sperm motility analysis was performed as previously described by Wilson-Leedy and Ingermann (2007), with minor modifications, namely a 53 Hz capture rate and a resolution of 329 × 247 pixels (2 × 2 binning). Sperm was collected from one testicle of 6 males exposed to the above-described treatments. Sperm motil-

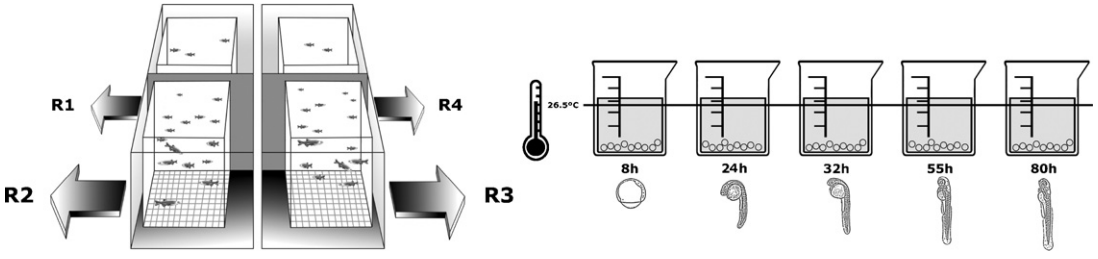

Fig. 1. Schematic representation of the experimental setup used for breeding trials of the F_1 generation and the embryonic assay with their offspring (F_2). After 8 mpf, breeding trials were performed using four replicates per treatment (R1, R2, R3, and R4). Viable eggs from each replicate were randomly distributed to 100 mL beakers (40 eggs per beaker). From each replicate, one beaker per stage (8 hpf, 24 hpf, 32 hpf, 55 hpf, and 80 hpf) was set, which means that during the five studied embryonic stages, a total of 20 beakers per treatment were used.

ity videos were acquired during 44 s and sub-sequentially split into 22 videos of 2 s. Image processing was performed with Aquacosmos 2.5 (Hamamatsu Photonics) and ImageJ (National Institutes of Health; available at http://rsb.info.nih.gov/ij/). Video and numerical data calculations were automated with ImageJ.

Several parameters were collected from the video analysis (see Wilson-Leedy and Ingermann, 2007), namely sperm motility (MOT), curvilinear velocity (VCL), velocity over a straight line (VSL) and progression (PROG).

2.9. Embryogenesis studies of F_2 generation

When reproductive trials of F_1 generation reached the third day, 200 viable eggs per replicate were kept for the embryogenesis studies. Eggs that had passed the 4 cell stage (1–1.5 hpf) were randomly distributed to 100 mL beakers (40 eggs per beaker) with daily replacement of water obtained from the flow through system where dosing of F_1 generation was taking place. Thus, embryos were exposed to the same treatments as the parental F_1 generation, mimicking a scenario where fish are exposed to ECs through the entire life-cycle. The beakers were randomly maintained on a water bath at 26.5 ± 0.5 °C and kept under the same photoperiod conditions as the adults. For each treatment, four beakers per stage were set (four replicates), which means that during the five studied embryonic stages, a total of 20 beakers per treatment were used (Fig. 1).

For embryogenesis studies embryo tests and endpoints were performed with slight modifications of the protocol proposed by Oberemm (2000). The following endpoints were evaluated during zebrafish egg/embryo development stages, briefly: at 8 hpf (65–75% – epiboly) progression of ectodermal front and homogeneity of front and tissue; at 24 hpf (prim 5–6), head and tail differentiation, length of tail, spontaneous movements and the presence of heart beat; at 32 hpf (prim 15–16) and 55 hpf (Long-pec, pec-fin), heart rate, malformations of the blood circulatory system, oedema and melanophores distribution; and at 80 hpf (protruding mouth), heart rate, expression of pigmentation, oedema and fin fold lesions; the mortality rate was assessed at all stages. Different embryos, even within a single clutch, develop at slightly different rates (Kimmel et al., 1995). Therefore, our time-point observations included eggs/embryos in slightly different phases, which have been indicated above. However, for simplicity, in the figures we have adopted the classification of Oberemm (2000), with a single stage per time-point observation. Inspection started by checking mortality and discharging dead eggs/embryos to avoid water quality decay. Then, 15 eggs/embryos from each beaker (60 per treatment/developmental stage) were randomly collected and placed on a Petri dish for observation under a stereo-microscope (SMZ1000, Nikon, magnification up to 80 times). Any modification from normal development and/or morphological anomalies were recorded by digital photography (Nikon Coolpix 5400) and scored as presence/absence. A detailed description and categorization of the different anomalies will be performed in the future. After each time-point observation, the beakers were removed, thus assuring the independence of data between developmental stages. The heart beat and spontaneous mobility were evaluated at 24 hpf in three embryos per replicate and the cardiac frequency was determined in one embryo per replicate at 32, 55 and 80 hpf, using a stop-watch, during two 15 s periods, restarting the counting if the embryo moved.

2.10. vtg1 gene transcription in F_1 zebrafish males after 8 months of EE2 exposure

For *vtg1* transcription in adult male zebrafish, after 8 months of exposure to EE2, total RNA was isolated from liver using "illustra RNAspin Mini kit" (GE Healthcare kit) according to the manufacturer's protocol, with on column DNase digestion during the extraction procedure. After column elution with 30 μL of RNase-free water, RNA concentration was determined by fluorescence (Fluoroskan Ascent, Labsystems) using the "Quant-iT™ RiboGreen® RNA Assay Kit" (Invitrogen) and RNA quality was checked in a 1% agarose gel. All RNA samples were stored at −80 °C until further use. For cDNA synthesis, the iScript cDNA synthesis kit (Bio-Rad) was used with 0.5 μg of total RNA.

For amplification and mRNA expression analysis through real-time PCR (RT-PCR) of *vtg1* (NM_170767), primers were designed in a region outflanking an intron using Beacons Design™ software (Premier Biosoft International) and synthesised by SigmaProligo:

Forward primer 5′-CTT ACG ACA CAG GAT TCA G-3′;
Reverse primer 5′-GTC TTC ATA GGT CTC AAT GG-3′.

The PCR reaction was prepared to a final volume of 25 μL with primers final concentration of 200 nM and using 2 μL of cDNA.

RT-PCR was initiated at 95 °C for 3 min. Thereafter, 35 cycles of amplification were carried out with denaturation at 95 °C for 10 s, annealing at 53 °C for 30 s and extension at 72 °C for 30 s (data collection), followed by a melting curve analysis to determine the specificity of the reaction. A standard curve consisted of eight 10-fold serial dilutions of positive control cDNA prepared from a total RNA sample isolated from a spawning female liver and used as reference sample for construction of a standard curve (reaction efficiency close to 100%, and $r^2 = 0.994$) and for *vtg1* relative concentration determination (ΔCt method), together with the male test samples and negative control (template-free). The RT-PCR products were analysed by agarose gel electrophoresis to confirm the presence of a single band with the expected size (200 bp) (data not shown). The identity of the PCR product amplified by the real-time primers was confirmed by cloning and subsequent sequencing. For sequencing, the PCR product was excised from the gel using the QIAquick Gel Extraction kit (Qiagen) and cloned into pGEM®-T Easy Vector (Promega). The ligation product was transformed into Novablue Singles cells I (Novagen) and grown over-night on IPTG/X-gal-coated LB-plates with ampicillin (50 μg/mL) at 37 °C. Three colonies were selected and cultured over-night in LB-medium with ampicillin (50 μg/mL) at 37 °C with orbital shaking. Plasmid preparations were prepared using QUIprep Spin Miniprep Kit (Qiagen).

2.11. Statistics

Data were analysed using Statistica 6.0 software. After testing for ANOVA assumptions (homogeneity of variances and normality of data), statistical differences in embryo mortality rate and abnormal eggs/embryos among treatments and stages were evaluated by a two-way factorial ANOVA, followed by Fisher LSD multiple comparison test. Mortality data were Arcsine-square root transformed before ANOVA analysis. Significant differences in the reproductive parameters, *vtg1* gene expression, and stereological analyses were evaluated by a one-way ANOVA, followed by Fisher LSD multiple comparison test. Sex ratio differences were tested by the χ^2 test, using the solvent control group as expected values. For sperm quality assessment, a preliminary analysis of the data showed a great degree of homogeneity among the analysed videos and, thus, values were averaged among each treatment (control, solvent control, and the three tested EE2 concentrations) only to determine the breakpoint of the curve that indicated the onset of the decay. A piecewise regression was used and the estimated breakpoint (38 s) was similar in all tested parameters (MOT, VCL, VSL and PROG) and treatments (data not shown). Thus, the values comprised between the onset of analysis and the calculated breakpoint were averaged (for each tested fish) and used in a MANOVA (4 parameters: MOT, VCL, VSL

Table 1
Reproductive parameters for all treatments groups (F_1 generation) at 8 mpf.

	Group		EE_2		
	Control	DMSO	0.5 ng/L	1 ng/L	2 ng/L
Reproductive parameters (8 mpf)					
Female					
% Females	60.9[a]	49.2	34.6[a]	57.1	55.8
Fecundity	183 ± 79	114 ± 24	75 ± 30	78 ± 33	140 ± 50
Viable eggs 1–1.5 hpf (%)	96 ± 1.0	99 ± 0.4	96 ± 0.3	94 ± 2.2	94 ± 2.8
% Previtellogenic follicles	5.4 ± 1.1	5.5 ± 0.3	6.8 ± 0.6	8.6 ± 1.4	6.2 ± 0.6
% Vitellogenic follicles	6.8 ± 0.6	7.8 ± 0.9	11.4 ± 1.7	11.2 ± 1.5	7.0 ± 0.9
% Preovulatory follicles	62.6 ± 2.8	62.8 ± 1.8	58.5 ± 2.4	53.2 ± 5.3	62.4 ± 3.8
Male					
% Males	39.1[a]	50.8	65.4[a]	42.9	44.2
% Spermatogonia	8.5 ± 1.0	8.4 ± 2.1	7.6 ± 1.2	7.0 ± 0.8	8.4 ± 1.6
% Spermatocyst	9.3 ± 0.9	12.6 ± 1.2	12.1 ± 1.7	12.0 ± 1.5	12.7 ± 1.5
% Spermatid	13.9 ± 1.4	11.9 ± 1.2	17.8 ± 1.4	18.1 ± 2.1	12.4 ± 1.5
% Spermatozoa	34.1 ± 2.7	40.7 ± 28.2	28.2 ± 1.4	32.4 ± 3.8	36.5 ± 2.6

[a] Significantly different from solvent control (DMSO). Values presented as mean ± standard error; $p < 0.05$, one-way ANOVA, followed by Fisher LSD multiple comparison test. % of females and males tested by χ^2 test.

and PROG; 5 factors: Control, solvent control group, three tested EE2 concentrations).

3. Results

3.1. Mortality of F_1 generation

The mortality rates, from embryo up to 20 dpf, varied between 36% and 55% and no differences were observed between control and EE2-exposed zebrafish groups (data not shown). No mortality occurred after this period. These mortality rates were within the normal expected values for zebrafish (Hill and Janz, 2003; Santos et al., 2006).

3.2. Weight, length, K and GSI of F_1 generation

With the exception of male total length, which was significantly increased ($p < 0.05$; one-way ANOVA, followed by Fisher LSD multiple comparison test) in all groups of zebrafish exposed to EE2 if compared with solvent control (4.37 ± 0.06 and 4.37 ± 0.04 cm for control and solvent control, and 4.60 ± 0.04, 4.52 ± 0.05 and 4.57 ± 0.05 cm for, EE2 0.5, 1 and 2 ng/L, respectively), the remaining tested parameters were not affected by EE2 exposure (data not shown).

3.3. Sex ratio of F_1 generation

At the end of the experiment, with the exception of EE2 0.5 ng/L, all groups showed a percentage of females between 49% and 61%, which is within the normal range in zebrafish raised in captivity (Hill and Janz, 2003) (Table 1). Despite this fact, control showed a percentage of females that was significantly higher than that of solvent control. Animals exposed to the lowest EE2 concentration showed a significant increase ($p < 0.05$, χ^2 test) in the percentage of males in comparison to solvent control.

3.4. Stereological analysis of gonads and sperm quality in F_1 generation

The statistical analysis of the data dealing with the relative volume percentage of the ovaries, occupied by each of the oocyte stages, showed no differences between treatments. Also, the testicular tissue occupied by the different spermatogenic stages, in 8 months old zebrafish, did not show significant differences between treatments ($p > 0.05$; one-way ANOVA, followed by Fisher LSD multiple comparison test) (Table 1). Likewise, no significant differences were observed in the sperm quality assessment (Table 2) MANOVA ($p = 0.55$) nor in any of the univariate results obtained for each of the selected parameters when comparing the different treatment groups ($p > 0.05$).

3.5. mRNA expression of vtg1 gene in F_1 zebrafish males after 8 months of EE2 exposure

The mRNA expression of *vtg1* in male liver, at the age of 8 months, was significantly induced in the highest concentration of EE2 (up to 40-fold) in comparison with all other treatments ($p < 0.01$; one-way ANOVA, followed by Fisher LSD multiple comparison test). A trend towards an increase in *vtg1* transcripts was already evident at nominal EE2 concentration of 1 ng/L, although it did not differ significantly from solvent control. The lowest EE2 concentration had no effect on the levels of *vtg1* transcripts (Fig. 2).

Table 2
Sperm quality parameters registered for F_1 males (motility, curvilinear velocity, velocity over and average path and progression) in all tested treatments (mean ± standard deviation).

Treatment	Motility (MOT; %)	Curvilinear velocity (VCL; μm/s)	Velocity over straight line (VSL; μm/s)	Progression (PROG; μm)	Average number of analysed spermatozoa per fish
Control	58.02 ± 17.24	88.92 ± 8.49	56.12 ± 6.37	2952.88 ± 357.51	100
DMSO	48.59 ± 22.04	80.42 ± 5.59	46.79 ± 5.73	2506.29 ± 267.45	127
EE_2 0.5 ng/L	52.96 ± 17.37	86.67 ± 14.11	49.30 ± 9.44	2648.79 ± 528.10	144
EE_2 1 ng/L	37.56 ± 16.12	79.07 ± 9.79	48.98 ± 7.90	2558.38 ± 388.43	88
EE_2 2 ng/L	47.03 ± 13.73	87.60 ± 13.12	51.48 ± 6.22	2733.30 ± 309.58	150

For simplicity purposes, presented values result from the averaged values between the onset of video recording, second 17 post-sperm activation, and second 38, the calculated breakpoint common to all treatments.

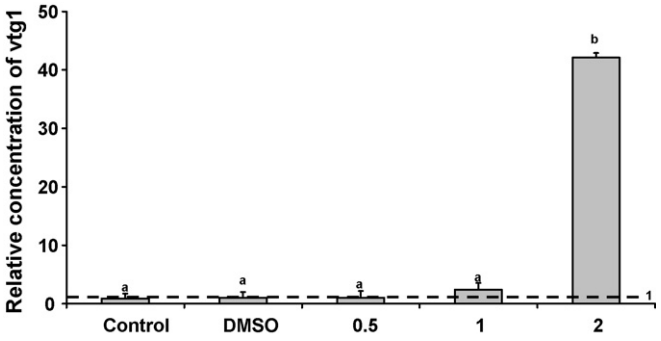

Fig. 2. Normalised fold expression of *vtg1* transcripts in 8 months zebrafish males ($n = 5$) after exposure to different EE2 concentrations (ng/L), solvent control (DMSO) group was used as the reference group (=1-fold). Values are presented as mean ± standard error. Different letters indicate significant differences among treatments ($p < 0.05$, one-way ANOVA, followed by Fisher LSD multiple comparison test).

3.6. Reproductive capacity of F_1

At 8 mpf, fecundity and % viable eggs (1–1.5 hpf) in females exposed to EE2 did not differ significantly from solvent control ($p > 0.05$; one-way ANOVA, followed by Fisher LSD multiple comparison test). However, a trend towards a decrease in fertility was observed in females of the groups exposed to EE2 at 0.5 and 1 ng/L when compared with solvent control females (Table 1).

3.7. Embryogenesis

3.7.1. Mortality

Cumulative mortality rates of embryos at the different phases of embryonic development are displayed in Fig. 3. Between 8 and 80 hpf, mortality rate ranged from 10% (solvent control group) to 60% (2 ng/L EE2 parental exposure). Even though the mortality rate of EE2 parental exposed embryos at 8 hpf did not differ significantly from solvent control, a trend towards an increase in mortality was evident in the highest EE2 treatment group. A significant increase ($p < 0.05$; two-way ANOVA, followed by Fisher LSD multiple comparison test) in the mortality rate of embryos from all EE2 parental exposed groups occurred between 8 hpf and 24 hpf, in a concentration-dependent manner. At 24 hpf, solvent control treatments displayed approximately 10% egg mortality (out of the initial fertilized eggs), whereas 0.5, 1 and 2 ng/L EE2 treatment groups showed, respectively, 25%, 30% and 60% egg mortality. After 24 hpf, cumulative mortality rates remained unchanged up to the end of the embryonic development. The mortality rate in control beakers at 24 hpf was slightly (but significantly) increased if compared to the same treatment at 55 and 80 hpf. This probably reflects the fact that different beakers were used for each time-point observation.

3.7.2. Abnormal development

During the different embryogenesis stages (8, 24, 32, 55 and 80 hpf) the presence of eggs/embryos showing anomalies were recorded within each group (Fig. 4). At 8 hpf, only the group exposed to the highest EE2 concentration showed a significant increase ($p < 0.05$; two-way ANOVA, followed by Fisher LSD multiple comparison test) in the percentage of eggs (approximately 43%) with abnormal development (Fig. 4). Most of these abnormal eggs had not yet entered gastrulation at the 8 hpf, and showed an disorganization of cells at the animal pole, which contrasted with the other treatments where most eggs had reached 65–75% epiboly at 8 hpf. At 8 hpf, the solvent control group showed slightly higher percentage of abnormal egg development if compared with control (not significant, $p > 0.05$). Rather than an effect of the solvent, this pattern seems to reflect an increase in the percentage of abnormal eggs in one out of the four replicates, which explains the high standard error. After 8 hpf, the rate of abnormal eggs/embryos was similar in all treatments and was kept at low levels (usually below 10%). Nevertheless, the percentage of abnormal eggs was increased in EE2 2 ng/L exposures if compared with solvent control at 32 hpf and both control and EE2 1 ng/L groups displayed an increase at 80 hpf if compared with solvent control.

3.8. Heart rate

The cardiac frequency at 32, 55 and 80 hpf was similar in all groups ($p < 0.05$, one-way ANOVA, followed by Fisher LSD multiple comparison test) (data not shown), which indicates that EE2 exposure did not have an effect in the heart rate in zebrafish embryos at the tested concentrations.

4. Discussion

The presence of ECs in the aquatic environment has raised considerable concern during the last decade (Sumpter, 2005). In an attempt to complement field data, and better predict the negative impact of ECs under controlled conditions, several laboratory studies have been performed using model fish species (Sumpter, 2005). The most noticeable effects reported after EE2 exposures were the decrease in fertility and fecundity after partial or full life-cycle exposure (Nash et al., 2004). In zebrafish, most full life-cycle studies on the effects of EE2 report a decrease in the number and percentage of fertilized eggs, at concentrations ranging from 1.1 to

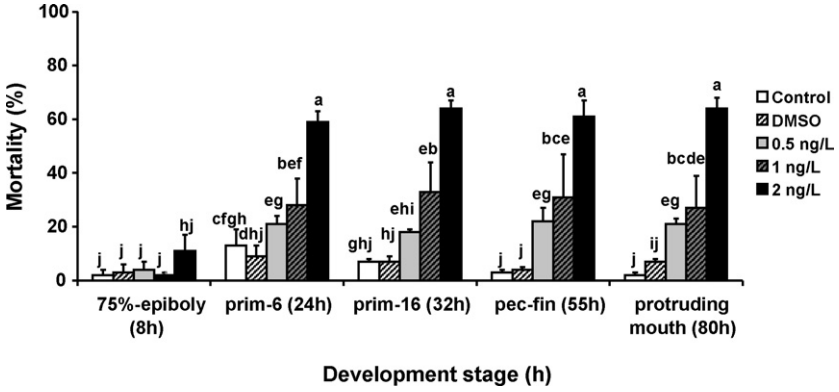

Fig. 3. Percentage of cumulative mortality, at several developmental stages: 75% epiboly (8 hpf); prim-6 (24 hpf); prim-16 (32 hpf); pec-fin (55 hpf) and protruding mouth (80 hpf) of eggs and embryos (F_2), descendents of zebrafish exposed to the different treatments for 8 mpf (F_1). Values presented as mean ± standard error. Different letters indicate significant differences among treatments ($p < 0.05$, two-way ANOVA, followed by Fisher LSD multiple comparison test).

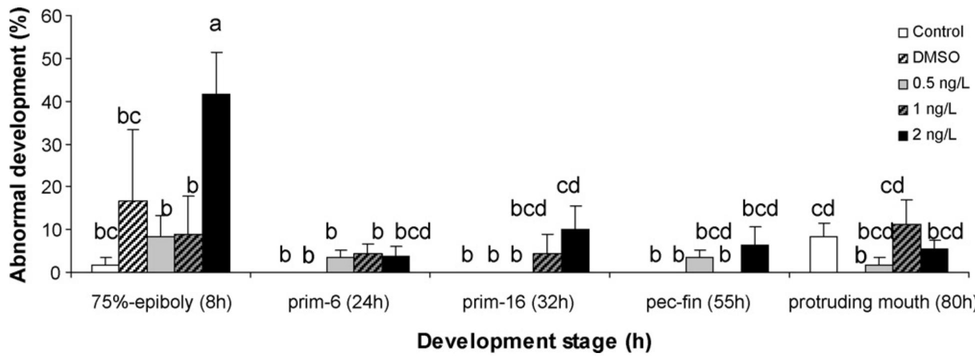

Fig. 4. Percentage of abnormal eggs/embryos (F_2), at several developmental stages: 75% epiboly (8 hpf); prim-6 (24 hpf); prim-16 (32 hpf); pec-fin (55 hpf) and protruding mouth (80 hpf) of offspring of zebrafish exposed to the different EE2 concentrations for 8 mpf (F_1). Values presented as mean ± standard error. Different letters indicate significant differences among treatments ($p < 0.05$, two-way ANOVA, followed by Fisher LSD multiple comparison test).

3 ng/L (Hill and Janz, 2003; Segner et al., 2003; Fenske et al., 2005; Schäfers et al., 2007), which is in agreement with our observations. Similarly, our data on sperm quality parameters, stereological analysis of female gonads and *vtg1* induction corroborates previous findings for the range of EE2 concentrations used in the present study (Hill and Janz, 2003; Xu et al., 2008). However, two recent studies with full life-cycle zebrafish exposure to EE2 have observed a decline in the fecundity and fertilization success at concentrations below the 1 ng/L (Larsen et al., 2008; Xu et al., 2008). These differences among studies may reflect the use of different zebrafish strains and/or exposure periods.

Recent studies indicate that the embryonic development of offsprings of EE2-exposed fishes may also be a target of EE2 (Nash et al., 2004; Brown et al., 2007). Yet, none of the previous studies have evaluated EE2 effects through the entire embryonic development. Hence, identifying the most sensitive embryonal development stage to EE2 is essential when trying to understand the mechanisms of action of this EC. In the present study, parental full life-cycle exposure to EE2 resulted in a concentration-dependent increase in egg mortality between 8 and 24 hpf. During zebrafish development this period includes late gastrulation and early organogenesis (Kimmel et al., 1995). After early organogenesis has been completed (24 hpf), no further mortality was observed (Fig. 3). This demonstrates that EE2 impact in embryo development occurs only up to early organogenesis for the range of concentrations tested in the course of the present study. In accordance with the results of egg mortality, an increase in abnormal egg development in the highest EE2 exposure concentration was observed at 8 hpf. These findings indicate that the abnormal development recorded at 8 hpf, together with the egg mortality observed up to 8 hpf, might explain the increase mortality at the 24 hpf time-point observation. Taken together, the data indicate that the highest EE2 level (actual concentration of 1 ng/L) impacts normal blastula, affects gastrulation and perhaps early organogenesis. In contrast to the highest EE2 exposure group, no increase in abnormal egg development could be recorded at 8 hpf (65–75% epiboly) in the other EE2 exposure groups. This might indicate that the two lowest concentrations of EE2 impact late gastrulation and/or early organogenesis only. In the present study, embryo tests were performed under similar EE2 concentrations as adults, thus mimicking a field scenario where full life-cycle takes place in EE2 contaminated ecosystems. Thus, it could be hypothesized that embryo mortality may also be related to exposure of embryos to EE2 in water. However, this is unlikely to be the case as a previous study with exposure of zebrafish embryos to EE2 concentrations ranging from 1 to 100 ng/L, led to no increase in mortality at 120 hpf (Versonnen and Janssen, 2004). The phase where EE2 disrupts the embryonic development of zebrafish matches the period where the development of zebrafish embryo is most sensitive under normal conditions. In our assay, the overall embryo mortality recorded in the control treatments was approximately 10%, which is within the lowest reported in the literature (Kishida et al., 2001), thus demonstrating the adequacy of the experimental setup. Similar to EE2-exposed embryos, mortality in control treatments was observed only up to 24 hpf, the period where the most important changes in embryo development take place. In a recent study, Brown et al. (2007) reported an increase in rainbow trout (*Onchorynchus mykiss*) embryo mortality at 19 dpf after male parental exposure for 56 days to EE2 at environmentally relevant concentrations (0.8 and 8.3 ng/L). The embryonic development of rainbow trout is longer than that of zebrafish, taking approximately 30 days at 11 °C. At 19 dpf, rainbow trout embryos have already gone through early organogenesis. However, since in the Brown et al. (2007) study no screening in embryo mortality was done between 9 and 19 dpf, the possibility that rainbow trout embryo survival was also impacted at early organogenesis cannot be excluded. This would agree with our findings for the two lowest concentrations of EE2, where an increase in embryonic mortality was observed at 24 hpf. Taken together, these findings indicate that EE2, at actual concentrations below 1 ng/L range, impact the embryonic development of two fish species with different life-strategies; while zebrafish is a short-lived species and fractional spawner, the rainbow trout is a long-lived species and a periodic annual spawner. This indicates that the effects of EE2 on embryonic development may affect fish species with different life strategies, thus suggesting that several wild fish populations may respond in a similar manner.

In a recent male and female zebrafish replacing experiment, Xu et al. (2008) found that male but not female replacement rescued embryos from mortality at 12 hpf if the parental population were exposed to EE2 from egg to 3-month-old fish. This led us to conduct a more detailed screening of male testis histology and spermatozoan quality at adulthood (8 months) of F_1 generation. At the end of the exposure period, no apparent differences could be observed in gonad histology between treatments. Furthermore, both stereological analysis of the testis and the tested sperm quality parameters did not differ among treatments. Similarly, no differences could be observed in female F_1 gonads at 8 mpf. Hence, the reduced embryo survival observed in their offspring is probably related with changes on the genetic information carried by male spermatozoa and/or alterations on the expression patterns of certain genes, rather than with the measured functional changes. We have recently observed that zebrafish EE2 exposure at 3.5 ng/L from 5 dpf up to 4 month induced an increase in genotoxic damage in erythrocytes both in adult males and females (Micael et al., 2007). This is consistent with the observation that EE2 hinders nucleotide excision repair in zebrafish liver cells (Notch and Mayer, in press). Likewise, Contractor et al. (2004) have shown that

EE2 induces methylation changes in the estrogen receptor and aromatase genes of medaka (*Oryzias latipes*). More recently, Brown et al. (2008) showed that when male rainbow trout were exposed to EE2 for 50 days during sexual maturation an increase of aneuploidy levels in sperm cells as well as in embryos could be observed. These findings further support the hypothesis that embryo mortality after parental EE2 exposure may be due to DNA damage. Thus, the question of whether this damage may be heritable through genetic or epigenetic mechanisms is of major importance. In mammals, the EC methoxychlor induces reproductive effects that can be inherited by future generations through an epigenetic mechanism (Anway et al., 2005; Crews et al., 2007). In an attempt to evaluate if EE2 effects are heritable in fish, Brown et al. (2009) tested if the offspring produced by male rainbow trout, exposed to EE2 during sexual maturation, showed the same decrease of progeny survival as the parental generation, when raised in water free from EE2. No heritable defects were detected. Whether the same applies to other fish species, particularly when the parental generation is exposed during the entire life-cycle, remains to be investigated.

The screening of *vtg* levels in plasma or *vtg* gene induction in male fish is the most common approach to evaluate the exposure of wild fish populations to ECs (Hutchinson et al., 2006; Ferreira et al., 2009). The current findings of increased embryo mortality at EE2 levels below those inducing reproductive impairment and *vtg1* gene induction in the male parental generation show that environmental risk assessment based on *vtg* induction only may not be sufficient to protect wild fish populations from EE2 exposure. Conversely, if our data is extrapolated to wild fish populations, an elevation of *vtg* induction could be an indication of a negative ecological impact. In the present study, a "no observed effect concentration" (NOEC) of EE2 on embryonic survival could not be determined, as the lowest concentration tested (actual EE2 levels of 0.19 ng/L) still significantly increased the mortality of embryos at 24 hpf (150% over the controls). This EE2 level is at present found in several rivers and estuaries in the vicinity of urban areas, thus raising considerable concern. In wild fish populations, the screening of ecological level effects of ECs is not an easy task if there is not a massive decline in population over a few generations (Sumpter, 2005). In fact, most field studies on the effects of ECs have failed to detect an ecological impact in fish populations (Sumpter, 2005). However, in most cases, only gonad histology and VTG induction were evaluated. As the field studies progress, an increasing body of literature indicates the presence of a population-level impact of ECs (Jobling et al., 2002; Kidd et al., 2007). Considering the present study, despite a thorough evaluation of reproductive endpoints and *vtg1* induction in the parental generation, no effects could be observed for the two lowest EE2 exposure levels. Hence, if our study reflected a real case scenario, and embryonic survival of the offspring would not have been screened, we could easily have missed the negative effects that could lead to population-level impacts. In the wild, many factors, such as predation and food availability, may interfere with embryo survival. Thus, future studies should investigate the population-level impact of reduced embryo survival after parental EE2 exposure, in comparison to other important causes of embryo mortality. Nevertheless, our results strongly support the inclusion of embryonic development studies in the screening of ED in wild fish populations.

Overall, the present study shows that life-cycle exposure of zebrafish to very low levels of EE2 severely impacts embryo survival at concentrations below those inducing a significant increase in *vtg1* gene transcripts in parental males. The window of embryo sensitivity to EE2 matches the most sensitive phase of zebrafish embryonic development under normal conditions, with the two lowest EE2 concentrations impacting late gastrulation and/or early organogenesis, whereas exposure to nominal 2 ng/L EE2 impact blastula and gastrula phases. Future studies should focus on the molecular changes upon EE2 exposure during embryonic blastula, gastrula and early organogenenis phases, as well as identifying the period within the 8 and 24 hpf where zebrafish embryos are more sensitive to very low levels (below 0.5 ng/L) of EE2. Likewise, the screening of male gonad DNA at adulthood may help to understand the mechanisms of EE2 disruption, and reveal more sensitive biomarkers of EE2 exposure based on adverse effect end points which can be directly related with population dynamics.

Acknowledgements

Nuno Monteiro's participation was funded by Fundação para a Ciência e a Tecnologia (FCT-SFRH/BPD/14992/2004), Ana Coimbra (FCT-SFRH/BPD/24439/2005) and Joana Soares (FCT-SFRH/BD/30211/2006). This work was funded by Fundação para a Ciência e a Tecnologia (POCI/MAR/60895/2004).

References

Ankley, G.T., Johnson, R.D., 2004. Small fish models for identifying and assessing the effects of endocrine-disrupting chemicals. ILAR J. 45, 469–483.
Anway, M.D., Cupp, A.S., Uzumcu, M., Skinner, M.K., 2005. Epigenetic transgenerational actions of endocrine disruptors and male fertility. Science 308, 1466–1469.
Ashby, J., Houthoff, E., Kennedy, S.J., Stevens, J., Bars, R., Jekat, F.W., Campbell, P., Van Miller, J., Carpanini, F.M., Randall, G.L., 1997. The challenge posed by endocrine-disrupting chemicals. Environ. Health Perspect. 105, 164–169.
Belfroid, A.C., Van der Horst, A., Vethaak, A.D., Schäfer, A.J., Rijs, G.B., Wegener, J., Cofino, W.P., 1999. Analysis and occurrence of estrogenic hormones and their glucuronides in surface water and waste water in The Netherlands. Sci. Total Environ. 225, 101–108.
Bigsby, R., Chapin, R.E., Daston, G.P., Davis, B.J., Gorski, J., Gray, L.E., Howdeshell, K.L., Zoeller, R.T., vom Saal, F.S., 1999. Evaluating the effects of endocrine disruptors on endocrine function during development. Environ. Health Perspect. 107, 613–618.
Brown, K.H., Schultz, I.R., Nagler, J.J., 2007. Reduced embryonic survival in rainbow trout resulting from paternal exposure to the environmental estrogen 17 alpha-ethynylestradiol during late sexual maturation. Reproduction 134, 659–666.
Brown, K.H., Schultz, I.R., Cloud, J.G., Nagler, J.J., 2008. Aneuploid sperm formation in rainbow trout exposed to the environmental estrogen 17(alpha)-ethynylestradiol. Proc. Natl. Acad. Sci. U.S.A. 105, 19786–19791.
Brown, K.H., Schultz, I.R., Nagler, J.J., 2009. Lack of a heritable reproductive defect in the offspring of male rainbow trout exposed to the environmental estrogen 17alpha-ethynylestradiol. Aquat. Toxicol. 91, 71–74.
Carvalho, A.P., Araújo, L., Santos, M.M., 2006. Rearing zebrafish (*Danio rerio*) larvae without live food: evaluation of a commercial, a practical and a purified starter diet on larval performance. Aquaculture Res. 37, 1107–1111.
Contractor, R.G., Foran, C.M., Li, S.F., Willett, K.L., 2004. Evidence of gender- and tissue-specific promoter methylation and the potential for ethinylestradiol-induced changes in japanese medaka (*Oryzias latipes*) estrogen receptor and aromatase genes. J. Toxicol. Environ. Health A 67, 1–22.
Crews, D., Gore, A.C., Hsu, T.S., Dangleben, N.L., Spinetta, M., Schallert, T., Anway, M.D., Skinner, M.K., 2007. Transgenerational epigenetic imprints on mate preference. Proc. Natl. Acad. Sci. U.S.A. 104, 5942–5946.
Desbrow, C., Routledge, E.J., Brighty, G.C., Sumpter, J.P., Waldock, M., 1998. Identification of estrogenic chemicals in STW effluent. 1. Chemical fractionation and in vitro biological screening. Environ. Sci. Technol. 32, 1549–1558.
Fenske, M., Maack, G., Schäfers, C., Segner, H., 2005. An environmentally relevant concentration of estrogen induces arrest of male gonad development in zebrafish, *Danio rerio*. Environ. Toxicol. Chem. 24, 1088–1098.
Ferreira, F., Santos, M.M., Castro, L.F.C., Reis-Henriques, M.A., Lima, D., Vieira, N.M., Monteiro, N.M., 2009. Vitellogenin gene expression in the intertidal blenny *Lipophrys pholis*: a new sentinel species for estrogenic chemical pollution monitoring in the European Atlantic coast? Comp. Biochem. Physiol. C. 149, 58–64.
Folmar, L.C., Hemmer, M., Hemmer, R., Bowman, C., Kroll, K., Denslow, N.D., 2000. Comparative estrogenicity of estradiol, ethynyl estradiol and diethylstilbestrol in an in vivo, male sheepshead minnow (*Cyprinodon variegatus*), vitellogenin bioassay. Aquat. Toxicol. 49, 77–88.
Freere, R.H., Weibel, E.R., 1967. Stereologic techniques in microscopy. J. Royal Microsc. Soc. 87, 25–34.
Guillette, L.J., 1995. Organization versus activation—the role of endocrine-disrupting contaminants (EDCS) during embryonic-development in wildlife. Environ. Health Perspect. 103, 157–164.
Hill Jr., R.L., Janz, D.M., 2003. Developmental estrogenic exposure in zebrafish (*Danio rerio*). I. Effects on sex ratio and breeding success. Aquat. Toxicol. 63, 417–429.
Hutchinson, T.H., Ankley, G.T., Segner, H., Tyler, C.R., 2006. Screening and testing for endocrine disruption in fish-biomarkers as "signposts," not "traffic lights," in risk assessment. Environ. Health Perspect. 114, 106–114.
Iguchi, T., Watanabe, H., Katsu, Y., 2006. Application of ecotoxicogenomics for studying endocrine disruption in vertebrates and invertebrates. Environ. Health Perspect. 114, 101–105.

Jobling, S., Coey, S., Whitmore, J.G., Kime, D.E., Van Look, K.J., McAllister, B.G., Beresford, N., Henshaw, A.C., Brighty, G., Tyler, C.R., Sumpter, J.P., 2002. Wild intersex roach (*Rutilus rutilus*) have reduced fertility. Biol. Reprod. 67, 515–524.

Johnson, A.C., Belfroid, A., Di Corcia, A.D., 2000. Estimating steroid oestrogen inputs into activated sludge treatment works and observations on their removal from the effluent. Sci. Total Environ. 256, 163–173.

Kidd, K.A., Blanchfield, P.J., Mills, K.H., Palace, V.P., Evans, R.E., Lazorchak, J.M., Flick, R.W., 2007. Collapse of a fish population after exposure to a synthetic estrogen. Proc. Natl. Acad. Sci. U.S.A. 104, 8897–8901.

Kimmel, C.B., Ballard, W.W., Kimmel, S.R., Ullmann, B., Schilling, T.F., 1995. Stages of embryonic development of the zebrafish. Dev. Dyn. 203, 253–310.

Kishida, M., McLellan, M., Miranda, J.A., Callard, G.V., 2001. Estrogen and xenoestrogens upregulate the brain aromatase isoform (P450aromB) and perturb markers of early development in zebrafish (*Danio rerio*). Comp. Biochem. Physiol. B 129, 261–268.

Kolpin, D.W., Furlong, E.T., Meyer, M.T., Thurman, E.M., Zaugg, S.D., Barber, L.B., Buxton, H.T., 2002. Pharmaceuticals, hormones, and other organic wastewater contaminants in U.S. streams, 1999–2000: a national reconnaissance. Environ. Sci. Technol. 36, 1202–1211.

Lai, K.M., Scrimshaw, M.D., Lester, J.N., 2002. The effects of natural and synthetic steroid estrogens in relation to their environmental occurrence. Crit. Rev. Toxicol. 32, 113–132.

Länge, R., Hutchinson, T.H., Croudace, C.P., Siegmund, F., Schweinfurth, H., Hampe, P., Panter, G.H., Sumpter, J.P., 2001. Effects of the synthetic estrogen 17 alpha-ethinylestradiol on the life-cycle of the fathead minnow (*Pimephales promelas*). Environ. Toxicol. Chem. 20, 1216–1227.

Larsen, M.G., Hansen, K.B., Henriksen, P.G., Baatrup, E., 2008. Male zebrafish (*Danio rerio*) courtship behaviour resists the feminising effects of 17alpha-ethinyloestradiol morphological sexual characteristics do not. Aquat. Toxicol. 87, 234–244.

Metcalfe, C.D., Metcalfe, T.L., Kiparissis, Y., Koenig, B.G., Khan, C., Hughes, R.J., Croley, T.R., March, R.E., Potter, T., 2001. Estrogenic potency of chemicals detected in sewage treatment plant effluents as determined by in vivo assays with Japanese medaka (*Oryzias latipes*). Environ. Toxicol. Chem. 20, 297–308.

Micael, J., Reis-Henriques, M.A., Carvalho, A.P., Santos, M.M., 2007. Genotoxic effects of binary mixtures of xenoandrogens (tributyltin, triphenyltin) and a xenoestrogen (ethinylestradiol) in a partial life-cycle test with Zebrafish (*Danio rerio*). Environ. Int. 33, 1035–1039.

Mills, L.J., Chichester, C., 2005. Review of evidence: are endocrine-disrupting chemicals in the aquatic environment impacting fish populations? Sci. Total Environ. 343, 1–34.

Monteiro, S.M., Rocha, E., Mancera, J.M., Fontaínhas-Fernandes, A., Sousa, M., 2009. A stereological study of copper toxicity in gills of *Oreochromis niloticus*. Ecotoxicol. Environ. Saf. 72, 213–223.

Nash, J.P., Kime, D.E., Van der Ven, L.T., Wester, P.W., Brion, F., Maack, G., Stahlschmidt-Allner, P., Tyler, C.R., 2004. Long-term exposure to environmental concentrations of the pharmaceutical ethynylestradiol causes reproductive failure in fish. Environ. Health Perspect. 112, 1725–1733.

Notch, E.G., Mayer, G.D., in press. 17alpha-Ethinylestradiol hinders nucleotide excision repair in zebrafish liver cells. Aquat. Toxicol. 10.1016/j.aquatox.2009.01.001.

Oberemm, A., 2000. The use of a refined zebrafish embryo bioassay for the assessment of aquatic toxicity. Lab. Animal 29, 32–40.

Sanderson, J.T., 2006. The steroid hormone biosynthesis pathway as a target for endocrine-disrupting chemicals. Toxicol. Sci. 94, 3–21.

Santos, M.M., Micael, J., Carvalho, A.P., Morabito, R., Booy, P., Massanisso, P., Lamoree, M., Reis-Henriques, M.A., 2006. Estrogens counteract the masculinizing effect of tributyltin in zebrafish. Comp. Biochem. Physiol. C 142, 151–155.

Schäfers, C., Teigeler, M., Wenzel, A., Maack, G., Fenske, M., Segner, H., 2007. Concentration- and time-dependent effects of the synthetic estrogen, 17alpha-ethinylestradiol, on reproductive capabilities of the zebrafish, *Danio rerio*. J. Toxicol. Environ. Health A 70, 768–779.

Segner, H., Navas, J.M., Schäfers, C., Wenzel, A., 2003. Potencies of estrogenic compounds in in vitro screening assays and in life cycle tests with zebrafish *in vivo*. Ecotoxicol. Environ. Saf. 54, 315–322.

Segner, H., Eppler, E., Reinecke, M., 2006. The impact of environmental hormonally active substances on the endocrine and immune system of fish. In: Reinecke, M., Zaccone, G., Kapoor, B.G. (Eds.), Fish Endocrinology, vol. 2. Elsevier Science, New York, pp. 809–866.

Sumpter, J.P., 2005. Endocrine disrupters in the aquatic environment: an overview. Acta Hydrochem. Hydrobiol. 33, 9–16.

Svenson, A., Örn, S., Allard, A.S., Viktor, T., Parkkonen, J., Olsson, P.E., Förlin, L., Norrgren, L., 2002. Estrogenicity of domestic and industrial effluents in Sweden. Aquat. Ecosyst. Health Manage. 5, 423–434.

Ternes, T.A., Stumpf, M., Mueller, J., Haberer, K., Wilken, R.D., Servos, M., 1999. Behavior and occurrence of estrogens in municipal sewage treatment plants. I. Investigations in Germany, Canada and Brazil. Sci. Total Environ. 225, 81–90.

Thorpe, K.L., Cummings, R.I., Hutchinson, T.H., Scholze, M., Brighty, G., Sumpter, J.P., Tyler, C.R., 2003. Relative potencies and combination effects of steroidal estrogens in fish. Environ. Sci. Technol. 37, 1142–1149.

Versonnen, B.J., Janssen, C.R., 2004. Xenoestrogenic effects of ethinylestradiol in zebrafish (*Danio rerio*). Environ. Toxicol. 19, 198–206.

Weber, L.P., Hill Jr., R.L., Janz, D.M., 2003. Developmental estrogenic exposure in zebrafish (*Danio rerio*). II. Histological evaluation of gametogenesis and organ toxicity. Aquat. Toxicol. 63, 431–446.

Westerfield, M., 2000. The Zebrafish Book—A Guide for the Laboratory Use of Zebrafish (*Danio rerio*), fourth ed. University of Oregon Press, Eugene.

Wilson-Leedy, J.G., Ingermann, R.L., 2007. Development of a novel CASA system based on open source software for characterization of zebrafish sperm motility parameters. Theriogenology 67, 661–672.

Xu, H., Yang, J., Wang, Y., Jiang, Q., Chen, H., Song, H., 2008. Exposure to 17alpha-ethynylestradiol impairs reproductive functions of both male and female zebrafish (*Danio rerio*). Aquat. Toxicol. 88, 1–8.