

AN ENVIRONMENTALLY RELEVANT CONCENTRATION OF ESTROGEN INDUCES ARREST OF MALE GONAD DEVELOPMENT IN ZEBRAFISH, *DANIO RERIO*

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Abstract—The aim of the present study was to elucidate how full life-cycle exposure to estrogens impacts zebrafish development and reproduction, compared to partial life-cycle exposure only, and whether the estrogen-induced effects in zebrafish are reversible or irreversible. Zebrafish were exposed in a flow-through system to an environmentally relevant concentration (3 ng/L) of the synthetic estrogen 17 α -ethinylestradiol (EE₂) either from fertilization until the all-ovary stage of gonad development (i.e., 42 d postfertilization [DPF] in our experiment) or from fertilization until the reproductive stage (i.e., 118 DPF). Reversibility of the estrogen-induced effects was assessed after 58 d of depuration in EE₂-free water until 176 DPF. Early life exposure led to a lasting induction of plasma vitellogenin (VTG) in adult females but altered neither the sex ratio nor the reproductive capabilities. Full life-cycle exposure resulted in elevated VTG concentrations and caused gonadal feminization in 100% of exposed fish and thus inhibited reproduction. Two types of ovaries were observed in continuously exposed adult fish, immature ovaries with primary growth stage oocytes only and mature ovaries containing the full range of all oocyte maturation stages. Fish with immature ovaries had plasma VTG levels like control males, while fish with mature ovaries had female-like VTG levels. The effects of full life cycle exposure were at least partly reversible, and 26% of fish of the previous all-female cohort developed fully differentiated testes. These findings suggest that continuous estrogen exposure had arrested the developmental transition of the gonads of genetic males from the early all-ovary stage to functional testes. After the exposure had ceased, however, these males apparently were able to accomplish testicular differentiation.

Keywords—Zebrafish Endocrine disruption Estrogen Life-cycle exposure Arrested testis differentiation

INTRODUCTION

It has been demonstrated in a number of studies that short-term exposure of fish to (xeno-) estrogens can induce activation, that is, transitory, responses such as vitellogenin (VTG) induction (e.g., [1–7]). However, to fully understand the potency of environmental estrogens to disrupt the development and the reproductive function of fish, not only activation but also lasting (i.e., organizational) effects have to be assessed [8,9]. Experimentally, organizational effects may be evaluated either by short-term exposure during critical periods of development or by long-term exposure during the full life cycle. Fish species with a relatively short life cycle are most favored for full life-cycle approaches, as their use shortens the duration of experimental studies on development and reproduction, thus making fish tests more manageable and cost effective. One of the candidate species under consideration for tests on endocrine-disrupting compounds is the zebrafish (*Danio rerio*). This species develops from fertilization to the reproductive stage within three to four months only, and it can be stimulated to breed throughout the year. This short life cycle is a main asset of the zebrafish as a model species for assessing developmental and reproductive effects of endocrine-disrupting compounds. The sexual differentiation of zebrafish differs from other species used in endocrine-disrupting compounds testing, such as fathead minnow (*Pimephales pimephales*) or

medaka (*Oryzias latipes*) in that zebrafish develop not as a differentiated gonochorist but as a “juvenile hermaphrodite” or undifferentiated gonochorist [10,11]. During ontogeny, zebrafish, regardless of the genetic sex, pass through a non-functional female phase when the gonads of the developing fish consists solely of primary growth stage oocytes. The non-functional ovaries later resolve into either functional ovaries or testes. It is not clear whether this distinctive type of sexual differentiation is of importance for the response of zebrafish to estrogen exposure. Several studies have investigated the effects of estrogens on zebrafish, but they performed partial life-cycle studies only, exposing either during early life stages [12–15] or during the adult stage [16]. None of these studies has evaluated the effects of full life-cycle exposure. The present study goes beyond the state of current knowledge of estrogenic responses of zebrafish. It investigates the effects of full life cycle estrogen exposure on vitellogenin, gonadal differentiation, and reproduction and compares the responses with those after early life exposure. The study also researches into the reversibility or irreversibility of the estrogen-induced effects and aims to verify the importance of the undifferentiated gonochorism in zebrafish on the nature of its response to estrogens. The synthetic steroid ethinylestradiol (EE₂) was selected as the test chemical. One of the most commonly used oral contraceptives is EE₂. In Europe and America, EE₂ has been detected in many sewage treatment work effluents and in many surface waters at concentrations within the ng/L range [17–20]. The reported EE₂ concentrations in effluents range from nondetectable up to 62 ng/L [17–20], while the reported surface water concentrations ranged from below limit of de-

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tection up to 15 ng/L [19,21,22]. Thus, the concentration of 3 ng EE₂/L used in the present study can be considered environmentally relevant. Further, according to the results of a previous study of our group [23] on the concentration dependency of chronic EE₂ effects on zebrafish, 3 ng EE₂/L is above the lowest-observed-effect concentration value for reproductive effects. Zebrafish were exposed in flow-through systems either from fertilization until the reproductive stage (i.e., full life cycle) or from fertilization until the nonfunctional ovary stage. The endpoints used to assess the impact of EE₂ on (sexual) development and reproduction comprised survival, vitellogenin gonadal differentiation, fecundity (female egg production), and fertilization success.

MATERIALS AND METHODS

The early life stage and the life-cycle test were performed with the zebrafish (*D. rerio*) at the Fraunhofer Institute for Molecular Biology and Applied Ecology in Schmallenberg, Germany. The experimental design of this study was based on results from a previously performed multigeneration study in which concentration-dependent effects of EE₂ exposure on zebrafish reproductive capability were examined. The study revealed a lowest-observed-effect concentration value for EE₂ effects on zebrafish reproduction of measured 1.1 ng EE₂/L (nominal 1.67 ng/L) [23]. For the induction of plasma VTG in adult male fish, the lowest-observed-effect concentration was at nominal 1.67 ng EE₂/L [5]. These findings led to the selection of 3 ng EE₂/L as the exposure concentration to be used in the present study.

Culture and husbandry of fish

Zebrafish (*D. rerio*) were derived from the breeding stock of the Fraunhofer Institute for Molecular Biology and Applied Ecology in Schmallenberg, Germany. The zebrafish strain used descended from the West Aquarium (Bad Lauterberg, Germany). Fish were kept in tap water filtered with activated charcoal and passed through a limestone column. Water temperature in the breeding stock tanks was kept at 26 ± 1°C by heating elements. Breeding and all exposure experiments were conducted in temperature controlled rooms with the temperature kept between 24 and 26°C. Spawning eggs were collected in glass spawning trays, covered by lattice lids (stainless steel), and placed into the tanks. Artificial spawning trees (i.e., commercial plastic water plants that had been leached out for several days in separate tanks of the flow-through system) were attached to the lids to initiate spawning.

Under experimental conditions fish were maintained in glass test tanks placed in a temperature-controlled water bath, providing for a consistent water temperature in all tanks during test conduction. All vessels were aerated individually. During the first period of test, until 42 DPF, glass tanks of 29 × 22 × 21 cm (length × depth × height; total volume = 13.4 L) with inserted cages of 20 × 9 × 9 cm (length × depth × height) for the eggs were used as test vessels. The embryos and larvae were kept in the cages before the cages were removed and the larvae released into the 13.4-L glass tanks two to three weeks postfertilization. At the end of the early life stage period at 42 DPF, juvenile fish were transferred from the 13.4-L tanks into 29-L tanks (40 × 27 × 27 cm; length × depth × height) and kept in these tanks until the end of the study. All fish were held under the same photoperiodic regime of 12:12-h light:dark. Fish were fed twice a day, once with TetraMin® dry flake food (Tetra, Melle, Germany) ad libitum

and once with *Artemia* sp. nauplii as live food supplement. Shortly after hatch, fry were fed twice daily with a commercial larval food AZ 100 (Tetra) until the end of the yolk-sac stage. The AZ 100 diet had gradually been replaced by TetraMin and was supplemented with live *Artemia* sp. nauplii.

Chemical analysis of the water

Chemical analysis of the test substance and concentrations was performed at the Fraunhofer Institute (Schmallenberg, Germany). The limit of detection for the EE₂ was 0.05 ng/L (signal-to-noise ratio 1:10) [24]. Samples from all test replicates, including controls, were taken every fourth day, acidified (100 µl of 30% hydrochloric acid/L) for preservation and extracted by solid-phase extraction on 3M EMPORE® C18 extraction discs (Varian, Palo Alto, CA, USA). Analysis of EE₂ was performed by ion trap gas chromatography/mass spectrometry [24] after derivatization with *N*-methyl-*N*-(trimethylsilyl)trifluoroacetamide (Sigma-Aldrich, Steinheim, Germany). Quantification was achieved by comparison to the internal calibration standards (17α-ethinylestradiol-2,4,16,16-d₄) EE₂-D₄ (C/D/N Isotopes, Pointe-Claire, Quebec, Canada).

Water temperature, pH value, ammonia, and dissolved oxygen were monitored throughout the studies. Temperature, dissolved oxygen content, and pH in all test tanks were measured every third day during the whole test periods with portable digital pH and oxygen meters (WTW Wissenschaftlich-Technische Werkstätten, Weilheim, Germany). Ammonia was determined by the use of a colorimetric ammonia test kit (Aqua-merck®, Merck, Darmstadt, Germany).

Chemical dosing and water quality

The tests with EE₂ were carried out in a flow-through system. The test substance 17α-ethinylestradiol (17α-ethinyl-1,3,5 [10]-estratriene-3, 17β-diol; 98% purity) was obtained from Sigma-Aldrich (Steinheim, Germany). For the dosage of EE₂ during exposure, primary stock solutions of 1 g/L EE₂ were prepared in acetone (100%), and 250 µl of the primary stock solution were transferred into a 250-ml volumetric glass flask that was then topped up with sterile pure water and stirred for 24 h in the dark to achieve a nominal concentration of 1 mg/L EE₂. With this stock, a final stock solution of EE₂ (30 µg/L) was made up in pure water and filled into glass syringes (B. Braun Melsungen AG, Melsungen, Germany). By means of electric propulsion, the EE₂ stock was dosed with 500 µl/h from the syringe into mixing/dilution vessels (DURAN®-Woulff bottles, Schott AG, Mainz, Germany) in which the test concentration of 3 ng/L was made up by an adequate inflow of dilution water (5 L/h). Dilution water was fed from an elevated reservoir tank via flow control devices. The control tanks contained dilution water only. A solvent control was not used because the maximum fractional concentration of solvent (acetone) in the EE₂ exposure tanks at any time was 3 nl/L (v/v), and at this concentration no solvent-derived effects were to be expected. Aqueous EE₂ stock solution was renewed weekly; syringes were refilled every third day. Flow rates in the test tanks were adjusted to provide two tank volume changes each day.

During the course of the study, the temperature in the test tanks was 25.6 ± 0.35°C, and the pH value ranged between 7.6 and 8.1. Dissolved oxygen in the test tanks was between 6.0 and 8.6 mg/L. The mean measured concentrations of EE₂ per treatment group, analyzed regularly during the course of

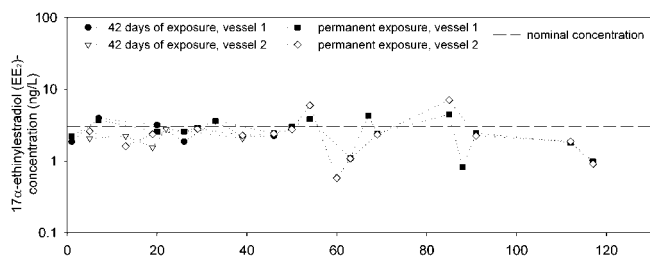


Fig. 1. Measured concentrations of 17 α -ethinylestradiol (EE₂) during exposure periods of 42 and 118 d postfertilization. The nominal concentration was 3 ng/L of EE₂ (dashed horizontal line). The EE₂ was measured by ion trap gas chromatography/mass spectrometry/mass spectrometry.

the experiment, ranged between 80 and 120% of the nominal 3 ng/L (see Fig. 1).

Experimental design of the exposure studies

The experimental design for the life-cycle exposure of zebrafish to 3 ng EE₂/L is shown in Figure 2. For each treatment and control group and for each sampling time, two replicate tanks were used, and each tank was initially stocked with 120 fertilized eggs. The early life stage exposure started 2 h post-fertilization and was conducted according to the Organization for Economic Cooperation and Development Guideline 210 [25] but extended until 42 DPF, when the nonfunctional "all-ovary" developmental stage [11] was reached. At 42 DPF, the stocking density in each tank was randomly reduced to 60 fish; tanks with less than 60 fish remaining were restocked with fish from the replicate tank. Exposure was ceased in two replicate tanks each at 42 and 75 DPF, and fish were then maintained in flow-through of uncontaminated (EE₂-free) water until the end of exposure at 118 DPF. At 75 DPF, the number of fish per tank was reduced once again, and the test was continued with 30 fish per tank until the onset of reproduction. As soon as regular spawning success had been observed in the control replicates, reproductive performance was assessed quantitatively: Fecundity and fertilization capacity were recorded daily over a period of 19 to 22 consecutive days between day 92 and day 114.

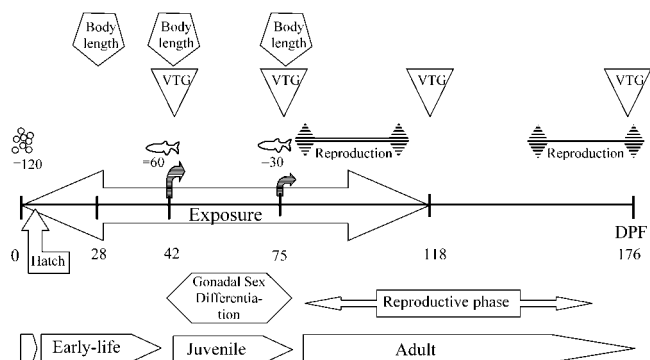


Fig. 2. Experimental scheme: The figure shows the time course of 17 α -ethinylestradiol exposure (upper part of the figure) and the respective developmental period of zebrafish (lower part of the figure). Fish were exposed to 3 ng 17 α -ethinylestradiol/L from fertilization to either 42 d postfertilization (DPF) or 118 DPF; after these periods, the fish were kept until 176 DPF in noncontaminated water. The fish symbols indicate the reduction of the numbers of fish per tank. The inverted triangles and trapezoids indicate the time points of sampling for the different effect endpoints (as indicated). For further details, refer to the description in the text. VTG = vitellogenin.

At 118 DPF, exposure was ceased and the full life cycle experiment terminated for most of the tanks except on replicate control and one exposure tank. These fish were maintained in uncontaminated water for depuration. Fecundity and fertilization success were determined in these tanks between 146 and 176 DPF. The test was completed at 176 DPF.

Effect endpoints

Survival and body length were determined at 28, 42, and 75 DPF. The individual body size of fish was measured by image analysis of digital photographs of the fish.

Vitellogenin analysis

After 42, 75, and 118 d of EE₂ exposure and at the end of study at 176 DPF, fish were sampled from all treatment groups for the quantification of VTG and for the analysis of gonad histology. Before sampling, fish were anaesthetized either in an aqueous solution of benzocaine (Sigma-Aldrich) (~0.04–0.08 g/L) or in ice water, and each specimen was measured and weighed. Because of the small size of fish, plasma collection was not feasible at 42 DPF. Instead fish were decapitated, and trunks were transferred to 2.0-ml Eppendorf tubes (Hamburg, Germany) and snap frozen in liquid nitrogen for later analysis of VTG. From 75 DPF onward, blood was collected from fish by cardiac puncture into chilled heparinized 1.0-ml syringes. The blood was diluted in equal volumes (30 μ l at 75 DPF and 50 μ l at 118/176 DPF) of 0.01 M phosphate-buffered saline (PBS) enzyme-linked immunosorbent assay (ELISA) buffer (pH 7.3) containing the protease inhibitor aprotinin (0.7–1.0 trypsin inhibitor units/ml; Merck), centrifuged at 2,900 g for 25 min at 4°C, and the supernatants were withdrawn and snap frozen in liquid nitrogen and stored at –80°C until analyzed for vitellogenin. After blood collection, the fish were either dissected or directly fixed for gonad histology. For the VTG analysis, tissue samples (trunks) were homogenized in ice-cold PBS containing 0.1% Tween-20 (Sigma-Aldrich) PBS-T ELISA buffer in a 1:2 wet-weight:buffer-volume ratio, or 1:3 where appropriate because of the increase in size of fish with age, using a glass homogenizer (motor driven) and centrifuged at 15,000 g for 3 min. The supernatants were collected and immediately analyzed. Vitellogenin was analyzed in the homogenate or plasma samples using a homologous competitive VTG ELISA for zebrafish [5]. Plasma and homogenate samples were directly applied to the assay at appropriate dilutions. Vitellogenin concentrations measured were normalized to the volume (ml) of the corresponding sample, taking the dilution of the sample into account.

Histological analysis

At 75, 118, and 176 DPF, fish were sampled for histology. Body length and weight of fish sampled were recorded, and after blood sampling (at 118 and 176 DPF), fish were dissected and gonads fixed for 24 h in Bouin's fluid. At 75 DPF, fish were not dissected but fixed as whole or decapitated specimens. Fixed tissues were dehydrated through a graded series of ethanol and embedded in paraffin wax (Leica Histowax, Leica Microsystems, Wetzlar, Germany). Serial paraffin sections were cut at 7 μ m, collected onto glass slides, and stained with Periodic-Acid Schiff's or hematoxylin-eosin. Analysis of slide preparations was performed with an Olympus BX 60 microscope. The oocytes of the zebrafish were classified according to Selman et al. [26].

Table 1. Survival and body length of control and 17 α -ethinylestradiol-exposed zebrafish after various exposure periods: Exposure was started with 120 fertilized eggs per replicate tank. At 42 d postfertilization (DPF), fish were reduced to 60 per replicate tank, after acquisition of survival and fish length. * = significant difference to control (analysis of variance and *t* test; *¹ *p* = <0.001; *² *p* = 0.05). SD = standard deviation

	Tank	0–28-DPF exposure			28–42-DPF exposure			42–75-DPF exposure	
		Survival (%) (post-fertilization)	<i>n</i>	Mean length (mm) \pm SD	Survival (%)	<i>n</i>	Mean length (mm) \pm SD	Survival (%)	Mean length (mm) \pm SD
Control	0/1	72	86	7.44 \pm 2.49	92	79	13.11 \pm 3.43	97	26.85 \pm 3.81
	0/2	61	73	6.80 \pm 2.55	93	68	12.98 \pm 3.55	97	27.70 \pm 3.05
0–42-DPF exposure	3/1	75	90	7.40 \pm 2.41	82	74	13.09 \pm 3.80	100	26.25 \pm 3.43
	3/2	56	67	8.35* ¹ \pm 2.37	87	58	12.47 \pm 3.35	100	26.60 \pm 3.36
0–75-DPF exposure	4/1	84	101	8.00* ¹ \pm 2.24	91	92	12.00* ² \pm 2.65	100	25.96 \pm 3.45
	4/2	72	86	8.19* ¹ \pm 2.39	90	77	12.81 \pm 3.05	98	25.66 \pm 3.47

Reproductive parameters

To investigate fecundity and fertilization success of mature zebrafish, spawning trays were placed into all test tanks to induce spawning behavior. One hour after the light had gone on in the morning, spawned eggs were collected from the spawning trays and counted. Eggs were examined under a dissecting microscope for cell cleavage to verify fertilization. Egg production and fertilization rate were measured daily for each tank, and total fecundity (as cumulative egg production) was also determined for the whole reproductive test period for each tank. At the end of the experiment, breeding fish were sexed by means of gonad histology, and the number of daily spawned eggs was then related to the number of reproductively active females in each tank.

Statistical analysis

Performing standard transformations of VTG data could not always provide for the assumption of normality and equal variance, and therefore data were analyzed by the less powerful Kruskal–Wallis one-way analysis of variance on ranks, followed by Dunn's method multiple comparison test. Additionally, different groups of exposure were pairwise compared by a Mann–Whitney rank sum test. Data on survival, growth, and reproductive performance were checked for assumptions of normality and homogeneity of variance and then transformed when necessary. Survival rates (as *p* values 0–1) and proportions of fertilized eggs were *z*-transformed (arcsine [square root *p*]) and analyzed for significant differences by analysis of variance, followed by Dunnett's test. Fish length data of replicate tanks were pooled and subjected to analysis of variance and Dunnett's test. In cases of unequal variance, Dunnett's T3 test was performed, and χ^2 analysis was applied to test differences in the ratios of female to male gonads in exposed compared to control fish at different developmental stages. Statistical analyses of data were carried out either in SigmaStat® 2.03 (Systat Software, Erkrath, Germany, formerly Jandel Scientific) or by using the SPSS (Statistical Package for Social Sciences) program package (SPSS, Chicago, IL, USA).

RESULTS

Survival and body length

Hatching success of zebrafish embryos was not affected by EE₂ exposure (data not shown). Within the first 28 DPF, survival of hatched zebrafish varied between 56 and 84% (Table 1), with no significant differences between control and exposed fish. However, body length of EE₂-treated zebrafish was significantly higher at 28 DPF. At 42 and 75 DPF, no significant differences between control and exposed fish were observed in terms of survival or body length (Table 1), except for tank 4/1 in the 0- to 75-DPF exposure group, where the mean body length was reduced.

Gonad histology

75 DPF: Controls. At 75 DPF, 14 out of 24 histologically examined control fish showed gonads with ovarian morphology; the other 10 fish showed gonads with testicular morphology (Table 2). In the ovaries, the larger proportion of the gonad tissue was occupied by perinucleolar oocytes, while oogonia and early oocyte stages were restricted to the caudal and cranial peripheries (Fig. 3a). One individual among the 14 female fish contained cortical alveolar and vitellogenic oocytes in addition to the primary growth stage oocytes and was thus classified as mature ovary (Table 2). Among the 10 fish with male gonads, individual variation in the degree of testicular differentiation was observed, with some fish displaying the full range of sperm cell differentiation stages and others showing early differentiation stages only. The latter testes were classified as immature testes, contrary to mature testes, which contain all stages of sperm maturation.

75 DPF: Exposure to 3 ng EE₂/L from 0 to 42 DPF. Neither the ovary/testis ratio (Table 2) nor the histological appearance of the ovaries (in %) was different from control fish. However, testes were less developed than in control fish; only two out of nine male fish possessed mature testes (Fig. 3b), while the other seven fish had immature testes (Fig. 3c).

75 DPF: Exposure to 3 ng EE₂/L from 0 to 75 DPF. In this group, only ovary-containing fish were observed (Table 2). The ovarian histology did not differ to the control group.

118 DPF: Controls. Fourteen out of 29 histologically ex-

Table 2. Histological appearance of gonads in control zebrafish and in 17 α -ethinylestradiol-exposed zebrafish; *n* = number of animals per treatment (total of two independent replicates; the data of the replicates were combined since they did not differ significantly)

Age	75 DPF ^a			118 DPF			176 DPF		
	Control (<i>n</i> = 24)	0–42-DPF exposure (<i>n</i> = 24)	0–75-DPF exposure (<i>n</i> = 20)	Control (<i>n</i> = 29)	0–42-DPF exposure (<i>n</i> = 30)	0–118-DPF exposure (<i>n</i> = 27)	Control (<i>n</i> = 25)	0–42-DPF exposure (<i>n</i> = 29)	0–118-DPF exposure (<i>n</i> = 27)
% Ovaries/% testes (gonad ratio)	58/42	62.5/37.5	100/0	48/52	56.7/43.3	100/0	48/52	55/45	74/26
No. of fish with immature ovaries ^b	13 (54%)	15 (62.5%)	20 (100%)	0 (0%)	6 (20%)	14 (25%)	0 (0%)	8 (27.5%)	1 (4%)
No. of fish with mature ovaries ^c	1 (4%)	0 (0%)	0 (0%)	14 (48%)	11 (36.7%)	13 (48%)	12 (48%)	8 (27.5%)	19 (70%)
No. of fish with immature testis ^d	4 (17%)	7 (29%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)
No. of fish with mature testis ^e	6 (25%)	2 (8.5%)	0 (0%)	15 (52%)	13 ^f (43.3)	0 (0%)	13 (52%)	13 (45%)	7 ^f (26%)

^a DPF = days postfertilization.

^b Immature ovary: The only oocyte stage found in early ovaries are oogonia and primary growth stage oocytes, mainly perinucleolar oocytes.

^c Mature ovary: The ovary contains, in addition to primary growth stage oocytes, the more advanced stages of oocyte maturation are present (cortical alveolar, vitellogenic, and mature oocytes).

^d Immature testis: Contains mainly spermatogonia, no or few spermatocytes/spermatids, and no spermatozoa.

^e Mature testis: Contains spermatozoa as well and an increased percentage of spermatocytes and spermatids.

^f One male fish with testis-ova; primary growth stage oocytes were intermingled in the testicular tissue.

aminated control fish showed fully developed ovaries containing all stages of oocyte maturation, that is, oogonia, primary growth stage oocytes (stage I), cortical alveolar oocytes (stage II), vitellogenic oocytes (stage III), and preovulatory, maturing oocytes (stage IV) (Fig. 4a). In addition, postovulatory follicles were present. These ovaries were classified as mature ovaries. The other 15 fish of the control group displayed mature testes, containing all stages of sperm maturation, that is, spermatogonia, spermatocytes, spermatids, and spermatozoa in the lumina of the seminiferous tubuli. The number of spermatozoa varied among individuals but was generally much higher than in testes of 75 DPF males.

118 DPF: Exposure to 3 ng EE₂/L from 0 to 42 DPF. From 30 histologically examined fish, 17 individuals possessed ovaries and 13 testes. The histological appearance of the ovaries showed pronounced interindividual variation: In 11 phenotypic females, mature ovaries were observed, whereas in six of the 17 ovary-containing individuals, immature ovaries were found, containing oogonia and primary growth stage oocytes but no vitellogenic or mature oocyte (Fig. 4b). The occurrence of two types of ovaries was visible macroscopically during dissection of the fish: While in the case of mature ovaries numerous eggs were externally visible, immature ovaries appeared small, with whitish tissues and no macroscopically recognizable substructure, and by mistake they could easily be classified as testes.

The differentiated, mature testes contained numerous spermatozoa. One male had testis-ova, where a low number of primary growth stage oocytes dispersed in differentiated testicular tissue.

118 DPF: Exposure to 3 ng EE₂/L from 0 to 118 DPF. In fish continuously exposed to EE₂, all 27 individuals examined possessed ovaries, and none of the fish had gonads of testicular morphology. Again, both mature and immature ovaries were present: Thirteen individuals had developed ovaries, with all oocyte stages and postovulatory follicles, while 14 fish had immature ovaries with exclusively oogonia and primary growth stage oocytes or, in a few individuals, intermingled with single cortical alveolar and early vitellogenic oocytes. The body weight of the individuals with immature ovaries was significantly lower than that of fish with mature ovaries (fish

with mature ovaries: 462 ± 102 mg wet wt; fish with early ovaries: 282 ± 65 mg wet wt). Intersex gonads (i.e., testis-ova) or pathological alterations of ovarian or testicular histology were not observed.

In the 13 fish with mature ovaries, oocyte maturation was less progressed than in mature ovaries of control fish: In ovaries of control fish, the percentages were 33 ± 10% for stage I oocytes, 17 ± 3% for stage II, 33 ± 6% for stage III, and 17 ± 5% for stage IV. In developed ovaries of continuously exposed fish, however, these numbers were 42 ± 6% for stage I, 27 ± 4% for stage II, 21 ± 3% for stage III, and 10 ± 5% for stage IV.

176 DPF: Control. Twenty-five fish were examined histologically, and 13 of these fish displayed fully differentiated testes; 12 fish had mature ovaries (Table 2).

176 d pf: Exposure to 3 ng EE₂/L from 0 to 42 DPF. Thirteen fish of this treatment possessed normally differentiated testes, with all sperm stages being present. The amount of sperm cells in the seminiferous tubuli varied between individual males. The remaining 16 out of 29 fish examined showed gonads with ovarian morphology, whereby eight individuals had mature and the other eight had immature ovaries. Again, the body weight of the latter group (342 ± 81 mg wet wt) was significantly lower than the body weight of fish with mature ovaries (547 ± 114 mg wet wt).

176 DPF: Exposure to 3 ng EE₂/L from 0 to 118 DPF. In this group, six out of 27 fish had fully differentiated testes, and one male displayed testis-ova. The other 20 fish possessed ovaries, of which 19 were developed as ovaries and one ovary was immature, containing oogonia and primary growth stage oocytes but with no further stages of oocyte maturation.

Vitellogenin

Vitellogenin was measured in different sample matrices, either in body homogenates (42 DPF) or in plasma samples (75, 118, 176 DPF). The analysis of VTG levels in zebrafish of 42 and 75 DPF was carried out on individuals of unknown sex. Because of the lack of pronounced sexual dimorphism in this species and because of the mode of gonadal sex differentiation [10,11], it was impossible to identify the sex of non-

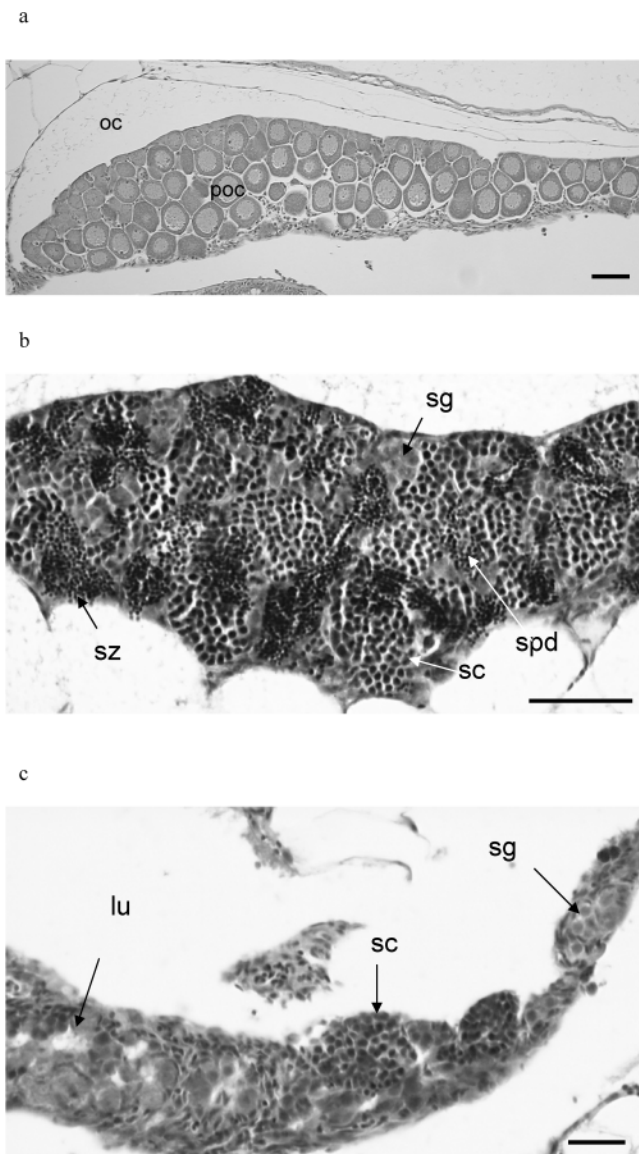


Fig. 3. Gonads of zebrafish at 75 d postfertilization (DPF). The oocytes were staged according to Selman et al. [26]. (a) Immature ovary of a control fish. The ovary contains densely packed previtellogenic oocytes. (b) Testis of a 75-DPF fish exposed to 3 ng 17 α -ethinylestradiol (EE₂)/L from fertilization until 42 DPF. The testis is well differentiated showing all major stages of sperm maturation. (c) Testis of a 75-DPF fish exposed to 3 ng EE₂/L from fertilization until 42 DPF. In this individual, testis differentiation is much less progressed than in the fish shown in (b). Early stages of sperm maturation, particularly spermatogonia, are predominant. Bar equals 50 μ m. oc = ovarian cavity, poc = previtellogenic oocytes; sz = spermatozoa, sc = spermatocyst, sg = spermatogonia, spd = spermatids, lu = lumen.

reproducing fish only by means of their appearance or the external gonad morphology. The gonad histology of these fish could not be examined, as the whole bodies were used up for the VTG analysis. Therefore, the VTG concentrations of 42- and 75-d-old fish represent mean values of samples from both (genetic) male and female fish.

At 42 DPF, no statistical difference was observed in the body homogenate VTG concentrations between the EE₂-exposed and the control fish (Fig. 5). Both in the control and in the exposed groups, a pronounced interindividual variation of VTG concentrations was observed, ranging from values of 0.09 up to 3.36 μ g/ml in the controls and from 0.05 to 7.75

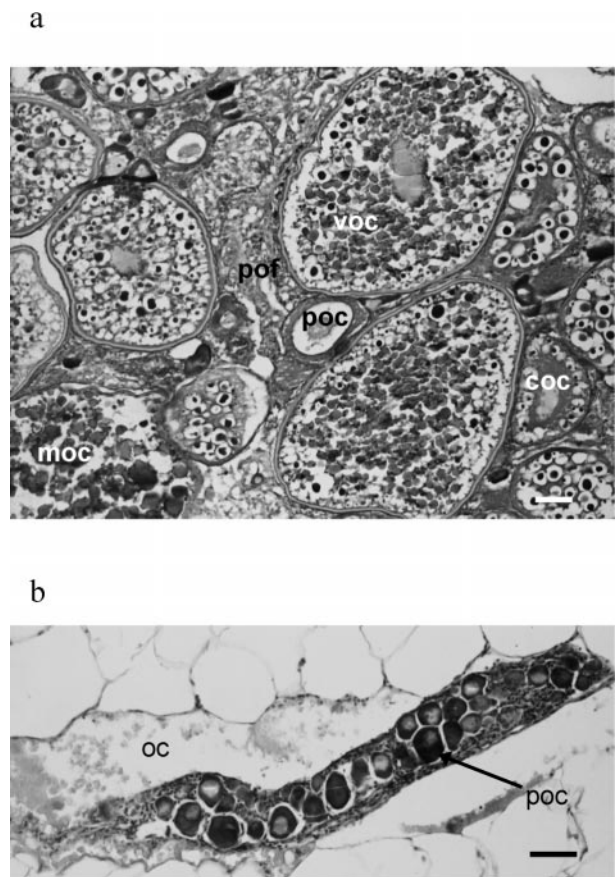


Fig. 4. Gonads of zebrafish at 118 d postfertilization (DPF). The oocytes were staged according to Selman et al. [26]. (a) Part of an ovary of zebrafish reared in control water: All oocyte maturation stages are present. (b) Ovary of a permanently exposed fish. The ovary contains previtellogenic oocytes but no further stages of oocytes. A high number of somatic cells is visible in the ovary. Bar equals 50 μ m. oc = ovarian cavity; poc = previtellogenic oocytes; coc = cortical alveolus stage oocyte; voc = vitellogenic oocyte; moc = mature oocyte; pof = postovulatory follicle.

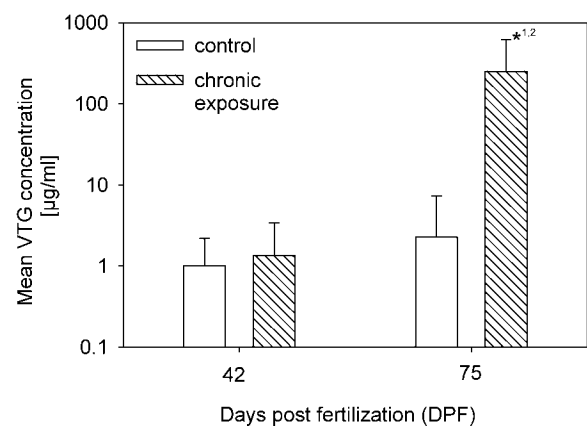


Fig. 5. Vitellogenin (VTG) concentrations in (decapitated) body homogenates of 42 d postfertilization (DPF) zebrafish and in plasma samples of 75-DPF zebrafish, of water controls or of continuous exposure to 3 ng/L of 17 α -ethinylestradiol. Values are means \pm standard deviation. Number of samples (fish) analyzed at 42/75 DPF were $n = 14/n = 17$ (control groups) and $n = 17/n = 19$ (exposure groups). Asterisks (*) indicate mean values that were significantly different from the control group (*^{1,2} $p < 0.001$). y-axis is log scaled.

$\mu\text{g/ml}$ in the exposed fish. Since we could not distinguish between male and female fish at this stage of development, it remained uncertain to what extent the individual VTG levels were linked to the sex of the fish.

In 75-DPF fish continuously exposed to 3 ng EE_2/L , the mean plasma VTG concentration was significantly ($p < 0.05$) elevated over control values (Fig. 5). Interindividual variation was still high, with VTG concentration in exposed fish ranging between 14.76 and 1356.21 $\mu\text{g/ml}$. The continuously EE_2 -exposed and phenotypically feminized adult fish at 118 DPF did not have uniformly female-like VTG levels. A high variability between individual VTG plasma concentrations was found. Closer analysis revealed remarkable agreement between the VTG level and the status of ovarian maturation of individual fish. Seven fish with mature ovaries showed plasma VTG concentrations in the range of 94.8 to 725.8 $\mu\text{g/ml}$, corresponding to VTG concentrations usually found in the control females. Fourteen individuals from this exposure group with immature ovaries had VTG concentrations that resembled those found in estrogen-exposed male zebrafish (5), ranging from nondetectable to 1.5 $\mu\text{g/ml}$. The remaining five fish had ovaries containing mainly primary growth stage oocytes and additionally some vitellogenic oocytes. The VTG concentrations in these fish ranged from 2.4 to 7.02 $\mu\text{g/ml}$. Given these findings, the VTG plasma concentration data of exposed fish, age 118 and 176 DPF, were analyzed differently, and fish were sexed according to their VTG response, regardless of the gonadal sex (Fig. 6). The seven continuously exposed female fish, possessing mature ovaries, showed no significant induction of plasma VTG at 118 DPF (Fig. 6a). Adult female zebrafish exposed to 3 ng EE_2/L from fertilization until 42 DPF only, however, showed significantly elevated plasma VTG levels at 118 DPF (Fig. 6a). In male fish of the same age, VTG was significantly increased after continuous exposure, but between the mean VTG plasma concentrations of 42 DPF-exposed males and the control males, no difference was observed (Fig. 6b). All fish of this exposure group (42 DPF) that showed immature ovaries in the histology (Table 2) had very low, male-like plasma VTG levels, and their VTG response was therefore rated as male response (Fig. 6b).

After depuration, at 176 DPF, plasma VTG concentrations were approaching control levels in most exposed fish, and the agreement between the gonadal sex (Table 2) and the VTG level of individual fish was much higher than at 118 DPF. However, females that were exposed to 3 ng EE_2/L until 42 DPF showed still significantly higher plasma VTG levels than control females. Five fish still contained immature ovaries and displayed a "male" VTG response in the ELISA.

Overall, VTG plasma concentrations measured in EE_2 -exposed fish were between 180 and over 8,600 times higher in females than in males or potential males without differentiated testes. Estrogen exposure led to an induction of VTG in male fish, but even if VTG was induced, the VTG levels measured in such males never reached levels similar to those in females.

Reproduction

Exposure from fertilization until 42 DPF did not alter significantly the initiation of spawning. The first spawning event occurred in both exposure replicates at 83 DPF, while control replicates started spawning between 80 and 82 DPF. The continuously exposed fish, however, never spawned while under exposure (i.e., until 118 DPF). From the time when they were transferred into EE_2 -free water for depuration, a recovery of

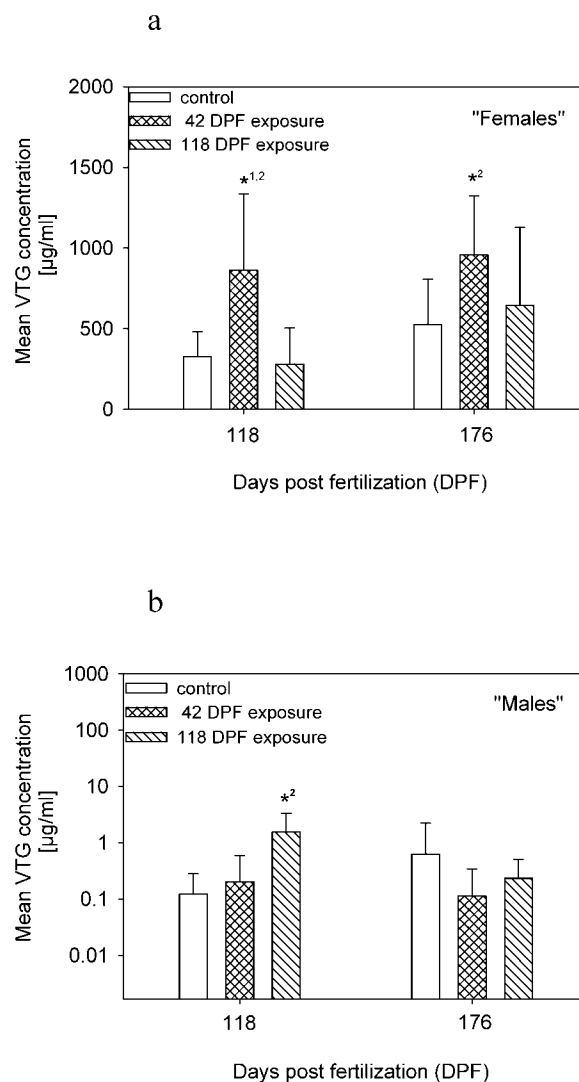


Fig. 6. Vitellogenin (VTG) plasma concentrations in adult zebrafish at 118 and 176 d postfertilization (DPF): Values distinguish between female (a) and male (b) fish for the water control treatment; for fish exposed to 17 α -ethinylestradiol between 0 and 42 DPF or continuously, fish with female-like (a) VTG levels (and mature ovaries) were distinguished from fish with male-like (b) VTG levels (and immature ovaries or testes). Values are means \pm standard deviation. Number of samples (fish) analyzed on days 118/176 postfertilization (PF) were $n = 12/n = 12$ (control females), $n = 16/n = 15$ (control males), $n = 10/n = 8$ (42-DPF exposure females), $n = 20/n = 18$ (42-DPF exposure males), $n = 7/n = 17$ (chronic exposure females), and $n = 21/n = 10$ (chronic exposure males). Asterisks (*) indicate mean values that were significantly different from the control mean ($*p < 0.05$). y-axis in (b) is log scaled.

courtship and mating behavior was observed, and first spawning occurred at 140 DPF.

Once spawning had been established in the various treatments, egg number per female (fecundity) and fertilization success were measured daily between 92 and 114 DPF in the control and in the 0- to 42-DPF EE_2 exposure replicates, then between 146 and 176 DPF again in the control and in the 0- to 118-DPF EE_2 exposure group, as no spawning occurred in this group before the end of exposure. In the control group, a mean number of 24.7 (± 10.7) eggs per female and day was measured in the period between 92 and 114 DPF, with a fertilization success of 90.1% ($\pm 4.67\%$) (Fig. 7). In the group exposed to EE_2 from 0 to 42 DPF, the number of eggs per day

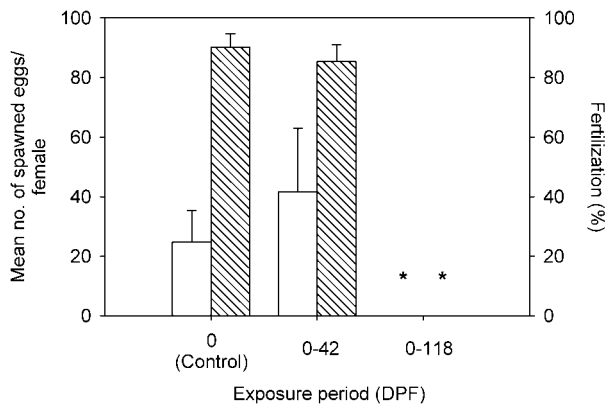


Fig. 7. Mean number of spawned eggs per female and spawning day (bars/left y-axis) and mean proportion of fertilized eggs (expressed as percentage [%]/right y-axis) between 91 and 114 d postfertilization (DPF) in zebrafish control group and treatment groups exposed to 3 ng/L of 17 α -ethinylestradiol for different time periods. Values are means \pm standard deviation. Asterisks (*) indicate mean values that were significantly different from the control mean (* p < 0.001).

and female was not significantly different to control fish, and no differences were observed in the fertilization success ($85.3\% \pm 5.8\%$ fertilization compared to 90.1% in the control) (Fig. 7). In fish exposed from 0 to 118 DPF, the egg number per female and the fertilization success could not be determined since these fish did not spawn during exposure. Only after depuration was spawning activity resumed, and the first spawning event was recorded at 140 DPF. This was a delay of six weeks in the initiation of spawning compared to the fish exposed temporarily during early life only. The reproductive performance of the chronically exposed fish was quantified between 146 and 176 DPF (Fig. 8). Although improving with time, the reproductive output proved to be significantly poorer than in the control fish: Previously exposed fish laid 27.5 ± 10.6 eggs per female compared to 57.5 ± 15.4 in the control, while fertilization success was only $21.7 \pm 10.9\%$ compared to $91 \pm 6.06\%$ in the control group (Fig. 9).

DISCUSSION

Exposure to 3 ng EE₂/L for 42 d, from fertilization until the protogynic stage, affected neither sex ratio nor fecundity or fertilization success of adult zebrafish. However, it persistently elevated plasma VTG levels in female zebrafish, and it partly changed gonadal development: Whereas in adult control

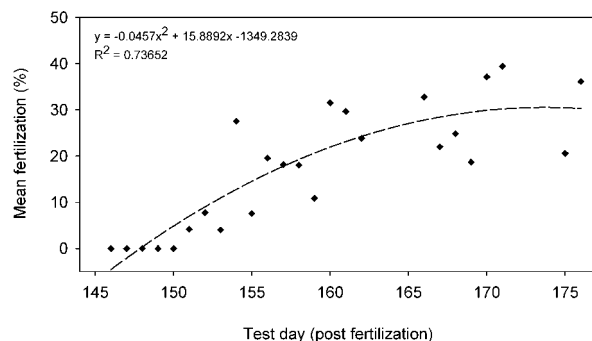


Fig. 8. Recovery of reproductive capacity of zebrafish, chronically exposed to 3 ng/L of 17 α -ethinylestradiol until 118 d postfertilization (DPF), expressed as the time-dependent increase of the proportion of fertilized eggs (expressed as percentage) between 146 and 176 DPF. Onset of spawning was at 140 DPF.

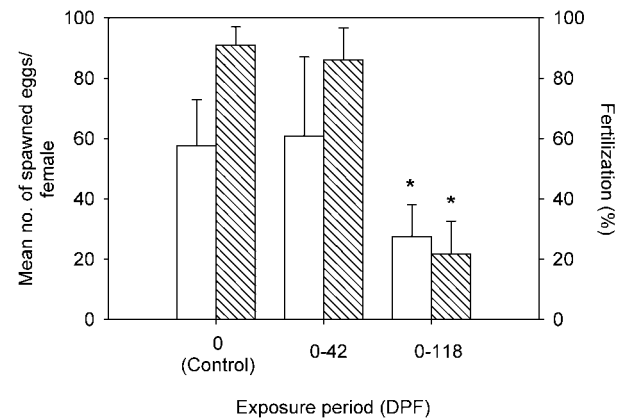


Fig. 9. Mean number of spawned eggs per female and spawning day (bars/left y-axis) and mean proportion of fertilized eggs (expressed as percentage [%]/right y-axis) between 150 and 176 d postfertilization (DPF) in zebrafish control group and treatment groups exposed to 3 ng/L of 17 α -ethinylestradiol for different time periods. Values are means \pm standard deviation. Asterisks (*) indicate mean values that were significantly different from the control mean (* p < 0.001).

fish all ovaries were mature, 20% of the 118-d-old fish of the early life exposure group had immature ovaries. The mechanism behind the persistent elevation of plasma VTG is not understood, but it points to the possibility that early exposure of female zebrafish resulted in a lasting change of any physiological process that is involved in VTG regulation, although this change is apparently not relevant for fecundity or fertility.

The effects of 42 DPF early life exposure on zebrafish gonad development (i.e., 20% of immature ovaries) differ from those reported for fathead minnow and medaka. In the fathead minnow (*Pimephales promelas*), exposure to 10 ng EE₂/L during early life was found to cause no significant shift in the sex ratio but was able to change the gonadal duct formation and to alter spermatogenesis [27]. A high incidence (64.3%) of ovarian-like cavities in 100-d-old male fathead minnows was found in fish that were exposed between 10 and 15 d posthatch (DPH), the time span when the histological ovarian differentiation of the fathead minnow [28] occurs. In the medaka, early life stage exposure to 10 ng E₂/L for one month posthatch led to a total feminization of the population [29]. Even if the exposure to estrogens is restricted to the pre-hatch embryonic phase, a significant feminization of adult medakas can occur [30,31]. The lasting effects of larval and early life estrogen treatment on medakas' sexual differentiation may be explained by the earlier onset of gonad differentiation in this species [31]. In medaka, the critical window of gonad differentiation, when it is particularly susceptible to estrogens, occurs around the time of hatch [30,32], while in the zebrafish, gonad differentiation occurs only during the juvenile stage [33]. Full life-cycle EE₂ exposure clearly impacted the sexual differentiation and reproduction of zebrafish. Continuous exposure induced plasma VTG and a complete feminization of gonad morphology at 75 DPF, after completion of gonad differentiation, and at 118 DPF, at maturity. Because of the feminized gonads, no mating or spawning took place. At first glance, these findings point to a 100% feminization elicited by estrogen exposure. However, a closer look revealed that two different types of female fish were present in the exposed population: females with mature ovaries and with VTG plasma levels comparable to control females and females with immature ovaries and with VTG plasma levels similar to those

found in estrogen-exposed male zebrafish [5,34,35]. One possible explanation for this dimorphic feminization caused by life-cycle exposure might be the nature of gonad development in zebrafish. Zebrafish are undifferentiated gonochorists, and during early development, gonad differentiation passes through a hermaphroditic or nonfunctional, protogynic phase, when all gonads develop into early ovaries [10]. The ovaries are immature (i.e., nonfunctional) and consist of oogonia and primary growth stage oocytes only. With proceeding development, some of these immature ovaries differentiate into mature ovaries, and the others eventually transform into testes [10,11,33]. A trigger for the differentiation of the early, protogynic gonad into either an ovary or a testis is likely to depend on the endogenous levels of androgens and estrogens. Evidence for the importance of sex steroids to determine the phenotypic sex of zebrafish during the gonad transition phase was presented by Fenske and Segner [36] only recently. Their study showed that manipulation of the enzyme aromatase, which converts androgens into estrogens, leads to gonadal feminization or masculinization, and studies in other fish species have shown similar findings [37,38]. Low estrogen concentrations versus androgens may trigger testicular development, and, vice versa, high estrogen concentrations versus androgens may trigger ovarian development. On the basis of the particular nature of gonadal sex differentiation in zebrafish, we propose that the estrogen exposure in our study suppressed normal differentiation of male gonads and caused an arrest in gonadal differentiation of genetic males at the protogynic stage. This implies that the 100% phenotypic females at 118 DPF comprised both genetic females (with mature ovaries and high VTG levels) and genetic males (with male gonad development arrested at the immature ovarian stage and with male-typical low, VTG levels). The mechanisms of gonadal feminization in zebrafish, induced by estrogens, therefore would differ from other small fish species, like medaka or fathead minnow, that are differentiated gonochorists. In these fish species, absence of testicular tissue in treated fish was not caused by arrested male development but is the result of a phenotypic sex reversal (i.e., the conversion of genetically male gonadal tissue into phenotypically female tissue) [27,39]. The reversal of the gonadal sex would explain the occurrence of intersex gonads or ovotestes after estrogen exposure in differentiated gonochoristic species [34,40]. Interestingly, in zebrafish, no strong evidence yet exists for the occurrence of ovotestes after estrogen exposure [13,14,41,42]. To date, only Andersen et al. [12] have reported a high incidence of ovotestes in zebrafish, but the intersex gonads were described as gonads going through transformation from the juvenile ovary into testes.

Assuming that the occurrence of immature ovaries in EE₂-exposed adult fish was caused by an arrest in testicular differentiation of genetic males, a resumption and completion of testes development after withdrawal of exogenous estrogen and depuration of fish was to be expected. In fact, after depuration of zebrafish for 58 d in estrogen-free water, 26% of fish of the previous all-female cohort displayed normally differentiated testes at 176 DPF. Similar observations were made by Hill and Janz [13]. They found no testes in 60-DPH fish exposed to 1 to 10 ng EE₂/L, but after depuration until 160 DPH, the gonadal sex ratio in these fish had recovered and was no longer different from the control. Despite the apparent morphological reversibility of effects after depuration, the authors report a sustained impairment of the reproductive capabilities. Similarly, our fish overcame the arrest in testicular

development, but a fully functional recovery was not achieved, at least not until 176 DPF. The fertilization of eggs, for instance, remained lower than in the other exposed fish. However, it is important to point out that the ability to recover may depend on time, duration, and concentration of estrogen exposure.

The life-cycle exposure to EE₂ also affected the cellular differentiation of the female gonads. The impact of exposure became apparent as a regression in oocyte maturation in the developed ovaries. Similar histological findings were reported by Weber et al. [42], who depicted a concentration-related suppression of gametogenesis in the ovaries in zebrafish after 60 DPH exposure to 10 ng EE₂/L. The oocyte development was described as retarded, accompanied by an increasing incidence of previtellogenic ovarian follicles. Similarly, Van den Belt et al. [16] noticed a cumulative occurrence of oocytes in gonads of adult zebrafish that no longer matured beyond the previtellogenic stage, depending on the duration of EE₂ exposure. In our study, retardation appeared to be less pronounced, and the regressive effect on oocyte maturation varied among individuals. Some females showed almost no mature stage IV oocytes but showed increased percentages of primary, cortical-alveolar, and vitellogenic oocytes, whereas in other females the fraction of mature ovarian follicles did not differ from control females. The main reason for these differences between our study and the other two studies is likely to be the exposure concentration since Van den Belt et al. [16] and Weber et al. [41] used higher EE₂ exposure regimes (25 and 10 ng EE₂/L, respectively). Hill and Janz [13] concluded from their partial life cycle results that low estrogen concentrations affect only male gonadogenesis, while alterations in female development require higher estrogen concentrations.

The impact of life-cycle exposure to EE₂ on the gonad differentiation drastically impaired the reproductive capability of zebrafish. Reproduction was completely suppressed under exposure conditions, and only after cessation of exposure did the fish start regaining their reproductive capabilities. Twenty-six percent of fish were able to overcome the arrested testis development until the end of the study and transform their immature ovaries into histologically normal, differentiated testes. Although a full recovery of the reproductive strength could not be achieved after 58 d of depuration, the study clearly provided evidence for the capacity of a functional and morphological recovery of zebrafish from estrogen exposure. Early life EE₂ exposure of zebrafish that did not take beyond the protogynic stage did not impair reproduction. In the adult fish, we found lasting effects of early life exposure on vitellogenesis in female fish and, in a small percentage of fish, on gonad differentiation as well, but they obviously had no negative impact on the reproductive success. We therefore propose that early life exposure of zebrafish that does not go beyond the protogynic stage of development is unlikely to be adequate for the assessment of estrogenic reproductive effects in this species.

CONCLUSION

Full life-cycle exposure of zebrafish to an environmentally relevant concentration of EE₂ (3 ng/L) resulted in an all-ovary population of fish and in a complete inhibition of reproduction. The underlying mechanisms seem to be linked to the distinctive nature of gonadal sex differentiation of zebrafish as an undifferentiated gonochorist. We hypothesize that exposure of genetic males to exogenous estrogens throughout gonad dif-

ferentiation led to an arrest in the developmental transition of the immature ovaries, as present during the nonfunctional protogynic phase, to testes. The discovery of male-like VTG levels in feminized individuals further corroborates our hypothesis that the life-cycle exposure to EE₂ did not induce a phenotypic feminization or sex reversal in the zebrafish. Furthermore, withdrawal of the exogenous estrogen allowed for at least a partial recovery of male gonad differentiation, according to our hypothesis, by resuming the arrested testis development. The findings of this study suggest that zebrafish differs from species developing as differentiated gonochorists, such as medaka and fathead minnow, where life-cycle exposure to estrogens apparently induces a full phenotypic sex change. The distinctive nature of zebrafish development, which affects its response to estrogens, will have to be taken into consideration when designing experimental protocols for tests on endocrine-disrupting compounds with this fish species or when interpreting experimental results.

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