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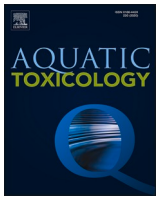

Estrogens as immunotoxicants: 17α-ethinylestradiol exposure retards thymus development in zebrafish (*Danio rerio*)

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ABSTRACT

Estrogenic endocrine disrupting compounds (EEDCs) can cause alterations in sexual development and reproductive function of fish. Growing evidence suggests that EEDCs can also interfere with development and function of innate immunity of fish. The present study examined a potential disruptive effect of EEDCs at field-relevant concentrations on the development of adaptive immunity, more specifically the thymus. Zebrafish (*Danio rerio*) were exposed from fertilization until 64 days post-fertilization (dpf) to environmentally relevant (3 and 10 ng/L) concentrations of the synthetic estrogen 17α-ethinylestradiol (EE2). The exposure duration covered the period of initial thymus differentiation to maximum growth. Thymus development was assessed by histological and morphometric (thymus area) analysis, thymocyte number, and transcript levels of thymocyte marker genes. Additionally, transcript levels of the estrogen receptors (*esr1* and *esr2a)* were determined. The EE2 exposure altered sexual development (gonad differentiation, transcript levels of hepatic vitellogenin and estrogen receptors) of zebrafish, as expected. At the same time, the EE2 treatment reduced the thymus growth (thymus area, thymocyte number) and transcript levels of thymus marker genes. The expression of the thymic estrogen receptors responded to the EE2 exposure but in a different pattern than the hepatic estrogen receptors. After the 64-day-exposure period, the juvenile fish were transferred into clean water for another 95 days to assess the reversibility of EE2-induced effects. The thymic alterations were found to be reversible in female zebrafish but persisted in males. The present study provides the first evidence that the development of the fish adaptive immune system is sensitive to EEDCs, and that this takes place at concentrations similar to those that disrupt sexual development.

1. Introduction

The freshwater environment is a major sink for anthropogenic contaminants. With respect to the ecotoxicological consequences of aquatic pollution, attention has shifted from industrial bulk chemicals to micropollutants, which display specific modes of action rather than systemic toxicity (Schwarzenbach et al., 2006; Segner 2011; Arnold et al., 2014). In particular, contaminants that are able to interfere with the endocrine system, the so-called endocrine disrupting compounds (EDCs) have become to be recognized as one of the most pressing issues of environmental contamination over the last decades (e.g., Sumpter and Johnson 2005; UNEP Report 2012). Of these, estrogen-active EDCs (EEDCs), which interfere with the endogenous estrogen system by binding to and activating the estrogens receptors (ERs) (Darbre, 2019), are among the best studied.

The reproductive system is the primary physiological target of estrogens in vertebrates. Therefore, research on EEDC toxicity to fish has largely focused on alterations of sexual development and adult reproduction (e.g., Jobling et al., 2002; Fenske et al., 2005; Aris et al., 2014; Bhandari et al., 2015). However, estrogen actions are not restricted to the reproductive system. Estrogens have multifaceted physiological functions and influence diverse organ systems (Filby et al., 2006; Shved

Abbreviations: DPF, days post fertilization; E2, 17α-ethinylestradiol; EEDC, Estrogenic endocrine disrupting compound; EDC, Endocrine disrupting compound; ER, Estrogen receptor; FSDT, Fish Sexual Development Test; H&E, hematoxylin and eosin staining; VTG, vitellogenin; WPF, weeks post-fertilization.

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et al., 2008; Segner et al., 2013; 2017). Recently, there is growing evidence that EEDCs can modulate the immune system of fish and their defense capacity towards pathogens (Segner et al., 2006; Iwanowicz and Ottinger 2009; Milla et al., 2011; Chaves-Pozo et al., 2018; Ye et al., 2018). Importantly, immunotoxic effects of EEDCs appear to be active at the same low exposure concentrations that compromise the reproductive system in fish (Segner et al., 2013; Rehberger et al., 2020).

The majority of studies on the immunotoxic activities of EEDCs in fish investigated the innate immune functions such as phagocytosis activity, inflammatory cytokine expression or respiratory burst activity (Wenger et al., 2011; Cabas et al., 2012; Massart et al., 2014; Shelley et al., 2013; Bado-Nilles et al., 2014; Gomez-Gonzalez et al. 2017; Maciuszek et al., 2020). In contrast, virtually no information is available on the sensitivity of the adaptive immune system of fish to environmental EEDCs (Milla et al., 2011; Cabas et al., 2018). An essential organ of the adaptive immune system is the thymus, which is the site of T cell maturation (Ma et al., 2013; Takaba and Takayanagi 2017; Ronza et al., 2020; Granadier et al., 2021). T-cells coordinate immune responses through cytokine secretion, and the elimination of infected cells through cytotoxic activities (Fischer et al., 2013; Nakanishi et al., 2015). A role of estrogens in thymus development and function is well established in mammals. For instance, estrogens are involved in the transient thymus regression during pregnancy and in the permanent thymus regression during aging (Zoller and Kersh, 2006; Hince et al., 2008; Gui et al., 2012; Dooley and Liston 2012; Giefing-Kröll et al., 2015; Laan et al., 2016). In addition, estrogens play a critical role in the development of the thymus in early life of mammals (Staples et al., 1999; Erlandsson et al., 2001; Yellayi et al., 2000). Estrogen actions on the mammalian thymus appear to be mediated at least partly through the nuclear and membrane ERs which are expressed in thymus cells (Mor et al., 2001; Okasha et al., 2001; Yellayi et al., 2002; Zoller and Kersh, 2006; Bernardi et al., 2015; Wang et al., 2008; Notas et al., 2020). Recently, it has been shown that the thymus of teleost fish also possesses ER transcripts and proteins (Paiola et al., 2017; Szwejser et al., 2017; Moreira et al., 2021), suggesting that the functional role of estrogens in thymus life-time development and function may be evolutionary conserved between fish and mammals. Further support for this assumption comes from the findings of Seemann et al. (2015) and Paiola et al. (2018). They demonstrated that exposure of juvenile sea bass (*Dicentrachus labrax*) to 17β-estradiol alters thymus growth, regionalization, as well as T-cell development and output. Taking this into account, it seems likely that environmental EEDCs could disrupt normal thymus development and function of fish.

In the present study we investigated whether EEDC exposure during early life alters thymus development of zebrafish (*Danio rerio*), and whether the EEDC-induced effects are reversible or persistent. The zebrafish was selected as experimental model, since its physiological thymus development is well described (Lam et al., 2002; Kernen et al., 2020), and transgenic lines with thymus markers (Langenau et al., 2004) are available. Exposure was performed during the developmental window from fertilization over the embryonic and larval stage until the juvenile stage (60 days post hatch, dph). During these stages, the adaptive immune system including the thymus of zebrafish differentiates and becomes functional, and the thymus undergoes a period of maximum growth (Willett et al., 1999; Lam et al., 2002; Danilova et al., 2004; Kernen et al. 2020). Besides, it is the period that is used in the Fish Sexual Development Test (FSDT, OECD test guideline 234; OECD 2011), which assesses mechanistic (vitellogenin) and apical (sex ratio) impact of EDCs on sexual differentiation of zebrafish.

During ontogenetic differentiation, organ systems may be particularly vulnerable to the impact of EDCs (“sensitive window concept”, Guilette et al. 1995), and early-life exposure may result in non-reversible, “organizational” changes. Such organizational effects of EDCs have been shown for the gonad development of zebrafish (Baumann et al., 2014a; Segner, 2009), and they are known for the developing immune system of mammals (Dietert 2009; DeWitt et al., 2012). Also for teleost fish, initial evidence exists that developmental exposure to contaminants can cause permanent dysfunction of the immune system (Milston et al., 2003; Ottinger and Kaatari, 2000, Luebke et al., 2006). To test for this possible outcome of early life EEDC exposure of zebrafish, the 64-day-treatment period was followed by a depuration period of 95 days in clean water, which covers the period from the juvenile stage of zebrafish to adulthood. The impact of EEDC exposure on thymus development of zebrafish was histologically determined, measuring thymus growth (thymus area, thymocyte numbers), as well as examining structural differences. Impact on thymocyte maturation was assessed by measuring transcript levels of cellular marker genes: *ikaros,* which is a regulator of lymphocyte lineage and, is expressed in early-stage thymocytes; and *tcrα* and *tcrδ*, which are members of the T-cell receptor family and are expressed in later thymocyte stages (Carpenter and Bosselut, 2010; Mitchell et al., 2016; Seelye et al., 2016; Willett et al., 2001). In addition, we examined the transcription levels of thymic nuclear estrogen receptors (ERs). The model EEDC used for the present study was the synthetic estrogen 17α-ethinylestradiol (EE2). Largely applied for contraception and hormonal therapy, EE2 is of particular environmental relevance because it degrades more slowly and it is more potent (higher ER affinity and efficacy) than natural estrogens (Aris et al., 2014; Legler et al., 2002; Ting and Praveena, 2017; Wise et al., 2011). Concentrations of EE2 found in freshwater habitats are usually in the low ng/L range (Aris et al., 2014). Accordingly, the exposure concentrations used in the present study were 3 and 10 ng/L. These low EE2 levels have been shown to disrupt normal sexual differentiation of developing zebrafish (Fenske et al., 2005; Schäfers et al. 2007) and they were found to be immuno-active in rainbow trout (*Oncorhynchus mykiss)* (Rehberger et al., 2020). To validate that the administered EE2 concentrations were indeed effective in disrupting the reproductive system, we examined the gonad histopathology, hepatic vitellogenin (*vtg*) and hepatic ER transcript levels.

2. Material & methods

2.1. Experimental animals

For the present study we used the lck:GFP transgenic zebrafish line developed by Nikolaus Trede’s laboratory (Harvard Medical School, Boston, US) (Langenau et al., 2004) and provided to our laboratory by Dr. Adam Hurlstone’s laboratory (Manchester University, UK). This line expresses fluorescence in T-cells. The thymus development pattern of this line is comparable to the one of wildtype zebrafish (Kernen et al. 2020). The fish were raised in the warm-water fish facility of the Center for Fish and Wildlife Health at Bern University (Switzerland) under standard conditions: temperature at 26.1 °C (± 0.97), conductivity at 621 μS (± 10.72), pH at 7.07 (± 0.22), hardness interval from 6 to 14 German degrees (°dH), minimum dissolved oxygen at 79%, light: dark cycle of 14: 10 h. Fish were fed three times per day; mornings and evenings with Gemma Micro 75, 150, or 300 dry food (fish size-dependent) (Skretting, US) and with living *Artemia spec.* nauplii in the afternoons. We conducted all experiments under institutional and national guidelines for the care and use of laboratory animals, license number BE 109/19.

2.2. Experimental design

Adult lck:GFP zebrafish groups were bred under standard conditions to produce eggs for the experiment. Within the first hours after fertilization, the eggs were exposed to the different treatments. Eggs were raised under semi-static conditions until 4 dpf and then under constant flow-through until 159 dpf (details in S1). The zebrafish were exposed from 0 to 64 dpf to the following treatments: (i) 3 ng/L or 10 ng/L of EE2 with ethanol used as a solvent; (ii) ethanol, also named "solvent control"; (iii) system water, also named "water control". After the exposure period of 64 days, the fish were further kept in clean water for 95 days until adulthood (159 dpf). Each treatment was conducted in duplicate (2

separate tanks), except the water control group, which was only performed in one tank due to place restrictions. The fish were sampled at two time-points, the first at 64 dpf under EE2 exposure (first sampling) and the second at 159 dpf, after the 95 days-recovery period (second sampling). A graphical overview is presented in Fig. 1.

2.3. 17α-ethinylestradiol (EE2) exposure

EE2 was purchased from Sigma-Aldrich (purity ≥ 98%, E487610G; Buchs, Switzerland). The stock solution of EE2 was prepared in molecular grade ethanol (Grogg Chemie, Stettlen-Deisswil, Switzerland) at 10 mg/ml. The final concentration in the tanks was 4 ng/L ethanol. Exposure in the petri dishes was performed under semi-static conditions, with 50% of daily water renewal with freshly prepared solutions of the control or EE2 solutions. When the larvae were placed in the aquaria, they were exposed under constant flow-through, which was regulated by using peristaltic pumps (Minipuls3, Gilson, Mettmenstetten, Switzerland) for the EE2/ethanol solutions and flow meters (Rotameter, Yokogawa, EU) for the system water. The stock solutions were freshly prepared every second day. The flow rates of the pumps and the water flow meters were monitored weekly.

2.4. Bioanalytical determination of 17α-ethinylestradiol (EE2) concentrations

The nominal EE2 concentrations in the exposure aquaria were 3 ng/L and 10 ng/L. Water samples from each tank were taken every 2–3 weeks over the entire exposure period. The measured concentrations were, on average, 3.1 ng/L and 10.9 ng/L, respectively, and are presented in Fig. S2. For ease of reading, the nominal values are used in this report.

EE2 was extracted from aquarium water using SPE C18 filter cartridges (Grogg Chemie, Stettlen, Switzerland). Details of the procedure are described in Rehberger et al. (2020) and can be found in S2.

2.5. Fish sampling

The fish were euthanized with an overdose (500 mg/L) of MS-222 (Syndel, Washington, USA) buffered with sodium bicarbonate (NaHCO3). Immediately afterwards, the total length, standard length, and weight were recorded for each fish. Fulton's condition factor (K) was calculated: (weight g / length cm)^3 × 100. Gonads of all fish were sampled for subsequent histopathological analyses and sex determination. Livers from each fish were sampled for RT-qPCR analyses. For the sampling of the thymus, the fish were split into two groups: in one group, both left and right thymus were sampled for RT-qPCR analysis, while in the other group, the left thymus was sampled for the thymocyte counting and the right thymus for histological analysis. Precise dissection of the small zebrafish thymus was possible because we used transgenic lck:GFP zebrafish line in which the thymus was detectable by its green fluorescence signal. Dissection was done under a Leica fluorescent microscope equipped with a green filter (excitation/emission wavelength of 488/507 nm). The number of fish sampled in the various treatments is given in Table S1.

2.6. Histological analyses

For histological analyses, the trunks, containing visceral organs and gonads, were placed in neutral buffered 4% formalin. The heads of the fish containing the right thymus were placed in Histochoice (Lucerna-Chem AG, Luzern, Switzerland) for three hours. Trunk and head samples from adult fish were additionally decalcified, for one hour in Decalcifier Rapid (Biosystems, Muttenz, Switzerland) or for 40 mins in Decalcifying Solution-Lite (Sigma-Aldrich, Buchs, Switzerland), respectively. The fixed samples were subjected to routine paraffin embedding procedures (OECD, Histopathology Guidance Document, 2010). Sections were cut at a thickness of 5 µm. Half of the thymus slides were stained with hematoxylin and eosin (H&E), while the other half were prepared for immunohistochemistry.

2.6.1. Gonad staging

The gonads were examined on H&E-stained coronal sections of the trunk part of the zebrafish. The staging of gonad maturation status was done according to the OECD Histopathology Guidance Document (2010).

2.6.2. Thymus morphometric measurements

The morphometric measurements of the thymus (right thymus of one fish) in histological sections were performed as previously described by Kernen et al. (2020). Briefly: the thymus growth was assessed by morphometric measurement of its area on histological sections and by determining the thymocyte numbers. The thymus area is presented both as absolute thymus area and relative thymus area, the latter being the absolute thymus area normalized by the head area in order to account for the fish body growth. The thymocyte number was normalized to milligram (mg) of fish weight. More details can be found in S3.

2.7. Determination of thymocyte number

Thymus samples for determination of thymocyte number per thymus (left thymus of one fish) were extracted and preserved using a procedure modified from Paiola et al. (2017). The left thymus of lck:GFP zebrafish was identified (fluorescence signal) and subsequently dissected under a Leica fluorescent microscope. The dissected thymus was placed in RPMI medium (Thermofisher Scientific, containing HEPES and sodium bicarbonate), and mechanically disaggregated. This was done by the same person for all samples to avoid technical variability. The resulting tissue slurry was passed through a 35 µm cell strainer to remove the non-thymocytes (Paiola et al., 2017). The isolated fluorescent thymocytes were counted in a Neubauer chamber. The counting was done in duplicate unless the deviation between the first two counts was higher than 30%, then a third count was conducted. The cell number was normalized by the fish weight to express the thymocyte number per mg of fish (larger fish had a higher absolute thymocyte count than smaller fish). On few selected samples, the purity of the thymocyte suspension was analyzed in a cell sorter (BD FACS ARIA III, Flow Cytometry and Cell Sorting Core Unit of the University of Bern). Typically, less than 5% of the cell suspension were non-thymocytes. This analysis was performed

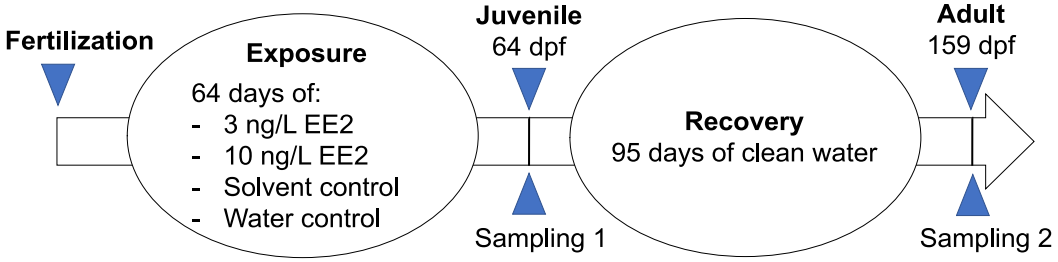

Fig. 1. Graphical presentation of the experimental design.

as prove of principle on selected samples but not routinely on all samples, as the sorting procedure is stressful for the cells and deteriorates their quality for the subsequent PCR.

2.8. RT-qPCR analyses

For molecular analyses, the method for liver RNA extraction was modified according to the age of the fish. At 64 dpf, the livers of the juvenile fish were transferred into lysis buffer (Promega, Dübendorf, Switzerland), mechanically lysed with a Tissue Lyzer (Qiagen, Hombrechtikon, Switzerland), and then stored at −80 °C. In adult fish, the livers were directly flash-frozen in liquid nitrogen (without lysis) and then stored at −80 °C until further processing.

Thymus samples for RT-qPCR analyses followed the same procedure for of both juvenile and adult fish<: the left and right thymus of one fish were dissected, placed in lysis buffer (PicoPure RNA Isolation Kit, Thermofisher Scientific, Reinach, Switzerland) for 30 mins at 42 °C and then stored at −80 °C for subsequent RT-qPCR processing.

RNA from the liver and thymus were isolated using the ReliaPrepTM RNA Tissue Miniprep System (Promega) and PicoPureTM RNA Isolation Kit (Thermofisher Scientific), respectively. The use of two different kits for the two different tissues was motivated by the fact that the thymus was very small and required a more sensitive kit to extract the RNA. We followed the manufacturer's instructions, with some minor modifications regarding the PicoPureTM RNA Isolation Kit, to include a DNase treatment step (Qiagen, Hombrechtikon, Switzerland). For each fish, 500 ng of liver RNA and 150 ng of thymus RNA were transcribed into cDNA using the GoScript Reverse Transcriptase Mix with Random Primers (Promega) following the manufacturer's instructions and then stored at −20 °C.

PCR measurements were performed with the Applied Biosystems 7500 FAST RT-qPCR system (Thermofisher Scientific), using SYBR Green PCR core Reagents (Promega). The primer sequences can be found in table S4. As a reference gene, the elongation factor alpha (*ef1α*) was selected for both liver and thymus, because it is stable under estrogenic exposure (McCurley and Gallard 2008). A detailed description of the procedure can be found in S4. Data were analyzed using the 2^{-ddCt} method (Livak and Schmittgen, 2001).

2.9. Statistical analyses

All statistical tests were performed with NCSS statistical software version 12, and the data were visually represented with GraphPad Prism version 8.4.0. The response variables were mortality, standard length, weight, condition factor K, thymus morphometric area, thymocyte number, thymic immune transcript, thymic/hepatic estrogen receptors, and hepatic *vtg* expression. For each response variable, we determined the statistical differences among the treatment groups using data from each single fish per concentration, not the replicate tanks. The response variable datasets were tested for normal distribution and equal variances between groups, using the Shapiro-Wilk test and the Levene test, respectively. As we could not conclude for normal distribution of the data and equal variances, we used the non- parametric Mann-Whitney Test (if two groups), or Kruskal-Wallis One-Way ANOVA on Ranks (if more than two groups) followed by the Dunn's-Bonferroni post hoc test. The X^2 test was applied to test differences in the mortality and gonad differentiation status ratios between the groups. Treatment differences were considered to be significant at $p < 0.05$, and highly significant at $p < 0.001$.

The data of the control water and solvent groups were pooled together as "controls" if no significant difference was observed between them (Kruskal-Wallis), which is an approach suggested by Green (2014). In the first sampling, the zebrafish were 64 dpf of age, which corresponds to the juvenile period (Singleman and Holtzman, 2014). At this age, not all fish were already differentiated into males or females and therefore, data from all fish were combined. At the second sampling (159 dpf) after the depuration period, data were analyzed for males and females separately. For each endpoint, we statistically tested the difference between the treatment in males and females separately.

3. Results

3.1. Survival

The average mortality from fertilization until 64 days post-fertilization (dpf) was 32% in the control groups (water, solvent), while in the 3 ng/L EE2-treated group it was 23% and in the 10 ng/L EE2 treatment it was 30%. The difference of mortalities was not significant between the controls and the 10 ng/L EE2 group, but the 3 ng/L EE2 group had a significantly lower mortality than the controls. Mortality of embryos and larvae only occurred during the early developmental phase until approx. 20 dpf, which is generally known to be sensitive for raising zebrafish (e.g. Norton et al., 2019). Afterwards, no more mortality or general health impact of the fish were observed. The overall mortality rates are not exceeding historical control data (e.g. Fenske et al., 2005; Schaefers et al., 2007; Holbech et al., 2013).

3.2. Body growth

Exposure of developing zebrafish for 64 days to 10 ng/L EE2 resulted in significantly lower standard length and body weight, and higher condition factor K compared to the other treatments (Fig. 2ADG). After the recovery period, mean standard length of male and female fish from both EE2 exposure groups was significantly lower than in the control groups (Fig. 2BC, Kruskal-Wallis, $p < 0.05$). Body weight was significantly decreased in male fish of both EE2 exposure groups (Fig. 2FI), whereas in female fish, only the 3 ng/L EE2 exposure group showed a significant reduction (Fig. 2EH). The condition factor K was only reduced in 3 ng/L EE2 exposed females. Overall, EE2 exposure had the tendency to reduce body growth of zebrafish.

3.3. EE2 effects on reproductive parameters

3.3.1. Histopathological analysis of the gonads

At 64 dpf, the gonads displayed the morphology of the undifferentiated stage, the (non-functional) protogynic stage, or they were already differentiated into either ovary or testis (Maack and Segner 2003). Most of the fish in the control groups displayed protogynic gonads: 70% in the water control and 67% in the solvent control. The percentage of fish with undifferentiated gonads in the water and solvent controls was 22% and 13%, respectively. The percentage of fish with undifferentiated gonads significantly increased under EE2 exposure: 33% in the 3 ng/L group, and 80% in the 10 ng/L group. In contrast, the percentage of protogynic gonads significantly decreased in the EE2 treatments to 49% in the 3 ng/L group, and to 17% in the 10 ng/L group.

After the recovery period, adult fish (159 dpf) of all groups presented fully developed mature gonads of either male or female morphology. No difference was observed between treatments, neither with respect to the presence of pathologies nor with respect to the gonad maturation stage or the overall sex ratio (data not shown).

Table 1.

3.3.2. Hepatic vitellogenin transcripts

After two months of EE2 exposure (at 64 dpf), the hepatic *vtg* transcript levels were significantly higher in the two EE2-treated groups (3 and 10 ng/L) compared to the controls (Fig. 3A). After the recovery period (at 159 dpf), the hepatic *vtg* transcripts in the previously EE2-exposed female and male zebrafish were no longer significantly different from the control groups (Fig. 3BC).

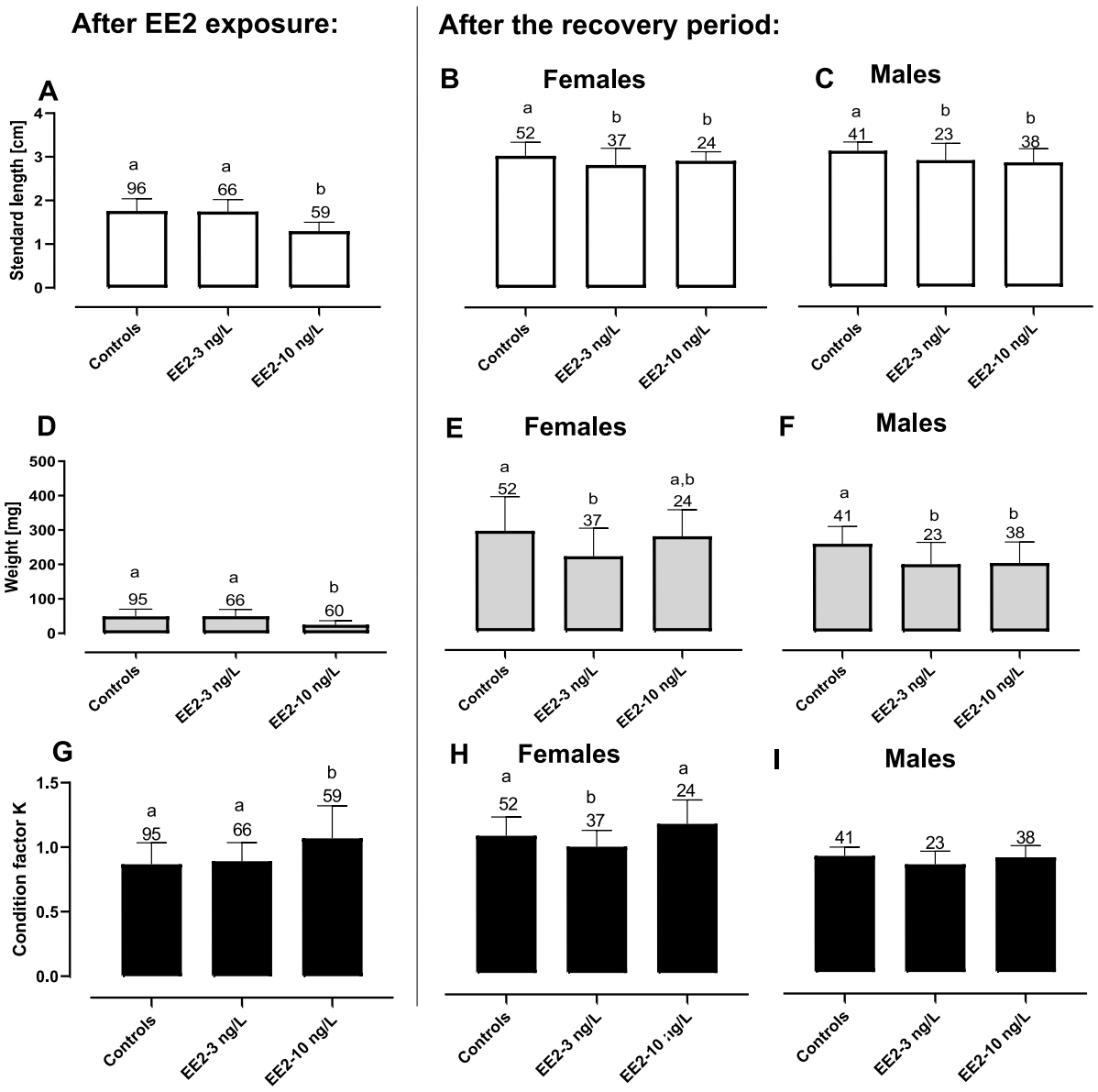

Fig. 2. Bar charts representing standard length (cm), weight (mg), and condition factor K of the lck:GFP zebrafish (mean ± SD) exposed to EE2 until 64 dpf (A, D, G) or kept in clean water from 64 until 159 dpf (B, C, E, F, H, I). Different letters indicate significant differences between the treatment groups (Kruskal-Wallis, $p < 0.05$). The fish n-numbers are given above the bar charts.

Table 1
Summary of histopathological analysis of zebrafish gonads after EE2 exposure from 0 to 64 dpf. Asterisks: X^2, $p < 0.05$.

	Water	Solvent	EE2–3 ng/L	EE2–10 ng/L
Undifferentiated	22%	13%	33%	**80%***
Protogynic	70%	67%	49%	**17%***
Males	8%	9%	18%	3%
Females	0%	11%	0%	0%
Total fish number	**23**	**45**	**45**	**36**

3.4. Hepatic estrogen receptor transcripts

Zebrafish possess three nuclear estrogen receptors, *esr1, esr2a,* and *esr2b*. The present study focused on *esr1* and *esr2a,* since *esr2b* is reported to be not responsive to estrogens (Chandrasekar et al., 2010). At the first sampling (at 64 dpf), the hepatic *esr1* transcript levels were significantly higher in the EE2-treated fish compared to the control groups (Fig. 4A). On the contrary, the hepatic *esr2a* expression was significantly downregulated in the EE2-treated fish compared to the control groups (Fig. 4D). After the recovery period in clean water (at 159 dpf), the hepatic *esr1* and *esr2a* transcript levels were no longer significantly different between the previously EE2-exposed fish and control fish, with the only exception of males of the 10 ng/L group: those fish still displayed significantly higher *esr1* transcript levels than the control fish (Fig. 4BCEF).

3.5. EE2 effects on the zebrafish thymus

3.5.1. Thymus growth: morphometric area and thymocyte number

Zebrafish that were exposed from fertilization until 64 dpf to 10 ng/L EE2 displayed a significantly reduced absolute and relative thymus area and possessed a significantly reduced number of thymoctyes per mg fish weight compared to control zebrafish (Fig. 5ADGJ). In zebrafish exposed to 3 ng/L EE2, a significant reduction was observed for the relative thymus area and the thymocyte number per fish weight, but not for the

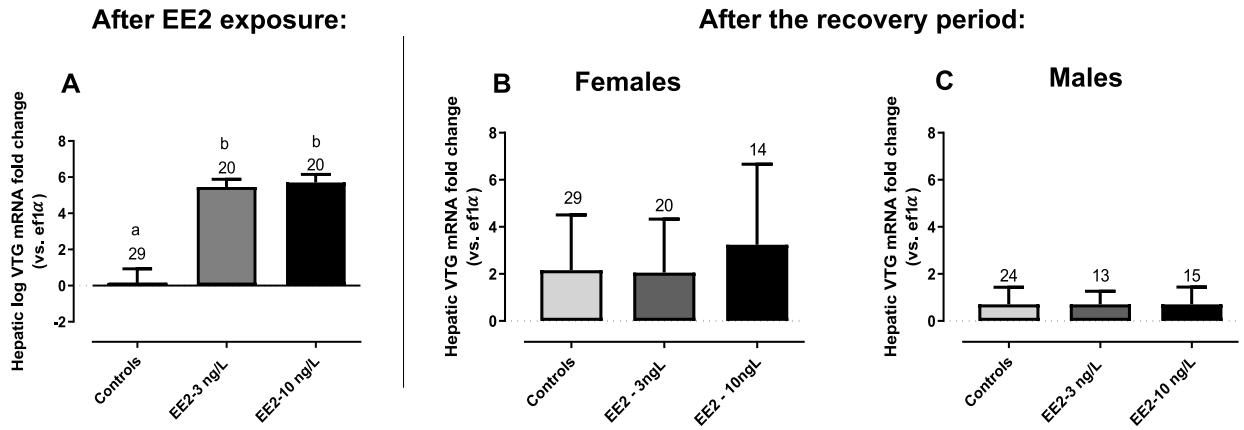

Fig. 3. Bar charts representing relative fold change of hepatic vitellogenin (*vtg)* (A) of the lck:GFP zebrafish (mean ± SD) exposed to EE2 until 64 dpf (A) or kept in clean water from 64 until 159 dpf (B, C).Different letters indicate significant differences between the treatment groups (Kruskal-Wallis, $p < 0.05$). The fish n-numbers are given above the bars.

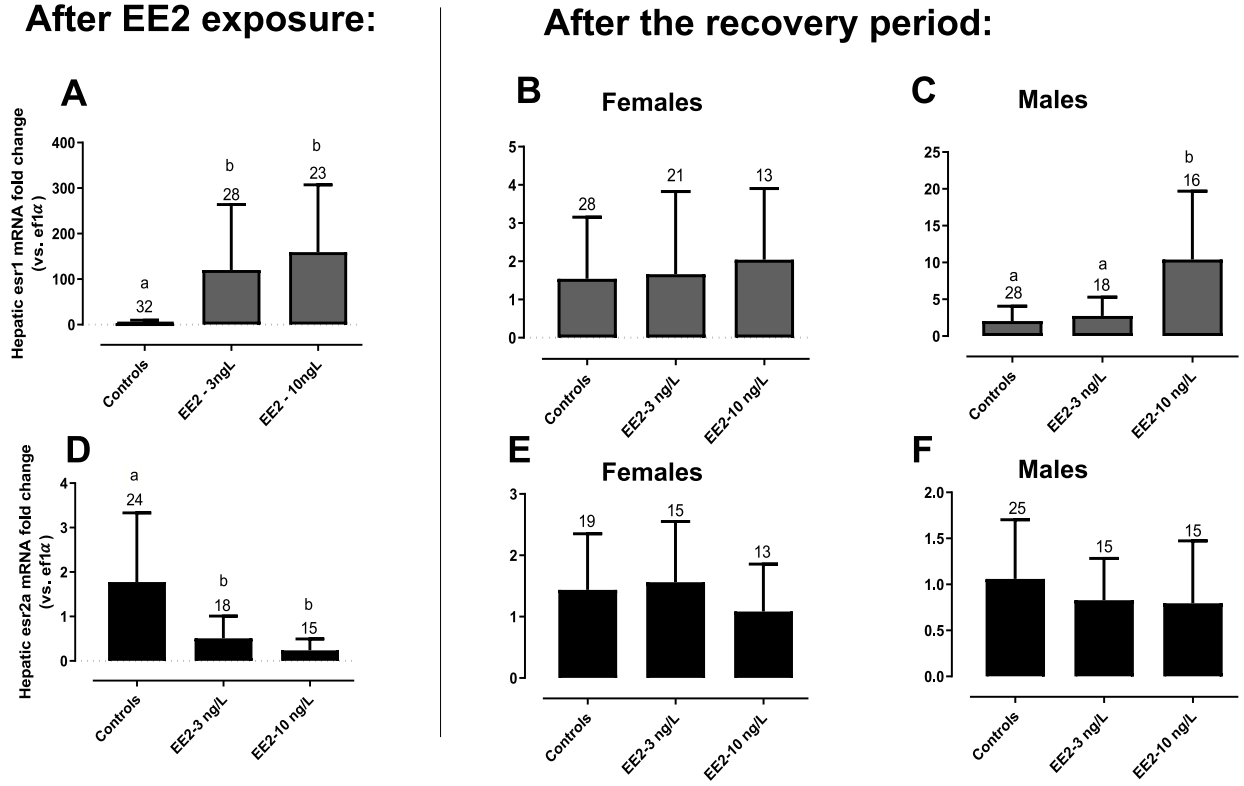

Fig. 4. Bar charts representing relative fold change of hepatic *esr1* (A), *esr2a* (D) of lck:GFP zebrafish (mean ± SD) exposed to EE2 until 64 dpf (A, D) or kept in clean water from 64 until 159 dpf (B, C, E, F). Different letters indicate significant differences between the treatment groups (Kruskal-Wallis, $p < 0.05$). The fish n-numbers are given above the bar charts.

absolute thymus area (Fig. 5DJ).

After the recovery period (at 159 dpf), male zebrafish from the 10 ng/L EE2 treatment still had a significantly reduced absolute and relative thymus area compared to control fish (Fig. 5CF). In contrast, in adult females no significant differences in the absolute and relative thymus area were found between fish previously exposed to EE2 and control fish (Fig. 5BE). Thymocyte numbers of adult zebrafish were not significantly different among treatment groups, neither in males nor in females (Fig. 5HIKL).

Although the EE2-treated fish had significantly less thymocytes than the controls after 60 days, at the adult stage, after the recovery period, these fish had no longer thymocyte numbers significantly different from controls. This interesting observation implicates that the rate of thymocyte loss form the juvenile stage (60 dpf) to the adult stage strongly differs among control fish and previously EE2-exposed fish: while the former undergo a pronounced decrease of thymocyte number from puberty to sexual maturity, the latter, starting at low levels of thymocyte numbers, experience an only weak loss of thymocytes during this period.

3.5.2. *Thymus histology*

The decrease of thymus size, as evident from the morphometric analysis, was well visible on the histological sections (Fig. 6, additional images in S5). In particular, the dorsal horn which extend from the

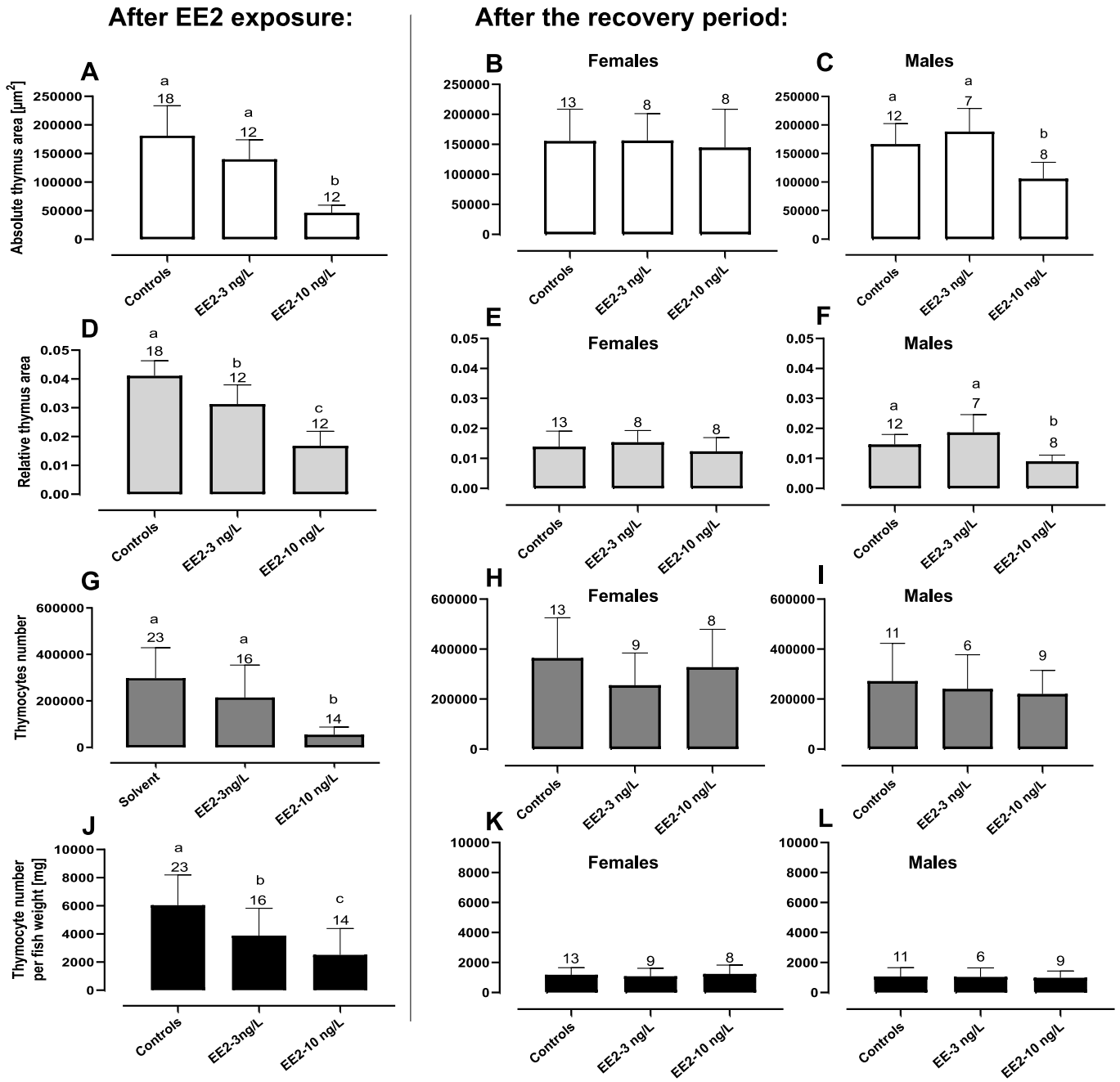

Fig. 5. Bar charts representing absolute (A), relative (D) thymus area, thymocyte number (G), and thymus number per body weight (mg) (J) of the lck:GFP zebrafish (mean ± SD) exposed to EE2 until 64 dpf (A, D, G, J) or kept in clean water from 64 until 159 dpf (B, C, E, F, H, I K, L). Different letters indicate significant differences among the treatment groups (Kruskal-Wallis, $p < 0.05$). The fish n-numbers are given above the bar charts.

thymus base close to the opercular cavity into the dorsal direction, was strongly reduced. Further analyses revealed that within the thymus, the cortex showed a strong reduction compared to the medulla. After the recovery period in clean water, the cortex-medulla ratio was no longer different between control fish and previously EE2-exposed fish, except for the males of the 10 ng/L EE2 group, where the cortex area was still partly reduced. Histopathological alterations of the thymus were not observed, neither in control fish nor in EE2-exposed fish.

3.5.3. *Thymus estrogen receptor transcripts*

The thymic gene expression of *esr1* was lower in the 10 ng/L EE2 group compared to the controls, but the difference was statistically not significant (Fig. 7A). The *esr2a* mRNA levels tended to be higher in the thymus of the EE2-treated groups compared to the control groups, but the difference was also not significant (Fig. 7D). After the recovery period in clean water (at the age of 159 dpf), thymic *esr1* and *esr2a* transcripts levels of female zebrafish were not different between controls and previously EE2-exposed fish, while in males the transcript levels of both receptors were significantly reduced in the fish recovering from the early life exposure to EE2 (Fig. 7BCEF). For *esr2a*, the males of all treatment groups possessed lower transcript levels in the thymus than the females.

3.5.4. *Thymus marker gene transcripts*

After 64 days of EE2 exposure, mRNA levels of *ikaros, tcrα* and *tcrδ* were lower in the 10 ng/L EE2 group compared to the control but the difference was statistically not significant (Fig. 8ADG). After the recovery at the age of 159 dpf, transcript levels of the *ikaros, tcrα,* and *tcrδ*

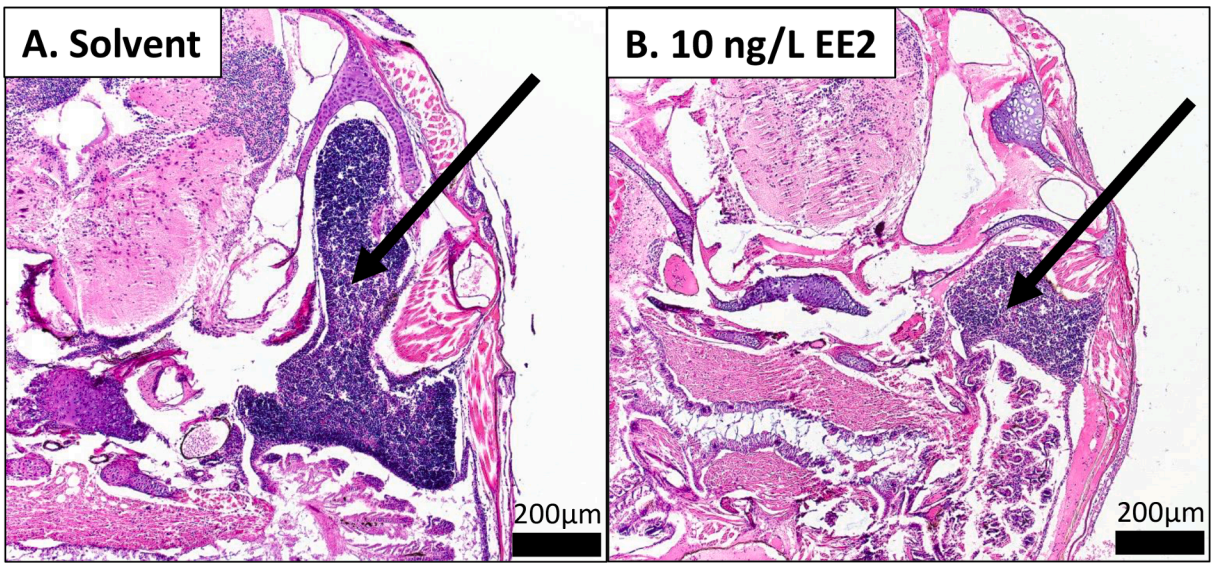

Fig. 6. Transversal histological thymus sections from lck:GFP zebrafish stained with Hematoxylin and Eosin (H&E, pictures A, B). The zebrafish were exposed to the concentration of 10 ng/L 17α-ethinylestradiol (EE2 and ethanol as solvent) or the solvent (ethanol) until 64 dpf. The thymus size is smaller in the EE2–10 ng/L fish (protogynic juvenile) compared to the solvent fish (undifferentiated juvenile). Arrows: thymus. Scale bar 200 µm.

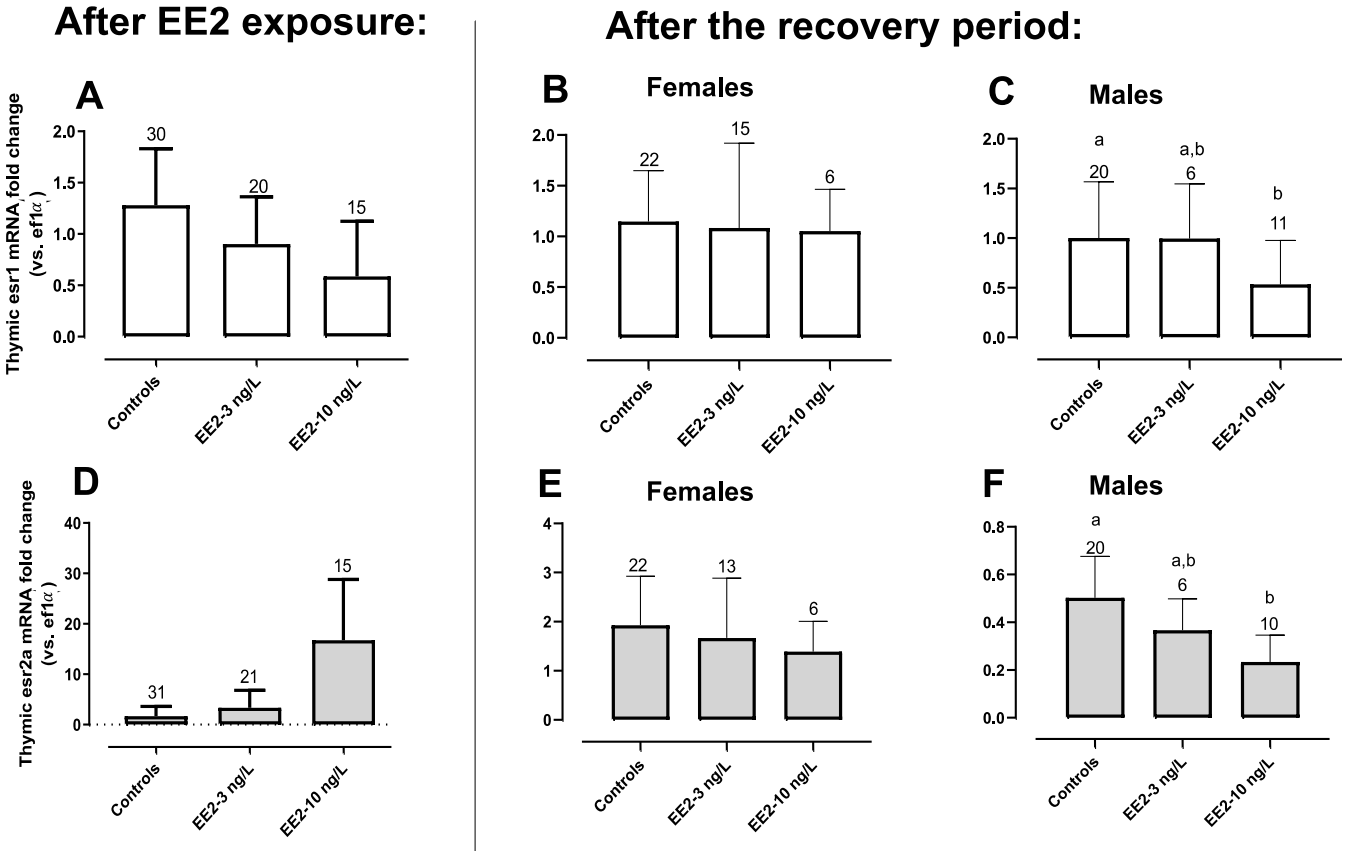

Fig. 7. Bar charts representing relative fold change of thymic *esr1* (A), *esr2a* (D) of lck:GFP zebrafish (mean ± SD) exposed to EE2 until 64 dpf (A, D) or kept in clean water from 64 until 159 dpf (B, C, E, F). Different letters indicate significant differences between the treatment groups (Kruskal-Wallis, $p < 0.05$). The fish n-numbers are given above the bar charts.

in the thymus of female fish were not different between controls and fish exposed to EE2 during early life (Fig. 8BEH). In contrast, adult males that had been exposed to 10 ng/L EE2 until 64 dpf exhibited *ikaros* and *tcrα* expression levels that were significantly lower than in control fish (Fig. 8CF).

4. Discussion

In the present study, we investigated whether (i) early life exposure of zebrafish to an estrogen-active compound (EE2), disrupts the thymus development, and whether (ii) the EE2-induced developmental

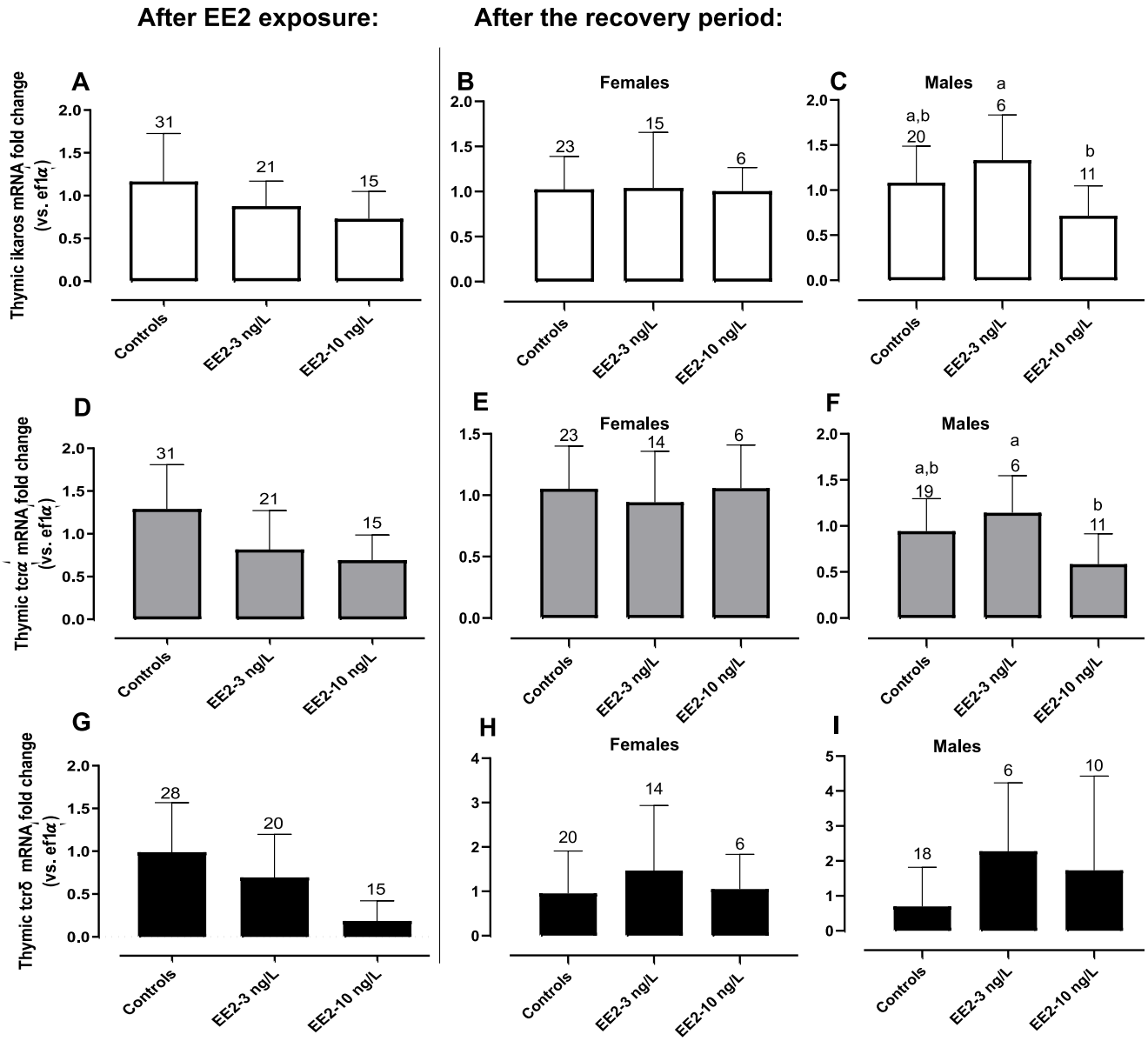

Fig. 8. Bar charts representing relative fold change of *ikaros, tcrα, tcrδ* in the thymus of the lck:GFP zebrafish (mean ± SD) exposed to EE2 until 64 dpf (A, D, G) or kept in clean water from 64 until 159 dpf (B, C, E, F, H, I). Different letters indicate significant differences between the treatment groups (Kruskal-Wallis, $p < 0.05$). The fish n-numbers are given above the bar charts.

alterations of the zebrafish thymus are persistent or reversible. Our results show that early life exposure to EE2 significantly disrupted thymus development of zebrafish. This effect occurred at the same low, environmentally relevant EE2 concentrations that are disruptive for the sexual development of zebrafish. In contrast to the sexual disruptive impact of EE2, the effects on thymus development were reversible. The key finding of the present study, i.e. that EE2 has a strong, but reversible impact on immune development of zebrafish, is novel and of high relevance for risk assessment of estrogenic compounds in the environment.

Previous studies have shown that the experimental conditions selected for the present study interfere with sexual development and growth of zebrafish (Fenske et al., 2005; Schaefers et al., 2007; Baumann et al., 2014). This could be confirmed for all analyzed growth- and reproduction-related parameters including hepatic vitellogenin and ER mRNA expression, and gonad differentiation status. Also the hepatic transcripts of the ERs *esr2a* and *esr2b* were regulated by the estrogenic exposure. Both isoforms in zebrafish have been reported to be estrogen-sensitive (Chandrasekar et al., 2010; Chen et al., 2019).

The novel question addressed in the present study was whether EE2 exposure conditions that disrupt the development of the reproductive system of zebrafish also disrupt the development of the thymus, a central organ of the immune system. Indeed, the treatment caused significant disruption of this central immune organ. The thymus is a primary lymphoid organ in all jawed vertebrates and generates T-cells in a dynamic process comprising T-cell receptor gene rearrangement and lineage commitment (Boehm and Swann 2014; Krueger et al., 2016). In the mammalian thymus, the maturing T-cells undergo positive and negative selection, and although there is no direct evidence that these processes also exist in the teleostean thymus, a number of observations suggest their presence in fish (Barazza et al. 2021). The thymic tissue contains differentiating T-cells called thymocytes, which are embedded in a three-dimensional network of stromal cells made up of thymic epithelial cells, dendritic cells and macrophages. In teleosts, the thymus is a paired organ located in the dorsomedial part of the opercular cavity (Bowden et al., 2005; Barraza et al., 2021; Bjorgen and Koppang, 2021).

A separation of the thymus tissue into a cortex and medulla is present in teleostean species like zebrafish or seabass (Menke et al., 2011; Paiola et al., 2017; Kernen et al. 2020), while for other species like flounder or Atlantic salmon, a lack of distinction between cortical and medullar zones has been reported (Barazza et al. 2021). The cortex contains a high density of developing thymocytes, while in the medulla, the number of thymocytes is relatively smaller and the network of epithelial cells is coarser. In the present study, the morphology of the thymus of EE2-exposed zebrafish was severely altered, which will most certainly have consequences for its functionality, i.e. immune response to pathogens.

The reason for the observed thymus development disruption in the present study seems to be the changes in estrogen signaling by the EE2 treatment. We know that estrogens play a critical physiological role in the ontogenetic development and function of the mammalian thymus. The mammalian thymus is a sexually dimorphic organ, and sex differences in the thymopoiesis, cell composition and thymus size have been consistently observed (Zoller and Kersh, 2006; Hince et al., 2008; Gui et al., 2012; Dooley and Liston 2012; Giefing-Kröll et al., 2015; Laan et al., 2016). The development of sexual differences of the mammalian thymus occurs during the critical perinatal and peripubertal periods and is governed by sex steroid-dependent mechanisms (Leposavic and Pilipovic 2018). Indeed, estrogens influence the highly active thymopoiesis during early life as well as the decline of thymopoiesis during aging (Staples et al., 1999; Erlandsson et al., 2001; Yellayi et al., 2000; Brown and Su, 2019; Leposavić and Pilipović, 2018). Estrogens induce apoptosis of double-positive thymocytes, and they downregulate *aire* in thymic epithelial cells to inhibit thymocyte negative selection (Brown and Su, 2019; Takaba and Takayanagi, 2017). The estrogenic actions on thymic cells are at least partly mediated through the nuclear ERs, but also through the membrane-bound GPR30 (Mor et al., 2001; Okasha et al., 2001; Zoller and Kersh, 2006; Bernardi et al., 2015; Wang et al., 2008). Given the influence of natural estrogens on the mammalian thymus, it is not surprising that exogenous administration of compounds interfering with estrogen signaling causes disturbances of thymopoiesis, thymus function and thymus involution (Yellayi et al., 2002; Selvaraj et al., 2005; Lynch et al., 2009; Kinsella and Dudakov 2020), as observed in the present study with fish.

Recently, it has been shown that also the teleostean thymus development is sensitive to natural estrogens, and that the estrogenic effects may involve ER signaling (Paiola et al., 2017; 2018; 2021; Kernen et al. 2020, Moreira et al., 2021; Seemann et al., 2015). These observations suggest that environmental EEDCs like EE2 may interfere with development and function of the fish thymus. This hypothesis was tested and confirmed in the present study. We assessed thymus development by several endpoints: size (morphometric area), thymus cellularity (thymocyte number), thymus histopathology and thymocyte marker genes (*ikaros*, a marker of early thymocyte progenitors, and *tcrα* and *tcrδ*, markers of later thymocyte stage and mature T-cells). We observed a general trend for reduction of these parameters under EE2 exposure compared to control zebrafish, with the reduction being significant for thymus size and cellularity. The transcript levels of thymocyte marker genes showed a (non-significant) trend for lower expression in the EE2–10 ng/L fish, which suggests a lower intensity of thymocyte maturation under EE2 exposure, which is in line with the finding of reduced thymus size. The decrease of thymus size was not accompanied by pathological alterations but might be caused by mechanisms that we did not investigate, for instance, an increase of apoptosis of the differentiating thymocytes, an arrest of thymocyte maturation, or insufficient supply with thymocyte progenitors (Chapman et al. 2015; Do et al., 2002; Gould et al., 2000; Manley et al. 2011; Zoller and Kersh, 2006). Taken together, our results clearly point to a retarding effect of EE2 exposure on thymus development in zebrafish early life stages.

The EE2-induced alterations of the developing zebrafish thymus might be mediated through ERs, as the zebrafish thymus expresses *esr1* and *esr2a*. The presence of ERs in thymic tissue has also been reported for sea bass (*Dicentrarchus labrax*) and carp (*Cyprinus carpio*) (Paiola et al., 2017; Szwejser et al., 2017; Moreira et al., 2021). The ER isotypes of the juvenile zebrafish thymus responded differently to the EE2 treatment: while the e*sr1* mRNA levels tended to decrease compared to the control groups, the *esr2a* transcript levels tended to increase. An upregulating effect of estrogens on *esr2a* is also indicated from the finding that adult female zebrafish – with high endogenous estrogen levels – display higher thymic *esr2a* transcript levels than males do. In the thymus of juvenile seabass, which expresses three ER isotypes (*esr1, esr2a, esr2b*), only *esr2*b appeared to be responsive to treatment with the natural estrogen 17β-estradiol (Moreira et al., 2021). These authors suggested that *esr2* may play an important role in thymus ontogenesis. Interestingly, the ER responses to EE2 in the juvenile thymus of our zebrafish were opposite to the pattern observed in the liver, where the *esr1* expression significantly increased under EE2 exposure, whereas the *esr2a* expression significantly decreased. A differential regulation of hepatic and immune ERs by estrogens has also been reported for rainbow trout (Casanova-Nakayama et al., 2018). The currently available information on ERs in piscine immune organs including the thymus is very recent and the observed response patterns are not yet consistent enough to enable a functional interpretation.

A further novel question addressed in the present study was whether the thymic alterations induced by developmental EE2 exposure of zebrafish are persistent or reversible after cessation of exposure. Regarding sexual development, Maack and Segner (2004) demonstrated the existence of a critical developmental window of estrogen sensitivity in zebrafish. It is the gonad transition period from the non-functional protogynic stage to the sexually dimorphic stage. Depending on the exposure duration and the EEDC concentration, the arrestment can be reversible or irreversible (Maack and Segner 2004; Fenske et al., 2005; Schäfers et al. 2007, Segner 2009; Baumann et al., 2014b). Vulnerable developmental periods during which toxicant exposure causes persistent functional alterations may exist in the immune system of fish as well (Milston et al., 2003; Ottinger and Kaatari, 2000). Therefore, we were interested to see whether EE2 exposure during early thymus development of zebrafish results in persistent or reversible alterations. In the present study, zebrafish were exposed over a period that includes the start of thymic rudiments formation (by 46–54 h post-fertilization, hpf), the start of lymphoid progenitor colonization (by 68 hpf), the start of lymphopoiesis (by 72 hpf), the start of emigration of the T-cells (by 6–9 dpf), the differentiation of the cortex and medulla (by 1–2 weeks post-fertilization, wpf) and followed by the thymus size expansion (until 10 wpf) (Bajoghli et al., 2019; Boehm et al., 2003; Danilova et al., 2004; Dee et al., 2016; Hess and Boehm, 2012; Lam et al., 2002; 2004; Langenau et al., 2004; Willett et al., 2001; Kernen et al. 2020). EE2-exposure over this period induced thymus alterations that were reversible in female zebrafish but only partly reversible in male zebrafish. These observations suggest a higher sensitivity of the male thymus to environmental estrogens than the females, which might result in higher susceptibility to pathogen infections. The underlying mechanism(s) of the sexual difference in the recovery potential remains to be elucidated.

A caveat in our study is the statistical analysis which may give reason for debate. For our statistical analyses, we used the number of fish rather than the number of replicate tanks per treatment. This can be considered pseudo-replication. In the ecotoxicological literature, remarkable methodological discrepancies about the statistical treatment of experimental data can be found and no current consensus seems to exist concerning fish and replicate numbers. In addition to strictly statistical considerations, ethical/3R concerns are relevant as well as biological considerations (the fishes in a tank are not clones but individuals). Although statistical significances in our study are likely to change with the choice of the statistical method, the key ecotoxicological message from this study will remain valid, namely that environmental EEDCs have the capacity to impact not only the sexual but also the immune development of fish.

5. Conclusions

Based on histological, cellular, and molecular analysis, the present study provides evidence that early-life exposure to environmentally relevant EE2 concentrations can alter the thymus development of zebrafish. This is the first study reporting that environmental EEDCs are able to compromise the adaptive immune system development in a fish species. Beyond, this study is the first to demonstrate a sexual dimorphism in the thymus recovery capacity. Taken together, the present findings highlight that low environmental concentrations of EEDCs that are able to disrupt the sexual development of fish may as well be able to disrupt their immune development. Our observation in a laboratory setting with a single contaminant exposure is corroborated by the study of Liney et al. (2006) who observed that fish exposed to complex wastewater treatment effluents display immunotoxic responses at higher effluent dilutions than those needed to trigger endocrine responses. Immune defenses exist to prevent the organism from the spread of neoplastic cells and to impede pathogenic infections. Accordingly, the Darwinian fitness of organisms is critically reliant on the normal development and function of the immune system (Schulenburg et al., 2009; Roast et al., 2020). If EEDCs and other environmental toxicants compromise immunity, one outcome of this could be an increased disease susceptibility (Acevedo-Whitehouse and Duffus 2009; Martin et al., 2010). As a matter of fact, the frequency of diseases is rising among wild populations of aquatic vertebrates (Harvell et al., 1999; Johnson and Paull 2011; Mason et al., 2013; Adlard et al., 2015). Thus, it appears timely that ecotoxicologists pay more attention to the potential immunotoxic effects of environmental contaminants. Consequently, the questions to be investigated in future (regulatory) studies are, on the one hand, what mechanisms underly the estrogenic effects on the thymus, in order to be able to establish an AOP connecting estrogen receptor activation in thymocytes and thymic atrophy, and on the other hand, whether the EEDC-induced thymus alterations compromise the immunocompetence of the fish, potentially leading to adverse consequences for the population. Facing the high prevalence of EEDC-pollution in the aquatic environment, these questions are crucial to understand immunotoxicity-related effects in fish populations. Ideally, endpoints for developmental immunotoxicity could be included in existing OECD test guidelines like the FSDT, in order to assess the full impact of EEDCs on exposed fish. Moreover, conceptual frameworks like AOPs (adverse outcome pathways) could be used to link the molecular events in the thymus to the altered overall pathogen resistance of the fish.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.aquatox.2021.106025.

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