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Research Article

Impact of 17-α-Ethinylestradiol on immune function and reproductive gene expression in Zebrafish (*Danio rerio*): implications for aquaculture of important commercial fish species

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Abstract

In the present study, we detected changes in immune parameters, antioxidant enzymes, and reproductive gene expression in adult female zebrafish (*Danio rerio*) exposed to different concentrations of 17-ethinylestradiol (EE2). A total of 300 healthy adult females were divided into 15 aquaria, which were allocated to five treatments with three replicates. The zebrafish were exposed to varying concentrations of EE2 including 0.5 (T0.5), 5 (T5), 50 (T50), and 500 (T500) ng L^{-1}, along with an unexposed control group (ultrapure water, T0) for 21 days. After 21 days, lysozyme activity, total immunoglobulin, superoxide dismutase, malondialdehyde (MDA), alkaline phosphatase (ALP), alanine aminotransferase, aspartate aminotransferase (AST) and reproductive gene expression were evaluated. The results showed that exposure to EE2 caused a significant decrease in lysozyme activity, and total immunoglobulin levels, but had no significant effect (p>0.05) on the SOD activity. The MDA level was significantly higher in the high-dose hormone treatments than in the control group (p<0.05). Different EE2 concentrations had a significant impact on ALP and ALT activities and they increased in fish exposed to EE2 compared to the control (p<0.05). The expression levels of *gnrh2*, *gnrh3*, *gnrhr2*, *gnrhr3*, *lhβ*, and *fshβ* genes significantly increased (p < 0.05) in the females. In contrast, the expression of the *hsd3b* gene significantly decreased (p>0.05). Significant decreases (p<0.05) were also observed in the expression of *lhcgr* and *fshr* genes in the female zebrafish. In conclusion, our results demonstrate that different concentrations of EE2 can disrupt steroidogenesis by interfering with the expression of genes related to sex steroid levels in the female broodstock of zebrafish.

Introduction

Endocrine-disrupting chemicals (EDCs) are among the important environmental pollutants that interfere with the synthesis, release, metabolism, and function of endocrine hormones; thereby affecting homeostasis (regulation and equilibrium), growth and development, behavior, and reproduction of living organisms (Huang *et al*., 2015; Carnevali *et al*., 2018). EDCs can interfere with the endocrine system of wildlife and humans by altering the metabolism of hormones or directly binding to hormone receptors as mimics of their natural ligands (Wang *et al*., 2011; Aris *et al*., 2014). Reported adverse effects of EDCs include population decline, an increasing incidence of cancer, inhibition of reproductive function, and developing disruption of the immune and nervous systems (Jin *et al*., 2010). Over the last decade, there has been growing evidence that EDCs could be active even at low exposure concentrations compromising the reproductive and immune systems in fish (Kernen *et al*., 2022; Razekenari *et al*., 2024).

Among EDCs, estrogen is the main cause of estrogenic activity, and the prototype of estrogenic endocrine disrupting compounds (EEDCs) is the synthetic estrogen ethinyl estradiol (EE2), which is a component of contraceptive pills reaching the aquatic environment mainly through sewage treatment plant effluents (Rehberger *et al*., 2020). In aquatic environments, EE2 is ubiquitous and present in concentrations varying from "not detected" to 50 ng/L. It has become a widespread problem in aquatic environments because of its high resistance to the degradation process, absorbability of organic matter, accumulation in sediments, and bioaccumulation in living organisms (De Wit *et al*., 2010; Aris *et al*., 2014). The destructive effect of EE2 on the endocrine system of living organisms through effects on altering sex determination, delaying sexual maturity, and reducing secondary sexual traits, even at low concentrations, was reported in various studies (Liu *et al*., 2012; Aris *et al*., 2014).

EE2 has key applications in aquaculture, primarily related to the management of fish populations and the enhancement of production efficiency. EE2 is used to produce monosex populations (Piferrer, 2001; Razmi *et al*., 2011; Juarez *et al*., 2017). The application of EE2 in higher concentrations could achieve successful feminization of the YY-genotype, which has been reported to be more difficult in comparison with the process of feminizing normal progeny (Juarez *et al*., 2017). While there are significant benefits to using EE2 in aquaculture, it's essential to consider potential environmental and regulatory concerns. The use of synthetic hormones can lead to ecological issues if they enter natural water bodies, affecting wild fish populations and aquatic ecosystems. Therefore, responsible practices and regulations are crucial to minimize risks associated with EE2 use in aquaculture. Previous studies often focus on low concentrations of EE2, which may not accurately reflect the real-world scenarios where aquatic organisms are exposed to higher concentrations. Our study examines various exposure levels to assess their biological effects on gene expression and reproductive health in female zebrafish,

which is vital for understanding environmental risks and the ecological impact of EE2. The research aims to provide insights into these mechanisms by exploring how EE2 impacts the immune, antioxidant capacity, and gene expression of reproduction. Zebrafish is considered an ideal model for endocrine system function research. Therefore, this study evaluated the effects of different EE2 levels on the immune and reproduction systems.

Materials and methods

Fish rearing and experimental design

For this experiment, a four-month-old female zebrafish, *Danio rerio* (Tuebingen strain) with an average weight 0.34±0.01 g was obtained from the Rouyan Institute (Tehran, Iran) and transferred to the laboratory of Kharazmi University (Karaj, Iran). The stock, comprising 1000 zebrafish, was subjected to acclimation and health monitoring for a duration of two weeks prior to the commencement of the experimentation. The fish were fed with a commercial diet (Biomar, 38.4% crude protein, 15.3% crude fat, 93.1% dry matter, and 11% ash) twice a day during the adaptation period. The fish were adapted at ambient temperature (25±0.5 °C) for two weeks with 14-h light and 10-h dark cycles and fed thrice a day *ad libitum* with a commercial diet. After two weeks, a total number of 300 healthy adult females were chosen and divided into 15 aquaria (a density of 20 fish per aquarium). The aquaria were allocated to five treatments with three replicates. The zebrafish were exposed to different EE2 (>98% purity, Sigma) nominal concentrations including 0.5 (T0.5), 5 (T5), 50 (T50), and 500 (T500) ng L^{-1} or the unexposed control (ultrapure water, T0) for 21 days. First, four concentrations were prepared using ethanol and stored in a dark place at 4°C. The fish were exposed to EE2 using the static exposure method and artificial aeration (Gárriz *et al*., 2017). The EE2 compound was added every 48 h based on the EE2 half-life (24-48 h) (Hashimoto *et al*., 2009; Roggio *et al*., 2014). Then, the EE2 concentration was re-determined by re-drawing water from the storage tank and added to the aquariums. The effects of chronic exposure of zebrafish with EE2 were investigated for 21 days. Water quality factors were monitored periodically, the pH was 7.4±0.04.

Sampling

After 21 days of exposure to EE2, all fish of each replication were anesthetized by using 0.02% MS-222 (Liu *et al*., 2010) and placed on ice. To examine genes related to reproduction the whole brain and gonad (10 females per tank) were removed and immediately stored in liquid nitrogen. The samples were transferred to the laboratory to extract RNA, and cDNA was synthesized to evaluate the expression of reproduction-related genes. Nine fish were immersed in a 25 mM Tris-Hydrochloric acid (Tris-HCl) solution at pH 7.2 to assess immunological and antioxidant factors (Sheikhzadeh *et al*. 2017).

Immune and antioxidant measurement

Lysozyme activity was determined based on the method outlined by Ellis (1990). A suspension of *Micrococcus luteus* bacteria in phosphate-citrate buffer (75 µL) was mixed with the samples (25 µL), and

absorbance was continuously measured at 450 nm for a duration of 5 min. The total immunoglobulin (total Ig) levels in whole-body homogenates were quantified following the procedure described by Siwicki and Anderson (1993). Lipid peroxidation in the fish samples was quantified by measuring malondialdehyde (MDA) levels, following Kei's (1978) method. A 20% trichloroacetic acid solution (1.25 mL) was mixed with fish homogenate (0.25 mL) and centrifuged at 2000 ×g for 10 min. The precipitate was treated with 1.25 mL of 0.05 M sulfuric acid and 1 mL of 0.2% thiobarbituric acid, then boiled for 30 min. After adding 2 mL of n-butanol and centrifuging again at 2000 g for 10 min, absorbance was measured at 532 nm. Superoxide dismutase (SOD) activity was evaluated using the method described by Nishikimi *et al*. (1972). Alkaline phosphatase (ALP), alanine aminotransferase (ALT), and aspartate aminotransferase (AST) were measured by using commercial kits (ParsAzmon, Iran).

Gene expression

Commercial kits were used for isolating total RNA and synthesizing complementary DNA (cDNA). The target genes are reported in Table 1.

Table 1: The list of primers and their nucleotide sequences used in this study for amplifying genes.

Gene	Primer	Length (pb)	NCBI Reference
gnrh2	F: 5'GGGACTACAGTAGAGGAGCT 3' R: 5'ATCAGCCCCATGACCAACAG 3'	20 20	NM_181439.4
gnrh3	F: 5'TTGGAGGTCAGTCTTTGCCA 3' R: 5' TCAGCAGGAATAGACAGCAC 3'	20 20	NM_182887.2
gnrhr2	F: 5'CACCTTCCTCTTCCTCTTAC 3' R: 5'CGTGCCTTTGGGATGTTGTT 3'	20 20	NM_001144979.1
gnrhr3	F: 5' GACCTCTGCGTTCATCCTGG 3' R: 5' TCCTCTGTGGTGCGGTCAGT 3'	20 20	NM_001177450.1
fshβ	F: 5' CGACTCACCAACATCTCCAT 3' R: 5' CCATTGTCCAGCATAGTCCT 3'	20 20	NM_205624.1
lhβ	F: 5' CAGAGACACTTACAACAGCC 3' R: 5' AGAAGACACCATTTCCAGCC 3'	20 20	NM_205622.2
fshr	F: 5' CGACTCACCAACATCTCCAT 3' R: 5' CCATTGTCCAGCATAGTCCT 3'	20 20	NM_205624.1
Ihcgr	F: 5' GAAGAATAGAAATCGCCCAG 3' R: 5' AGACGTTCGGCAGGTTATT 3'	20 19	NM_205625.1
hsd3β	F: GACATCGAGGCTGTATCTGAC 3' R: 5' TTGAAAGCCCGTGTCGAGT 3'	21 19	NM_212797.1

The relative transcript levels of brain GnRH variants (*Gnrh2*, *Gnrh3*, *Gnrhr2*, *Gnrhr3*), pituitary Gth-β subunits (*fshb*, *lhb*) and gonadal Gth receptors (*fshr*, *lhcgr*) were determined in each individual using real-time RT-PCR. They were subjected to real-time PCR analysis, with the housekeeping gene GAPDH as a reference. The amplification of genes through PCR was undertaken, and the quantification of RNA levels was determined employing the $2^{-\Delta\Delta CT}$ method as described by Livak and Schmittgen (2001).

Statistical analysis

This experiment was conducted with a completely randomized design with three replicates for all factors. The normality and

homogeneity of variance of the data were verified through the Kolmogorov-Smirnov test and Levene's test. The obtained data were analyzed statistically by the one-way analysis of variance (ANOVA) using SPSS 25 software. Tukey's post-hoc test was used to compare the means at the 95% probability level.

Results

After 21 days, exposure to EE2 caused a significant decrease (p<0.05) in lysozyme activity and total immunoglobulin levels, but had no significant effect (p>0.05) on SOD activity levels (Table 2). MDA levels were significantly higher in the high-dose hormone treatments than in the control group (p<0.05).

Table 2: Immune and antioxidant factors in zebrafish fish exposed to different concentrations of EE2. Different letters indicate significant differences among treatments.

	T0	T0.5	T5	T50	T500
Lysozyme (u/mg protein tissue)	18.95 ± 0.17^{c}	18.83 ± 0.18^{c}	18.02 ± 0.41^{b}	16.57 ± 0.15^{a}	15.01 ± 0.23^{a}
Total Immunoglobulin (mg/g tissue)	0.90 ± 0.07^{c}	0.65 ± 0.02^{b}	0.46 ± 0.03^{a}	0.43 ± 0.01^{a}	0.40 ± 0.03^{a}
Superoxide dismutase (u/mg protein)	2.48 ± 0.11^{a}	2.72 ± 0.15^{a}	2.97 ± 0.16^{a}	2.25 ± 0.18^{a}	2.87 ± 0.28^{a}
Malondialdehyde (nmol/mg protein tissue)	2.93 ± 0.15^{a}	3.22 ± 0.13^{b}	3.45 ± 0.10^{cb}	4.40 ± 0.12^{c}	5.57 ± 0.18^{c}

Different EE2 concentrations had a significant impact on ALP (Fig. 1A) and ALT (Fig 2B) activities and they increased in fish exposed to EE2 compared to the control (p<0.05). There was no significant impact on AST activities (Fig 1C) between treatments (p>0.05).

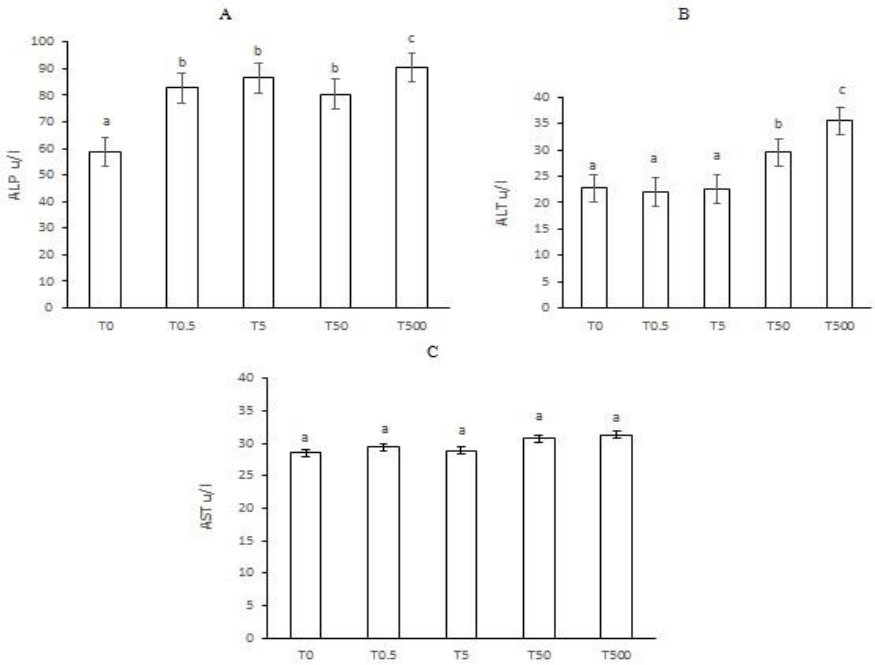

Figure 1: Liver enzyme activities in zebrafish fish exposed to different concentrations of EE2. Different letters indicate significant differences among treatments.

After exposure to different concentrations of EE2, the expression levels of *gnrh2* (Fig. 1A), *gnrh3* (Fig. 2B), *gnrhr2* (Fig. 2C), *gnrhr3* (Fig. 2D), *lhβ* (Fig. 2A), *fshβ* (Fig. 3B) increased significantly (p<0.05) in females. A significant (p<0.05) decrease was observed in *lhcgr* (Fig 3C), *fshr* (Fig. 3D), and *HSD3β* (Fig 3E) genes in female zebrafish.

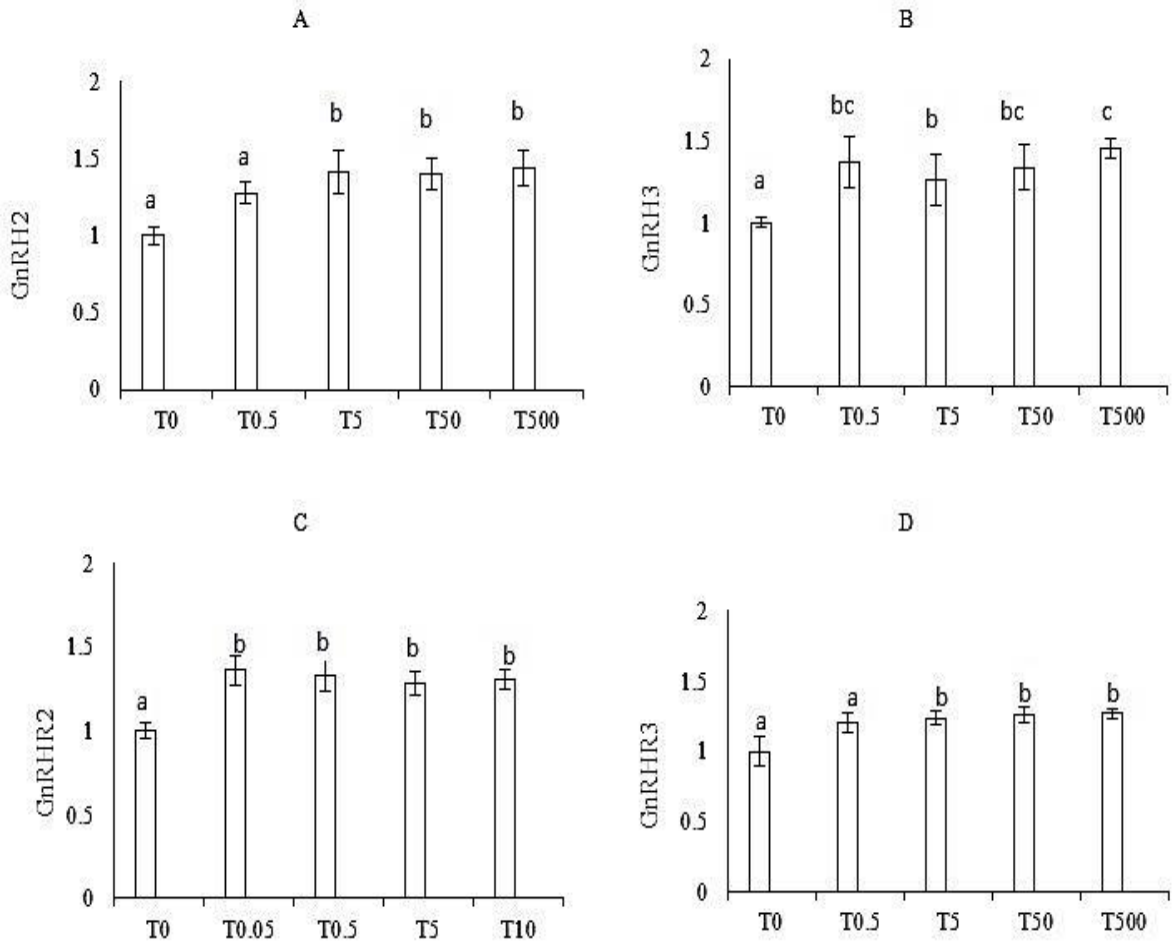

Figure 2: Gene expression of *gnrh2* (A), *gmrh3* (B), *gnrhr2* (C), and *gnrh3* (D) in zebrafish fish exposed to different concentrations of EE2. Different letters indicate significant differences among treatments.

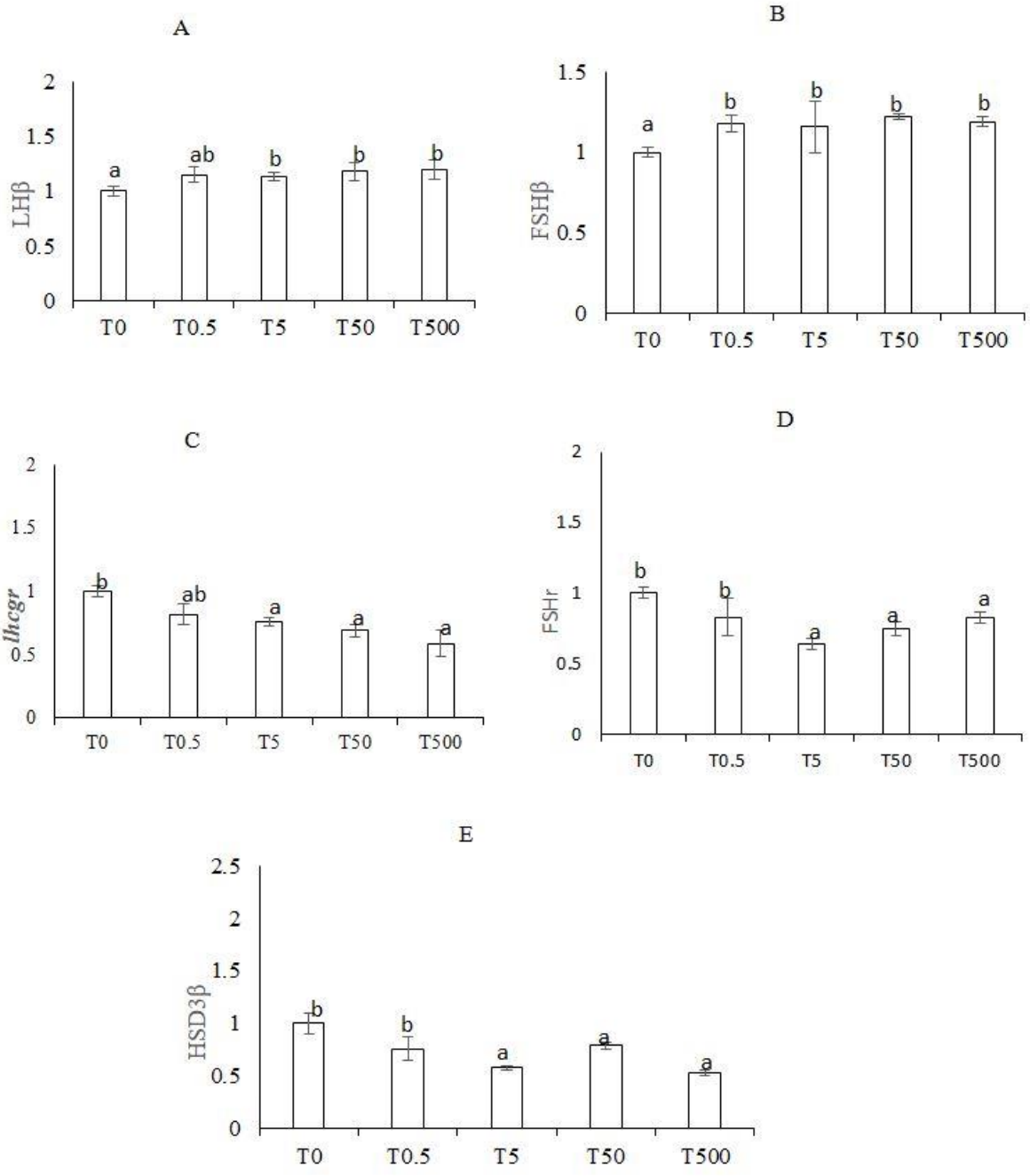

Figure 3: Gene expression of *lhβ* (Fig. 1A), *fshβ* (Fig. 2B), *lhcgr* (Fig 2C) and *fshr* (Fig 2D) in zebrafish fish exposed to different concentrations of EE2. Different letters indicate significant differences among treatments.

Discussion

The current investigation exhibited that different concentrations of EE2 possess the capability to weaken the body's immune system and disrupt the reproduction system of zebrafish, predominantly at the gonadal level.

Fish subjected to toxic environments can undergo various pathological changes, including liver damage (Toledo *et al*., 2016). In this study, fish exposed to EE2

showed a notable reduction in lysozyme and total Ig, along with a significant rise in MDA levels compared to the control group. Lysozyme is a crucial defense molecule in the innate immune system of fish, playing a vital role in protecting against infections from exogenous pathogens, including both Gram-positive and Gram-negative bacteria. As such, it serves as a key and conventional indicator of innate immunity and immune toxicity in fish. Thilagam *et al*. (2009) reported that plasma lysozyme activity decreased in sea bass (*Centropristis striata*) exposed to 0.2 and 2 ng L^{-1} EE2 over 30 days. A similar decline was noted in zebrafish larvae exposed to 0.1, 1, and 10 ng L^{-1} EE2 (Jin *et al*., 2010), as well as in rainbow trout (*O. mykiss*) exposed to 1 ng L^{-1} of EE2. MDA is a byproduct of lipid peroxidation that reflects the extent of lipid peroxidation; elevated levels of malondialdehyde indicate increased cellular toxicity, exacerbating cell and tissue damage. In this study, MDA levels significantly increased with higher EE2 exposure. Liu *et al*. (2015) reported that exposure to environmental toxins led to elevated hepatic malondialdehyde levels, which can cause tissue damage through lipid peroxidation. In this study, the MDA of fish exposed to EE2 was higher than that of the control fish, indicating that EE2 exposure can lead to ROS generation and malondialdehyde accumulation in the liver of Zebrafish.

This study found that exposure to EE2 led to significant increases in ALP and ALT. These enzymes are crucial for protein and amino acid metabolism, and they can be released into the plasma when tissue damage or dysfunction occurs. As a result, they are commonly used as indicators for diagnosing tissue damage in the liver, muscles, and gills (Atli *et al*., 2015). Zhao *et al.* (2016) reported an increase in ALT and AST activity when exposed to deoxynivalenol.

Endocrine disruption studies at the molecular level provide an accurate apprehension of the effects of this disruptor and make it possible to compare the performance of different EDCs (Sridevi *et al*., 2015). GnRHs and gonadotropins are important regulators of sex steroids and conversely these latter demonstrate both inhibitory and stimulatory feedback influences at the levels of the pituitary and hypothalamus to regulate the production and secretion of GnRH and Gths (Zohar *et al*., 2010). Since GnRHs act as neuromodulators and regulate reproductive behaviors and any change in the balance of GnRHs and GnRHRs can disrupt sex hormones balance in fish. Although numerous studies have been conducted on exposure to estrogen, only a limited number of them have focused on the disruption of *Gnrhs* or *Gnrhrs* expression analysis. In the present investigation, the expression of *gnrhs* and *gnrhrs* genes was found to increase in all treatments, indicating the presence of a positive feedback mechanism. However, a decrease in *gnrh3* expression was observed in goldfish (*Carassius auratus*) treated with 5 µg/L E2 (Golshan *et al*., 2015), and a decrease in *gnrh1* expression was observed in African catfish, *Clarias garjepinus* exposed to 1 µg/L of EE2 (Swapna and Senthilkumaran, 2009). Furthermore, Parhar *et al*. (2000) reported an increase in the number of POA-H GnRH neurons but no change in the levels of

midbrain *gnrh2* mRNA in sexually immature males of Nile tilapia, *Oreochromis niloticus* treated with 5 µg/g of E2. The expression pattern of *gn*rh gene and gonadotropins in response to EE2 differed significantly from other teleost exposed to estrogen disruptors. These contradictory findings, as stated by the authors, may be attributed to variations in fish reproductive stages, species, and experimental conditions in particular the doses used which are much lower in the present study.

However, there is a lack of information regarding the expression of the *lhb* and *fshb* genes. The conducted study exhibited substantial variability in the expression of both *gths* genes in the treated fish. A longer duration of exposure may have been necessary to identify significant differences. Nevertheless, the influence of estrogens was clearly evident in both Gths receptors, as there was a significant decrease in the transcript levels of *lhcgr* and *fshr* genes in the presence of EE2 in fish females. This decrease could potentially be attributed to a negative feedback mechanism. Similar outcomes were observed in Pejerrey fish *(Odontesthes bonariensis*) following exposure to E2 and EE2 (Garriz *et al*., 2017). The positive or negative feedback control of steroid hormones on pituitary *fshβ* and *lhβ* genes is mainly dependent on reproductive stages in teleost species.

In the present study, different levels of EE2 led to changes in the gene expression of steroidogenic enzymes in male and female zebrafish. The *hsd3b* is a key enzyme that catalyzes the synthesis of the potent steroid hormones (progesterone, 17-hydroxyprogesterone, androstenedione, and testosterone) from their hormonally active precursors (pregnenolone, 17-hydroxyprogesterone, 17-hydroxyprogesterone, and 17-hydroxyprogesterone) (Liu *et al*., 2012). The expression level of *hsd3b* gene decreased in females by the influence of different EE2 doses. Similar results were reported in adult rare minnow (*Gobiocypris rarus*) exposed to a dose of 25 ng/L (Liu *et al*., 2012). These results correspond to those reported in female *G. rarus* exposed to 25 ng/L of EE2 for 28 days (Zha *et al*., 2007). However, Filby *et al*. (2007) reported that the use of 10 ng/L of EE2 for 21 days in fathead minnow (*Pimephales promelas*) females could stimulate *cyp19a* and *cyp11a* gene expression. These discrepant results may be attributed to many factors, including species, developmental stages, EE2 concentration, and the exposure period. Inhibition of steroid enzymes by estrogenic compounds in male fish may occur due to the negative feedback of EE2 on FSH, which does not seem to be the case in the present study. Another pathway is the direct effect of EE2 on the testis of zebrafish, and the effect of E2 on testicular steroidogenic enzymes is independent of FSH stimulation (Liu *et al*., 2012).

The present results demonstrated that exposure to EE2 significantly weakens the immune abd antioxidant responses as well as disrupts reproductive gene expression in zebrafish. EE2 is used in aquaculture to produce monosex populations (Piferrer, 2001; Razmi *et al*., 2011; Juarez *et al*., 2017). Hence, EE2 can be found frequently in aquatic environments due to its presence

in wastewater effluents, can disrupt immune capability and increase oxidative stress, thereby increasing susceptibility to diseases and decreasing survival rates in fish populations. Moreover, the reproductive disorders due to disrupted gene expression raise concerns about the sustainability of breeding programs, which could lead to diminished genetic diversity and reduced reproductive success in farmed species. It is necessary to manage the use of endocrine disruptors in fish culture and aquatic systems and the importance of developing strategies to mitigate these risks, ensuring the health of aquatic ecosystems and the viability of the aquaculture sector. For future evaluation, the effects of EE2 on other commercially important fish species could be investigated to assess their vulnerability to similar disruptions. Several studies have shown that some feed additives can modulate the immunotoxin of endocrine disrupters (Kaya and Kaptaner, 2016; Abdo-Al-Ela *et al*., 2017). Therefore, future studies could investigate the effects of dietary supplements on mitigating the negative impacts of EE2 in fish.

Conflicts of 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.

References

Abo-Al-Ela, H.G., El-Nahas, A.F., Mahmoud, S. and Ibrahim, E.M., 2017. Vitamin C modulates the immunotoxic effect of 17α-methyltestosterone in Nile tilapia. *Biochemistry*, 56(**14**), 2042-2050. DOI:10.1021/acs.biochem.6b01284

Aris, A.Z., Shamsuddin, A.S. and Praveena, S.M., 2014. Occurrence of 17α-ethynylestradiol (EE2) in the environment and effect on exposed biota: a review. *Environment International*, 69, 104-119. DOI:10.1016/j.envint.2014.04.011

Atli, G., Ariyurek, S.Y., Kanak, E.G. and Canli, M., 2015. Alterations in the serum biomarkers belonging to different metabolic systems of fish (*Oreochromis niloticus*) after Cd and Pb exposures. *Environmental Toxicology and Pharmacology*, 40(**2**), 508-515. DOI:10.1016/j.etap.2015.08.001

Carnevali, O., Santangeli, S., Forner-Piquer, I., Basili, D. and Maradonna, F., 2018. Endocrine-disrupting chemicals in aquatic environment: what are the risks for fish gametes? *Fish Physiology and Biochemistry*, 44, 1561-1576.DOI: 10.1007/s10695-018-0507-z

De Wit, M., Keil, D., van der Ven, K., Vandamme, S., Witters, E. and De Coen, W., 2010. An integrated transcriptomic and proteomic approach characterizing estrogenic and metabolic effects of 17 α-ethinylestradiol in zebrafish (*Danio rerio*). *General and comparative endocrinology*, 167(**2**), 190-201. DOI:10.1016/j.ygcen.2010.03.003

Ellis, A.E., 1990. Lysozyme assay. In: Stolen J.S., Fletcher D.P., Anderson B.S., Robertson B.S. (eds) Techniques in fish immunology. SOS Publication, Fair Haven, NJ, USA. pp 101–103.

Filby, A.L., Thorpe, K.L., Maack, G., Tyler, C.R., 2007. Gene expression profiles revealing the mechanisms of anti-androgen-and estrogen-induced

feminization in fish. *Aquatic Toxicology*, 81(**2**), 219-231.DOI:10.1016/j.aquatox.2006.12.003

Gárriz, Á., Del Fresno, P.S. and Miranda, L.A., 2017. Exposure to E2 and EE2 environmental concentrations affect different components of the Brain-Pituitary-Gonadal axis in pejerrey fish (*Odontesthes bonariensis*). *Ecotoxicology and Environmental Safety*, 144, 45-53. DOI:10.1016/j.ecoenv.2017.06.002

Golshan, M., Hatef, A., Socha, M., Milla, S., Butts, I.A., Carnevali, O., Rodina, M., Sokołowska-Mikołajczyk, M., Fontaine, P., Linhart, O. and Alavi, S.M.H., 2015. Di-(2-ethylhexyl)-phthalate disrupts pituitary and testicular hormonal functions to reduce sperm quality in mature goldfish. *Aquatic Toxicology*, 163, 16-26. DOI:10.1016/j.aquatox.2015.03.017

Hashimoto, S., Watanabe, E., Ikeda, M., Terao, Y., Strüssmann, C.A., Inoue, M. and Hara, A., 2009. Effects of ethinylestradiol on medaka (*Oryzias latipes*) as measured by sperm motility and fertilization success. *Archives of Environmental Contamination and Toxicology*, 56, 253-259.DOI:10.1007/s00244-008-9183-9

Huang, Y., Wang, X.L., Zhang, J.W. and Wu, K.S., 2015. Impact of endocrine-disrupting chemicals on reproductive function in zebrafish (*Danio rerio*). *Reproduction in Domestic Animals*, 50(**1**), 1-6. DOI:10.1111/rda.12468

Juarez -Juárez, V., Alcántar-Vázquez, J.P., Antonio-Estrada, C., Marín-Ramírez, J.A. and Moreno-De La Torre, R., 2017. Feminization by 17α-ethinylestradiol of the progeny of XY-female Nile tilapia (*Oreochromis niloticus*). Effects on growth, condition factor and gonadosomatic index. *Turkish Journal of Fisheries and Aquatic Sciences*, 17(**3**), 599-607. DOI:10.4194/1303-2712-v17_3_16

Jin, Y., Chen, R., Liu, W. and Fu, Z., 2010. Effect of endocrine disrupting chemicals on the transcription of genes related to the innate immune system in the early developmental stage of zebrafish (*Danio rerio*). *Fish & Shellfish Immunology*, 28(**5-6**), 854-861. DOI:10.1016/j.fsi.2010.02.009

Kaya, Ö. and Kaptaner, B., 2016. Antioxidant defense system parameters in isolated fish hepatocytes exposed to bisphenol A—effect of vitamin C. *Acta Biologica Hungarica*, 67, 225-235. DOI:10.1556/018.67.2016.3.1

Kei, S., 1978. Serum lipid peroxide in cerebrovascular disorders determined by a new colorimetric method. *Clin. Chim. Acta*, 90(**1**), 37-43. DOI:10.1016/0009-8981(78)90081-5

Kernen, L., Phan, A., Bo, J., Herzog, E.L., Huynh, J., Segner, H. and Baumann, L., 2022. Estrogens as immunotoxicants: 17α-ethinylestradiol exposure retards thymus development in zebrafish (*Danio rerio*). *Aquatic toxicology*, 242,106025. DOI:10.1016/j.aquatox.2021.106025

Liu, Q., Chen, Y., Copeland, D., Ball, H., Duff, R.J., Rockich, B. and Londraville, R.L., 2010. Expression of leptin receptor gene in developing and adult zebrafish. *General and Comparative Endocrinology*, 166(**2**), 346-355. DOI:10.1016/j.ygcen.2009.11.015

Liu, S., Qin, F., Wang, H., Wu, T., Zhang, Y., Zheng, Y., Li, M. and Wang, Z., 2012. Effects of 17α-ethinylestradiol and bisphenol A on

steroidogenic messenger ribonucleic acid levels in the rare minnow gonads. *Aquatic Toxicology*, 122, 19-27. DOI:10.1016/j.aquatox.2012.05.010

Liu, S.Y., Zhang, J., Hu, C.X., Li, D.H. and Liu, Y.D., 2015. Antioxidative responses in zebrafish liver exposed to sublethal doses Aphanizomenon flos-aquae DC-1 aphantoxins. *Ecotoxicology and Environmental Safety*, 113,425-432. DOI:10.1016/j.ecoenv.2014.12.029

Livak, K.J. and Schmittgen, T.D., 2001. Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta CT}$ method. *Methods*, 25, 402-408. DOI:10.1006/meth.2001.1262

Nishikimi, M., Rao, N.A., Yagi, K., 1972. The occurrence of superoxide anion in the reaction of reduced phenazine methosulfate and molecular oxygen. *Biochemical and Biophysical Research Communications*, 46(**2**), 849-854. DOI:10.1016/S0006-291X(72)80218-3

Parhar, I.S., Soga, T. and Sakuma, Y., 2000. Thyroid hormone and estrogen regulate brain region-specific messenger ribonucleic acids encoding three gonadotropin-releasing hormone genes in sexually immature male fish, *Oreochromis niloticus*. *Endocrinology*, 141(**5**), 1618-1626. DOI:10.1210/endo.141.5.7460

Piferrer, F., 2001. Endocrine sex control strategies for the feminization of teleost fish. *Aquaculture*, 197(**1-4**), 229-281. DOI:10.1016/S0044-8486(01)00589-0

Razekenari, A.M., Fereidouni, A.E., Movahedinia, A. and Neyshabouri, E.Z., 2023. Impacts of sublethal concentrations of 17 α-ethinylestradiol (EE2) on growth, reproductive performance, and survival in red cherry shrimp Neocaridina davidi (*Crustacea*, *Atyidae*) during consecutive spawnings. *Aquatic Toxicology*, 259, 106519 P. DOI:10.1016/j.aquatox.2023.106519

Razmi, K., Naji, T., Alizadeh, M. and Hoseinzadeh Sahafi, H., 2011. Hormonal sex reversal of rainbow trout (*Oncorhynchus mykiss*) by ethynylestradiol-17α (EE2). *Iranian Journal of Fisheries Sciences*, 10(**2**),304-315

Rehberger, K., von Siebenthal, E.W., Bailey, C., Bregy, P., Fasel, M., Herzog, E.L., Neumann, S., Schmidt-Posthaus, H. and Segner, H., 2020. Long-term exposure to low 17α-ethinylestradiol (EE2) concentrations disrupts both the reproductive and the immune system of juvenile rainbow trout, *Oncorhynchus mykiss*. *Environment International*, 142, 105836. DOI:DOI: 10.1016/j.envint.2020.105836

Roggio, M.A., Guyón, N.F., Hued, A.C., Amé, M.V., Valdés, M.E., Giojalas, L.C., Wunderlin, D.A. and Bistoni, M.A., 2014. Effects of the synthetic estrogen 17α-ethinylestradiol on aromatase expression, reproductive behavior and sperm quality in the fish *Jenynsia multidentata*. *Bulletin of Environmental Contamination and Toxicology*, 92, 579-584. DOI: 10.1007/s00128-013-1185-2

Sheikhzadeh, N., Kouchaki, M., Mehregan, M., Tayefi-Nasrabadi, H., Divband, B., Khataminan, M., Khani Oushani, A., Shabanzadeh, S., 2017. Influence of nanochitosan/zeolite composite on growth performance, digestive enzymes and serum biochemical parameters in rainbow trout (*Oncorhynchus mykiss*). *Aquaculture Research*, 48(**12**), 5955-5964. DOI:10.1111/are.13418

Siwicki, A., Anderson, D., 1993. Nonspecific defense mechanisms assay in fish: In: Siwicki A., Anderson D., Waluga J. (eds). II. Potential killing activity of neutrophils and macrophages, lysozyme activity in serum and organs and total immunoglobulin level in serum. Fish disease diagnosis and prevention methods,. Wydawnictwo Instytutu Rybactwa Śródlądowego, Olsztyn, Poland. pp 105–112.

Sridevi, P., Chaitanya, R.K., Prathibha, Y., Balakrishna, S.L., Dutta-Gupta, A. and Senthilkumaran, B., 2015. Early exposure of 17α-ethynylestradiol and diethylstilbestrol induces morphological changes and alters ovarian steroidogenic pathway enzyme gene expression in catfish, *Clarias gariepinus*. *Environmental Toxicology*, 30(**4**), 439-451. DOI:10.1002/tox.21920

Swapna, I., Senthilkumaran, B., 2009. Influence of ethynylestradiol and methyltestosterone on the hypothalamo–hypophyseal–gonadal axis of adult air-breathing catfish, *Clarias gariepinus*. *Aquatic Toxicology*, 95(**3**), 222-229.DOI:DOI: 10.1016/j.aquatox.2009.09.008.

Thilagam, H., Gopalakrishnan, S., Bo, J. and Wang, K.J., 2009. Effect of 17β-estradiol on the immunocompetence of japanese sea bass (*Lateolabrax Japonicus*). *Environmental Toxicology and Chemistry: An International Journal*, 28(**8**), 1722-1731. DOI:10.1897/08-642.1

Toledo-Ibarra, G.A., Resendiz, K.D., Ventura-Ramón, G.H., González-Jaime, F., Vega-López, A., Becerril-Villanueva, E., Pavón, L. and Girón-Pérez, M.I., 2016. Oxidative damage in gills and liver in Nile tilapia (*Oreochromis niloticus*) exposed to diazinon. *Comparative Biochemistry and Physiology Part A: Molecular & Integrative Physiology*, 200, 3-8. DOI:10.1016/j.cbpa.2016.05.007

Wang, L., Ying, G.G., Zhao, J.L., Liu, S., Yang, B., Zhou, L.J., Tao, R. and Su, H.C., 2011. Assessing estrogenic activity in surface water and sediment of the Liao River system in northeast China using combined chemical and biological tools. *Environmental Pollution*, 159(**1**), 148-156. DOI:10.1016/j.envpol.2010.09.017

Zha, J., Wang, Z., Wang, N. and Ingersoll, C., 2007. Histological alternation and vitellogenin induction in adult rare minnow (*Gobiocypris rarus*) after exposure to ethynylestradiol and nonylphenol. *Chemosphere*, 66(**3**), 488-495. DOI:10.1016/j.chemosphere.2006.05.071

Zhao, L., Li, X., Ji, C., Rong, X., Liu, S., Zhang, J. and Ma, Q., 2016. Protective effect of *Devosia* sp. ANSB714 on growth performance, serum chemistry, immunity function and residues in kidneys of mice exposed to deoxynivalenol. *Food and Chemical Toxicology*, 92, 143-149. DOI:10.1016/j.fct.2016.03.020

Zohar, Y., Muñoz-Cueto, J.A., Elizur, A. and Kah, O., 2010. Neuroendocrinology of reproduction in teleost fish. *General and Comparative Endocrinology*, 165(**3**), 438-455. DOI:10.1016/j.ygcen.2009.04.017