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Effect of environmentally relevant concentrations of estrogens on the aggressive response of male zebrafish

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Environmental estrogens (EEs) are diverse and widespread in aquatic systems, influencing fish social behaviour. Prior studies have focused on individual EEs, but their combined effects, particularly at environmentally relevant concentrations, remain underexplored. In this study, adult male zebrafish were exposed to EE2-low (5.55 ng/L), EE2-high (11.1 ng/L), and Mix (4-NP, 62.2 ng/L; BPA, 250 ng/L; E1, 4.56 ng/L; E2, 5.53 ng/L; E3, 39.6 ng/L, with an estrogenic potency equal to EE2-low) for 60 days. Post-exposure assays (mirror test and dyadic interaction) revealed that Mix significantly reduced the frequency of approaching mirror, the attack mirror duration, and the frequency of chasing un-exposed fish, indicating a decrease in aggressive behaviour in Mix-fish. However, the ratio of 11-ketotestosterone (11-KT) to 17 β -Estradiol (E2) was observed in EE2-exposed fish in addition to Mix-fish, indicating that, in addition to sex hormones, stress hormones and neurotransmitters may also be involved in Mix-altered aggression in zebrafish. In contrast, an elevation in plasma cortisol levels and a reduction in serotonin (5-HT) and dopamine (DA) levels in the brain were found only in Mix-fish, accompanied by altered expression of genes involved in the hypothalamic-pituitary-interrenal (HPI) axis and the 5-HT/DAergic system in the brain. The data suggest that a mixture of EEs may inhibit aggression in male zebrafish by disrupting the HPI/cortisol axis and the 5-HT/DAergic system, thus causing serious ecological consequences. These findings suggest that EEs mixtures may inhibit aggression by disrupting key physiological systems. This, in turn, could undermine the competitive and then survival abilities of zebrafish, and potentially affect their population number and structure.

KEYWORDS

environmental estrogens, zebrafish, aggression, HPI/cortisol axis, serotonin, dopamine

1 Introduction

Environmental estrogens (EEs) are substances with estrogenic activity present in the environment that can interfere with the synthesis, secretion, and metabolism of endogenous hormones in animals, and can activate or inhibit the function of the endocrine system (Shen et al., 2013; Tan et al., 2024). These diverse and widely distributed substances exhibit various toxicities and are persistent in aquatic environments (Lei et al., 2009; Melo et al., 2021; Huang et al., 2013). Currently, EEs have been detected in major Chinese water systems, including the Yangtze, Yellow, and Jialing Rivers (Lei et al., 2009; Jian et al., 2009; Huang et al., 2013).

Numerous studies have demonstrated that EEs discharged into rivers impair gonadal development, reduced reproductive capacity, and induce androgynism in fish (Shen et al., 2013; Biswas et al., 2020; Gonsioroski et al., 2020; Melo et al., 2021). Additionally, EEs can cause neurological dysfunction, leading to abnormal social behaviours such as reproductive, courtship, and aggressive behaviours, thereby decreasing their mating competitiveness and the ability to defend territories (Larsen et al., 2008; Colman et al., 2009; Filby et al., 2012; Lu et al., 2024; Tan et al., 2024). While most studies have focused on the effects of individual or only a few EEs on the reproductive behaviour of fish (Baatrup and Henriksen, 2015; Melo et al., 2021), fewer have examined their impact on mood regulation (Filby et al., 2012). Given that EEs typically occur as low-concentration mixtures in natural waters, many researchers have believed that their impact on fish emotions may be minimal (Caldwell et al., 2012). For instance, Fenske et al. (2020) discovered that acute or chronic exposure to an environment containing 5 ng/L does not elicit an anxiety response in zebrafish. However, lifelong aquatic exposure suggests that environmentally relevant EEs could disrupt fish emotions over time.

Stress responses are critical emotional reactions in animals encountering competition, predators, or potential threats, including aggression, anxiety, and fear (Zabegalov et al., 2019). Aggressive responses in animals are hostile competitive acts that trigger physiological or psychological harm on conspecific or heterospecific competitors (Brodie et al., 2016). As such, aggressive responses play a significant role in both inter- and intraspecific competition among animals, where they play crucial roles in the reproduction and survival of animal populations (Filby et al., 2012; Liu et al., 2020b).

Aggressive responses are regulated by a combination of hormones, neurotransmitters, and neurons (Filby et al., 2012; Zabegalov et al., 2019). Sex hormones, particularly 11-ketotestosterone (11-KT) in fish, are crucial, with elevated plasma 11-KT levels linked to increased aggression (Filby et al., 2012; De Almeida et al., 2015). Conversely, 17 β -estradiol (E2) has been shown to attenuate aggression in male animals (Colman et al., 2009; Filby et al., 2012). In addition, higher brain expression of androgen receptor (AR) mRNA accompanies enhanced aggression in fish (Wacker et al., 2016).

In the brain, sex hormones regulate aggressive responses by modulating the expression of key regulators, including c-fos (FBJ murine osteosarcoma viral oncogene homolog), BTG-2 (B-cell translocation gene 2), Bdnf1 (Brain-Derived Neurotrophic Factor 1, BDNF1), and Dusp1 (Dual Specificity Phosphatase 1) (Karmakar et al., 2010; Allen et al., 2011; Ito et al., 2011; Vaarala et al., 2012; Malki et al., 2016). Androgens stimulate c-Fos and induce aggressive responses by binding to the JUN/AP-1 complex (Haller et al., 2006). Androgens also regulate BDNF1 expression, thereby modulating the release of dopamine (DA) and serotonin (5-HT) to indirectly influence aggression (Tyler and Pozzo-Miller, 2001; Verhovshek et al., 2013). Dusp1, an inducible immediate-early gene regulated by androgens, is implicated in modulating aggressive responses (Vaarala et al., 2012; Malki et al., 2016). Furthermore, Recent studies have shown that BTG-2 (an anti-proliferative factor that regulates the cell cycle) is positively correlated with aggressive behaviour and its synthesis is regulated by E2 (Malki et al., 2016; Karmakar et al., 2010).

Corticosteroids (e.g., cortisol in fish) are important steroid hormones regulating aggressive responses in vertebrates (Zabegalov et al., 2019). In zebrafish, cortisol synthesis is regulated by the hypothalamic-pituitary-interrenal (HPI) axis. Corticotropin-releasing hormone (CRH) stimulates the release of pro-opiomelanocortin (POMC) precursor via G protein-coupled receptors (CRHR1 and CRHR2), which is then processed into adrenocorticotrophic hormone (ACTH) by the action of prohormone convertases 1 and 2 (PC1 and PC2) (Herman et al., 2016; Zabegalov et al., 2019). ACTH subsequently acts on the interrenal tissue of fish, stimulating cortisol secretion (Oh et al., 2020). In contrast, CRH-binding protein (CRHBP) can inhibit CRH action by blocking its receptor binding, thus negatively regulating the HPI axis (Herman et al., 2016). Consequently, disruption of the HPI axis and plasma cortisol levels may alter the aggressive responses of fish.

Aggressive responses are not only controlled by the endocrine system, but also precisely modulated and executed by the nervous system. In vertebrates, neurotransmitters such as 5-HT and DA modulate aggressive responses (Chen et al., 2005; Kulikov et al., 2012; Liu et al., 2020a). 5-HT participates in many aspects of animal physiology, while its biosynthesis, metabolism, and activation have been demonstrated to modulate aggressive responses (Kulikov et al., 2012; Manuck et al., 2000). The synthesis of 5-HT is catalysed by tryptophan hydroxylase (TPH, encoded by tph1a and tph1b) and regulated by Pet-1 (Norton et al., 2008; Lillesaar, 2011). After its action, 5-HT is degraded by the monoamine oxidase MAO (Manuck et al., 2000; Fedotova et al., 2014) and the remaining dissociative 5-HT molecules are reabsorbed by the serotonin transporter (SERT) (Norton et al., 2008; Kulikov et al., 2012). DA, which is required for the regulation of reward, mood, and cognition, has also been implicated in aggressive responses and other aberrant behaviours (Chen et al., 2005; Narvaes and Almeida, 2014). DA is synthesized by tyrosine hydroxylase (TH), released at the presynaptic membrane by the vesicular monoamine transporter

(VMAT, encoded by SLC18A2), and exerts its effects by binding to its receptors (DRD1 and DRD2) (Chen et al., 2005; Narvaes and Almeida, 2014). DA is inactivated by catechol-O-methyltransferase (COMT), while the remaining dissociative DA molecules are reabsorbed by the DA transporter (DAT, encoded by SLC6A3) on the presynaptic membrane (Kahlig and Galli, 2003; Chen et al., 2005). Disruptions in any process of the 5-HTergic and DAergic pathways may alter the aggressive responses in fish.

Prior studies have shown that EEs such as bisphenol A (BPA), 4-nonylphenol (4-NP), estrone (E1), and estriol (E3) in the Jiangsu section of the Huai River, China, have reached medium-to-high risk levels, posing threats to aquatic life (Huang et al., 2019). To investigate the impact of EEs on the aggressive responses of fish, and considering that the impact of EEs is more pronounced in males, male zebrafish have been used as the experimental animals in this study. Adult male zebrafish were exposed to a mixture of EEs (Mix treatment) at environmentally relevant concentrations (based on the detected concentrations of EEs in the Huai River, Jiangsu Province, China, (4-NP, 62.2 ng/L; BPA, 250 ng/L; E1, 4.56 ng/L; E2, 5.53 ng/L; E3, 39.6 ng/L)) for 60 days (Huang et al., 2019). To compare the toxicity of Mix with that of a single environmental estrogen, adult male zebrafish were further exposed to two concentrations of EE2 (EE2-low (5.55 ng/L, with an estrogenic effect equivalent to Mix) and EE2-high (11.1 ng/L)). Aggressive responses were investigated using the mirror test and male dyadic interaction test. The plasma levels of cortisol, the ratio of 11-KT to E2 (11-KT/E2), and the brain levels of 5-HT and DA were subsequently analysed. Additionally, the mRNA level of genes involved in HPI/cortisol axis (*crha*, *crhb*, *crhbp*, *crhr1*, *crhr2*, *actha*, *acthb*, *pc1*, *pc2*), 5-HTergic (*tph1b*, *tph2*, *pet1*, *slc6a4a*, *slc6a4b*, *htr1aa*, *htr1ab*, *htr2b*, *mao*), and DAergic (*th1*, *comta*, *comtb*, *drd1b*, *drd2a*, *drd2b*, *slc6a3*, *slc18a2*) systems, as well as important local factors in the brain (*ar*, *c-fos*, *btg-2*, *bdnf1*, and *dusp1*) were analysed by quantitative reverse transcription polymerase chain reaction (qRT-PCR).

2 Materials and methods

2.1 Animals

Adult male zebrafish (AB strain, aged 4 months) sourced from the China Zebrafish Resource Center were acclimated for 14 d in a circulating water system with a constant photoperiod (light/dark: 14/10 h, illumination intensity 500 ± 20 Lux), at $28 \pm 0.5^\circ\text{C}$. During the domestication and treatment period, the water quality parameters were maintained as follows: pH, 7.4 ± 0.2 ; dissolved oxygen, 6.8 ± 0.5 mg/L. The fish were fed twice daily at 9:00 am. and 4:00 pm. with a flaky diet (Hua Pu Aquatic Development Co., Ltd., Xiamen, China) at a rate of 2% of their body weight per day.

All animal experiments were performed in accordance with the Guide for the Care and Use of Laboratory Animals and were approved by the Committee of Laboratory Animal Experimentation at Chongqing Normal University (Approval No. CKLCUFF20230803).

2.2 Exposure

After the acclimatisation period, four groups were established: Control, EE2-low (EE2, 5.55 ng/L), EE2-high (EE2, 11.1 ng/L) and Mix (4-NP, 62.2 ng/L; BPA, 250 ng/L; E1, 4.56 ng/L; E2, 5.53 ng/L; E3, 39.6 ng/L) (Huang et al., 2019). Three replicate tanks were established for each group, totalling 12 tanks. Subsequently, 120 acclimated male zebrafish (0.33 ± 0.05 g) were randomly selected and distributed among the individual exposure tanks, with 10 male zebrafish per tank (30 cm $\times$ 20 cm $\times$ 50 cm, with 20 L water, 20 cm deep). Each group thus comprised 30 male zebrafish. In this study, types and concentrations of EEs utilized in the Mix treatment group were based on the report of Huang et al. (2019) from their investigation in the Huai River Basin, Jiangsu Province, China. Details of the estrogenic potencies and EE2-equivalents of the EES compounds are listed in Supplementary Table S1. The control fish were exposed to the solvent (0.005% DMSO), which was also used for the remaining groups. All treatment groups were processed for 60 days in accordance, following the methods described by Tan et al. (2024). All chemicals were procured from Sigma-Aldrich. The purity of 4-NP (CAS No. 104-40-5), BPA (CAS No. 80-05-7), E1 (CAS No. 53-16-7), E2 (CAS No. 50-28-2), E3 (CAS No. 50-27-1), and EE2 (CAS No. 57-63-6) was $\geq 98\%$. Throughout the exposure period, the light exposure, water temperature, and water replacement were in accordance with the methodologies described in our previous study (Tan et al., 2024). The water for the exposed fish was measured weekly, and the relevant measured values are recorded in Supplementary Table S2. Besides, all male zebrafish remained healthy with no abnormalities or mortalities observed in the exposure period.

2.3 Aggressive response test

Following the exposure, aggressive responses were assessed using two methods: the mirror test and dyadic interaction test. Both methods, grounded in fish territoriality and aggressive responses to intruders, are designed as simulated aggressive response tests and have been proven effective (Kalueff and Stewart, 2012; Zabegalov et al., 2019). Besides, during behavioural testing, lighting, temperature, and water quality were maintained consistent with the exposure conditions.

Assessment I: The mirror test was conducted on the exposed fish following previously methods (Kalueff and Stewart, 2012; Liu et al., 2020b). In the aggressive test tank (15 cm $\times$ 20 cm $\times$ 25 cm, with a water level of 10 cm), a mirror was positioned on one side of the tank and a parallel line was drawn 5 cm away from mirror surface on the tank floor (Figure 1A). Initially, each experimental fish was individually acclimated in the experimental tank for 18 h and kept the mirror obscured by a baffle. An 18 h acclimation period was provided to ensure that the observed aggression could reflect the experimental conditions rather than being influenced by stress responses like anxiety from adapting to a new environment (Kalueff and Stewart, 2012). Subsequently, after the baffle was slowly

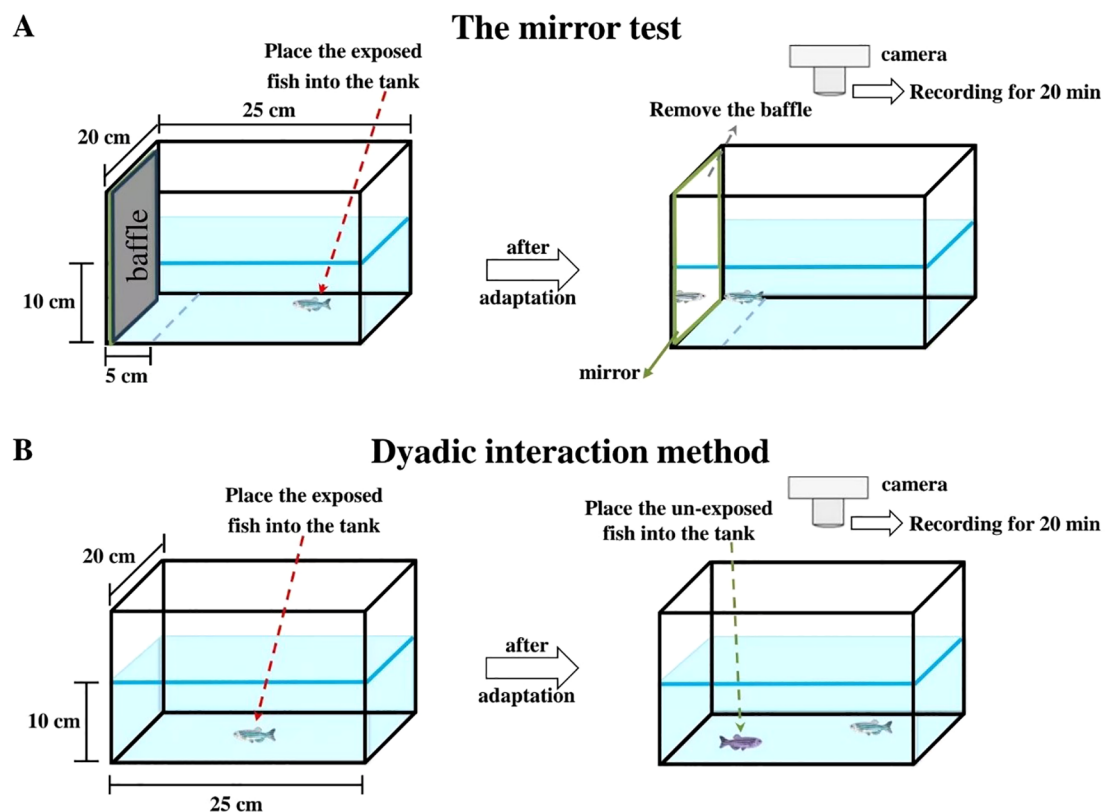


FIGURE 1
Schematic diagram of the operation for testing the aggressive response of zebrafish. (A) the mirror test; (B) the dyadic interaction method.

removed, the fish approached to (entering the area between the parallel line and mirror) the mirror image of itself and attacked it (hitting the mirror). Behaviour was recorded for 20 min using a camera (EOS R50, Canon, Jiangsu, China) mounted above the tank. Thirty fish from each group were tested ($N = 30$). The parameters of the aggressive response were subsequently analysed: 1) duration approaching mirror, 2) frequency approaching mirror (number of crossing the parallel line but without mirror contact), 3) attack duration, and 4) attack frequency (number of times the fish bite the mirror) (Kalueff and Stewart, 2012; Liu et al., 2020b).

Assessment II: In the dyadic interaction test (Figure 1B), exposed-fish were acclimatised individually in the test tank (15 cm $\times$ 20 cm $\times$ 25 cm, with a water level of 10 cm) for 18 h. Subsequently, an unexposed male zebrafish of similar size (0.32 ± 0.06 g, marked but not affecting conspecific recognition) was introduced into the tank. Behaviour was recorded for 20 min using a camera (EOS R50, Canon, Jiangsu, China) placed above the test tank ($N = 30$ per group). Generally, the exposed-fish exhibited aggression towards the intruder fish, such as chasing (the exposed fish suddenly accelerates toward against its opponent), pursuing and attempting to bite their opponents until submission was achieved (Zabegalov et al., 2019). Consequently, aggression was assessed by measuring: 1) frequency of chase (the exposed fish chased the unexposed fish) and 2) frequency of attack (the exposed fish bit the unexposed fish) (Liu et al., 2020b). After the

experiment, 120 unexposed male zebrafish were transferred to a circulating water system to be raised for use in other experiments.

To ensure objectivity and avoid bias, behavioural tests were conducted in a randomized order. Fish were randomly allocated to experimental conditions using a computer-generated randomization schedule, and tests were performed by researchers blinded to the treatment groups. The same behavioural data for individual fish were collected separately by two trained researchers (with a data discrepancy of less than 15%). The average value of each fish's same behavioural data was then calculated separately for subsequent data analysis. Following the aggression test, the fish used for behavioural testing were returned to the relevant tanks and allowed to acclimatise for two days before being sampled.

2.4 Sampling

Prior to sampling, zebrafish (10 per tank) were anaesthetised with MS-222 (0.1%) through glass capillary tubes located in the tank corners, in order to avoid additional disturbance. Subsequently, blood samples from four fish per tank were collected using heparinised capillary tubes to make one biological sample ($N = 1$). The blood samples were transferred to anticoagulant centrifuge tubes and centrifuged at 10,000 rpm for 10 min at 4°C. The supernatant was then collected and stored at -20°C to estimate the plasma levels of 11-

KT, E2, and cortisol. The brains of three fish per tank were also removed to make one biological sample ($N = 1$) for subsequent estimation of the 5-HT and DA levels in the brain, and stored at -80°C . The brains from 3 fish per tank were collected as one biological sample ($N = 1$), and immediately frozen in liquid nitrogen until total RNA extraction.

2.5 Measurement of plasma levels of 11-KT, E2, and cortisol

The plasma 11-KT, E2, and cortisol levels were measured using ELISA kits following the manufacturer's instructions (Cayman Chemical, USA). As previously described, blood sample was harvested from four fish per tank to prepare one sample ($N = 1$) and three samples ($N = 3$) for each group were prepared for analysis. The intra-assay and inter-assay coefficients of variation for hormone measurements were 5.6–6.7% and 4.2–9.4%, respectively. The sensitivity of the 11-KT, E2 and cortisol ELISA kit was 1.3 pg/mL, 20 pg/mL and 35 pg/mL respectively; and the detection ranges were 0.78–100 pg/mL, 0.61–10,000 pg/mL and 6.6–4,000 pg/mL respectively.

2.6 Measurement of brain level of 5-HT and DA

5-HT and DA levels in the brain were measured using ELISA kits (SINOBESTIO, Shanghai, China). Brains were rinsed with HBSS solution (Beyotime, China) at 4°C , and the weight of each sample (three brains pooled as one sample, $N = 1$) was accurately determined. Physiological saline was added to each sample at a ratio of 9:1 (mL/g). After thorough homogenization of the tissue, samples were centrifuged at 3000 r.p.m. for 10 minutes at 4°C . The supernatant was harvested and the concentrations of 5-HT and DA were immediately measured according to the manufacturer's instructions (SINOBESTIO, Shanghai, China). The intra- and inter-assay coefficients of variation for neurotransmitter measurements were 6.6–7.5% and 4.7–9.5%, respectively. The sensitivity of 5-HT and DA detection was < 0.1 ng/mL and 3.3 pg/mL, respectively. The detection ranges were 1.5–24 ng/mL for 5-HT and 0.5–80 ng/mL for DA, respectively.

2.7 qRT-PCR

qRT-PCR was performed as previously described (Tu et al., 2020). In summary, total RNA was extracted from samples using TRIzol reagent (Invitrogen, Carlsbad, CA, USA). Three brains were pooled to form one biological sample, with a total of three biological samples ($N = 3$) per group. RNA quality was verified by agarose gel electrophoresis, with clear 28S and 18S rRNA bands ($28\text{S}:18\text{S} > 1.5$) and assessed for concentration and purity ($A_{260}/A_{280} = 1.8\text{--}2.1$) using a Tnano-800 spectrophotometer (Tuohe Electromechanical Technology Co., Ltd., Shanghai, China). The genomic DNA was

removed using DNase I, and cDNA was synthesized thereafter using TakaRa Reverse Transcription Kit (TaKaRa, Dalian, China). The primers sequences for target genes and internal reference genes are listed in Table 1. qRT-PCR reaction was conducted using the SYBR[®] Premix Ex Taq[™] kit (TaKaRa, Dalian, China) on a CFX96[™] Real-time PCR Detection system (Bio-Rad, USA) in 96-well plates (Axygen Biosciences). Reactions were run in triplicate under the following conditions: 95°C for 30 s, followed by 40 cycles of 95°C for 5 s, 60°C for 30 s, and 72°C for 10 s. To confirm that the reactions were not contaminated, a dissociation curve and non-template controls were performed. Amplification efficiencies (95.7–104.2%) were calculated from Ct values of a serially diluted cDNA. The expression of each target gene was normalized to the geometric mean of *ef1 α* and *rpl13a* using the $2^{-\Delta\Delta\text{CT}}$ method (Livak and Schmittgen, 2001).

2.8 Statistical analysis

Statistical analysis was conducted using SPSS 27.0 (Chicago, Illinois, USA). The assumptions of normality and homogeneity of variances were assessed by using the Shapiro-Wilk test and the Levene test, respectively. As behavioural data did not meet these assumptions, a generalized linear model (GLM) was applied. Additionally, the GLM suitability was confirmed by the residualplot analysis (the Quantile-Quantile (Q-Q) plots and the standardized residuals versus predicted values plots (Supplementary Figures S1 and S2). Other data (hormones, neurotransmitters, and gene expression level) satisfied the assumptions, so one-way analysis of variance (ANOVA) was employed, followed by Fisher's Least Significant Difference (LSD) *post hoc* tests to determine intergroup differences. Data are presented as mean $\pm$ standard error (SE), with $P < 0.05$ indicating statistical significance. For all analyses, 95% confidence intervals were calculated. The GraphPad Prism 8.0 (San Diego, CA, USA) was used for graphing.

3 Results

3.1 Effect of EEs on aggressive response of zebrafish

The duration of approaching mirror and the frequency of attacking the mirror showed no significant effect between control and treatment groups ($P > 0.05$, Figures 2A, D). Compared with control, the frequency of approaching the mirror in male zebrafish was significantly reduced in Mix and EE2-high ($P_m = 0.043$ and $P_h = 0.037$, Figure 2B). The attack mirror duration of the Mix-fish was significantly decreased compared with the fish in all other groups ($P = 0.045$, Figure 2C). In dyadic interactions with unexposed males, the frequency of chase of the Mix-treated fish showed a sharp decrease compared with the control ($P = 0.042$, Figure 2E), but no significant difference compared with EE2-treated fish

TABLE 1 Primers and their sequences of the genes used in the present study.

Gene name (Protein name)	Primer sequences (5' to 3')	Accession NO.	Size (bp)	References
<i>thp1b</i> (tryptophan hydroxylase 1 b)	F: GTAAATCGAGACGGCGCAAC R: GGCACCTCAGTAAGATCGCT	NM_001001843	132	Tu et al., 2020
<i>thp2</i> (tryptophan hydroxylase 2)	F: CGTCTCATCCGTCTTCCAG R: GAGGGTGAGGATTGTGGGTG	NM_214795	139	Tu et al., 2020
<i>pet1</i> (plasmacytoma expressed transcript 1)	F: CCATTTCAGTTTTTCAGGTATTTCC R: GGCTGTGGTAGAGGGTTGGAG	EF370169	105	Tu et al., 2020
<i>slc6a4a</i> (solute carrier family 6 member 4a)	F: GTCCAACAGACGAGCCTTGA R: TAGGGAAAGCGCCACACATT	NM_001039972.1	113	Tu et al., 2020
<i>slc6a4b</i> (solute carrier family 6 member 4b)	F: TGTGTTGGGTTGCCATCAGT R: CTCCGATGATGTAGCCGACC	NM_001177459.1	132	in this study
<i>htr1aa</i> (5-hydroxytryptamine receptor 1 A a)	F: GCGAAGCGTAGAACGCAAAA R: GTTTATGACAGCGCCCAACC	NC_007119	143	Tu et al., 2020
<i>htr1ab</i> (5-hydroxytryptamine receptor 1 A b)	F: CTGTGTCGCTGCACCTTTTC R: AGTTTGGTGTGTGGGCAGA	NM_001145766.1	189	Tu et al., 2020
<i>htr2b</i> (5-hydroxytryptamine receptor 2 B)	F: CTGGGTCTCATCACTCTCGC R: GACGCGATCAGAAAGTTCCG	NM_001128709.1	104	in this study
<i>mao</i> (Monoamine oxidase)	F: TACTTGCCATACCCCTGGT R: TGCTGCCACAGTATCCCTTC	NM_212827.3	162	Tu et al., 2020
<i>th1</i> (Tyrosine hydroxylase)	F: AGCGAGCAGATCGTGTTTGA R: CCTCCAAGCCATCCTTTGGT	NM_131149.1	178	Tu et al., 2020
<i>drd1b</i> (Dopamine receptor B)	F: GAACAACTGCTGACAAGGTCT R: TACACGTGAATCGGAGCAACT	NM_001135976.2	161	Tu et al., 2020
<i>drd2a</i> (Dopamine receptor D2a)	F: TGCTCTCTGTGTGATTGCGA R: GCATGTGCGTTTGGTGTGA	NM_183068.1	151	Tu et al., 2020
<i>drd2b</i> (Dopamine receptor D2b)	F: TGTGGGGATGGAATGTTGG R: TGGACTGATATTCGGCGTGG	NM_197936.1	134	Tu et al., 2020
<i>slc6a3</i> (Dopamine Transporter)	F: TGATCATGGTGCCGCTCTAC R: CAGTCTCAGGAGTGATGGCG	NM_131755.1	93	Tu et al., 2020
<i>comta</i> (catechol-O-methyltransferase a)	F: GCGACGAGAAAGCCTCATA R: GCCAGCGTAGGCGATTATCT	NM_001030157.2	194	Tu et al., 2020
<i>comtb</i> (catechol-O-methyltransferase b)	F: TGTGAGATGAGAAAGGCTGT R: CGAGCGATGCGAACTGTAGA	NM_001083843.1	111	Tu et al., 2020
<i>slc18a2</i> (Vesicular monoamine transporter 2)	F: CCCGCCAACGAGGAAAAGAT R: GGACGCCTATTCAGGGCTTT	NM_001256225.2	132	Tu et al., 2020
<i>ar</i> (Androgen receptor)	F: GCGAATGGATGGATGTAAC R: TCATCAGAGCAGATTAGGC	NM_001083123	118	Liu et al., 2020b
<i>c-fos</i> (c-fos protein)	F: GGAGAGTCTTTGGACCTGC R: GCTGGAGTACAGGTACCAC	NC_019882	174	Liu et al., 2020b
<i>btg-2</i> (B-cell translocation gene 2)	F: GGACCACTCGCTGTCTTTCA R: AGAGGGGTCGTGAGGTATC	NM_131850	165	Liu et al., 2020b
<i>bdnf1</i> (Brain-derived neurotrophic factor)	F: GCCGCCGTTACTCTTTCTCTT R: CTGTCACCCACTGGCTAATACTG	FJ915060	140	Liu et al., 2020b
<i>dusp1</i> (dual-specificity phosphatase1)	F: ATATGTTGGAGGAGACGAC R: ACAGAAGCCTGTTCTGGTGT	NC_007125.7	165	in this study
<i>crha</i> (corticotropin releasing hormone a)	F: ATTTAGTCGAACCGCAGCCA R: ATCTCAGTCGGTGTCTCCA	XM_009298729.3	155	Tu et al., 2020
<i>crhb</i> (corticotropin releasing hormone b)	F: TTCCCGCGTATGAATGT R: TACTGGGATGTCTCGGGAA	NM_001007379.1	187	Tu et al., 2020

(Continued)

TABLE 1 Continued

Gene name (Protein name)	Primer sequences (5' to 3')	Accession NO.	Size (bp)	References
<i>crhbp</i> (corticotropin releasing hormone binding protein)	F: TCATCGGCGAACCTACTGAC R: CCTTCATCAGCCAGCCATCA	NM_001003459.1	105	Fuzzen et al., 2011
<i>crhr1</i> (corticotropin releasing hormone receptor 1)	F: CCACCGAATACCCGAAACA R: CACCCGAGGATGATCAGTG	XM_691254.6	151	Tu et al., 2020
<i>crhr2</i> (corticotropin releasing hormone receptor 2)	F: TGACGAGCCACCTGTTTCTC R: CACAGGTCAGGAGTTAGGCG	NM_001113644	143	Tu et al., 2020
<i>actha</i> (proopiomelanocortin a)	F: CCTTTCCTGTAGCACTCG R: CTTGATGGTCTGCGTTTGC	NM_181438.3	136	Tu et al., 2020
<i>acthb</i> (proopiomelanocortin b)	F: TATCGCATGACCCACTTCCG R: GGGGTTTGTGGGATTCGTCT	NM_001083051.1	91	Tu et al., 2020
<i>pc1</i> (prohormone convertase 1)	F: GAGTGAACACACCGACAT R: TTGTTGTCTGCTTGCATGGC	NM_001137662.1	158	in this study
<i>pc1</i> (prohormone convertase 2)	F: CATACCCCTTACCCGCGCTAC R: CCCACCCACAGATGTTGTT	NM_001142266.1	103	in this study
<i>ef1a</i> (eukaryotic elongation factor 1 α)	F: GATCACTGGTACTTCTCAGGCTGA R: GGTGAAAGCCAGGAGGGC	NM_131263	121	Tan et al., 2024
<i>rpl13a</i> (ribosomal protein L13 α)	F: CCCGCGTGTCTTCTTTTCC R: CTTGCTTGGCCACAATAGCG	NM_212784	111	Tu et al., 2020

($P > 0.05$, Figure 2E). In contrast, exposure to EE2 and Mix resulted no significant effect on the frequency of attack ($P > 0.05$, Figure 2F).

3.2 Effects of EEs on the ratio of 11-KT to E2 in plasma and the expression of aggression-related genes in the brain

Compared with the control, after treatment EE2 and Mix, the 11-KT/E2 ratio showed a significant reduction ($P_1 = 0.001$, $P_h < 0.001$, and $P_M = 0.001$). The 11-KT/E2 ratio had no significant effect between the EE2-low, EE2-high, and Mix groups ($P > 0.05$, Figure 3A).

Compared with the control, Mix significantly inhibited the mRNA expression levels of *ar*, *fos*, *btg-2*, *bdnfl*, and *dusp1* ($P = 0.002$, $P = 0.005$, $P = 0.031$, $P < 0.001$, and $P = 0.019$, respectively). In EE2-low and EE2-high, mRNA expression of *ar*, *btg-2*, and *bdnfl* was significantly reduced ($P_1 = 0.001$, $P_1 < 0.001$, $P_1 = 0.008$, $P_h = 0.001$, $P_h = 0.005$, and $P_h < 0.001$, respectively), and *dusp1* expression was significantly decreased only in EE2-high ($P = 0.027$, Figure 3B).

3.3 Effects of EEs on plasma levels of cortisol and expression of genes in HPI axis

Mix significantly elevated plasma cortisol levels in male zebrafish compared with control ($P < 0.001$), whereas EE2 exposure at neither concentration had no significant effect ($P > 0.05$, Figure 4A).

Compared with the control, Mix significantly upregulated the expression of *crhb*, *acthb*, *pc1*, and *pc2* ($P = 0.001$, $P = 0.023$, $P =$

0.031 , and $P = 0.012$, respectively), but sharply downregulated *crhbp* expression ($P < 0.001$). Besides, the results revealed a significant elevation in *crhb* expression ($P = 0.024$) and a significant reduction in *crhbp* ($P = 0.021$) in the EE2-high compared with the control. No significant difference in gene expression was observed in EE2-low compared with control ($P > 0.05$, Figure 4B).

3.4 Effects of EEs on brain levels of 5-HT and expressions of related genes in the brain

Compared with the control, brain 5-HT levels in male fish exhibited a significant decrease after Mix treatment ($P = 0.004$), while a slight, but not significant, decrease was found in fish treated with EE2 ($P > 0.05$). Additionally, no significant difference in brain 5-HT levels were observed between Mix- and EE2-treated groups ($P > 0.05$, Figure 5A).

Compared with the control, Mix treatment also significantly downregulated the mRNA expression levels of *tph1b*, *pet1*, *slc6a4a*, *slc6a4b*, *htr1aa*, *htr1ab*, and *htr2b* ($P = 0.003$, $P = 0.025$, $P = 0.023$, $P = 0.021$; $P = 0.008$, $P < 0.001$, and $P = 0.007$, respectively). In addition, expression levels of *tph1b*, *slc6a4a*, and *htr2b* were significantly lower in Mix than in both EE2-low and EE2-high ($P_1 = 0.017$, $P_1 = 0.029$, $P_1 = 0.014$, $P_h = 0.012$, $P_h = 0.031$, and $P_h = 0.048$, respectively; Figure 5B).

3.5 Effects of EEs on brain levels of DA and expressions of related genes in the brain

The brain level of DA showed a significant reduction in Mix-fish compared with control ($P = 0.039$). Besides, the brain DA levels

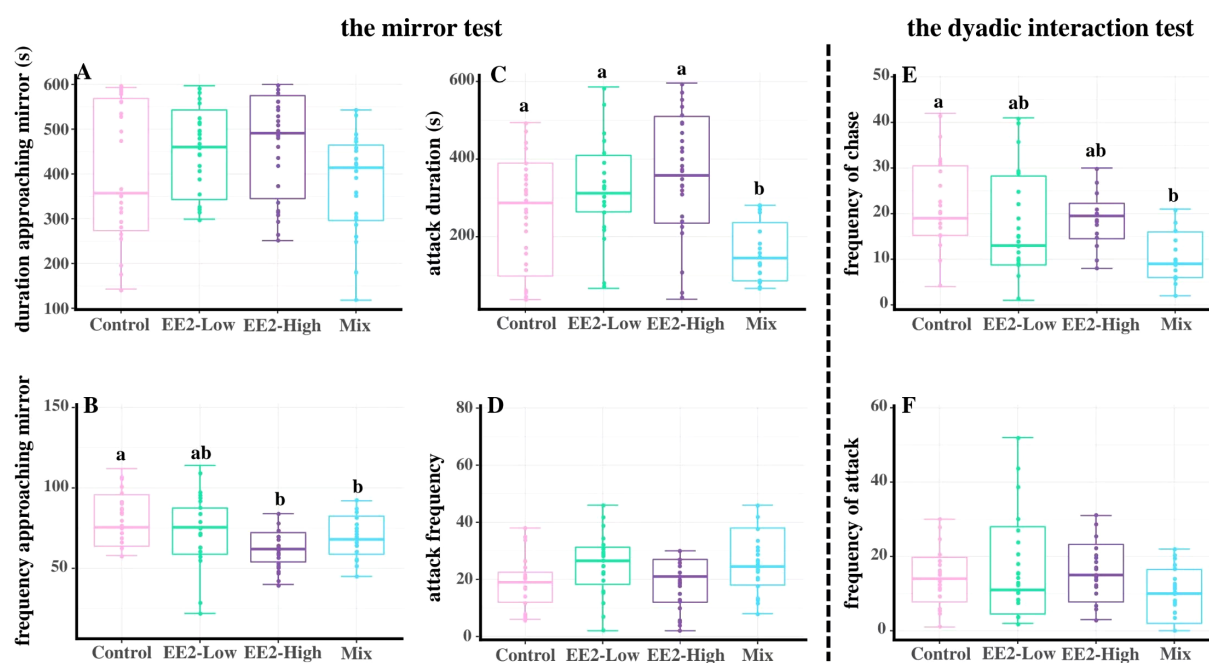


FIGURE 2

Effect of environmental relevant concentrations of EEs on aggressive response of zebrafish. (A–D) the mirror test; (E, F), dyadic interaction test. (A) duration approaching mirror; (B) frequency approaching mirror; (C) attack duration; (D) attack frequency; (E) frequency of chase; (F) frequency of attack. Different letters on boxplots (a, b) in each figure represent significant differences between groups ($P < 0.05$). Data are presented as mean $\pm$ SE, $N = 30$.

showed no significant effect between the Mix- and EE2-treated fish ($P > 0.05$, Figure 6A).

Comparative analysis revealed a significant reduction in the mRNA expression levels of *th1*, *drd1b*, *drd2a*, and *drd2b* in the brain of Mix-fish compared with control ($P < 0.001$, $P = 0.049$, $P = 0.045$, and $P = 0.043$, respectively). In both EE2 treatments groups, *th1* expression was significantly lower than in control ($P < 0.001$ and $P < 0.001$). Further, a significant decrease in *drd2b* expression was observed in EE2-low ($P = 0.010$, Figure 6B).

4 Discussion

4.1 Effect of environmental relevant concentrations of EEs on aggressive response of zebrafish

Although several studies have examined the effects of EEs on the aggressive response of fish, most have focused on the effects of a single high dose of EEs on fish aggression, with limited attention to environmentally relevant concentrations of EEs mixtures (Colman et al., 2009; Filby et al., 2012; Cooper et al., 2021; Melo et al., 2021). In natural aquatic environments, EEs are typically present at low concentrations, potentially causing minimal disruption to fish populations (Larsen et al., 2008; Caldwell et al., 2012). However,

EEs are persistent and degrade slowly, thus, behavioural anomalies in fish may emerge following long-term exposure (Huang et al., 2013; Melo et al., 2021; Lu et al., 2024). In this study, we investigated whether long-term exposure to several types of EEs at extremely low (environmentally relevant) concentrations disrupts aggressive responses in male zebrafish.

Our data revealed that only EE2-high fish exhibited a significant reduction in frequency approaching mirror following 60 d of treatment, suggesting that EE2 treatment had minimal impact on male zebrafish aggression. In contrast, three parameters (frequency approaching mirror, attack duration, and frequency of chase) were significantly reduced in Mix-treated fish, indicating attenuated aggression in Mix-exposed fish. Thus, these strongly suggest that although Mix, despite exerting an estrogenic effect similar to EE2-low, has a more pronounced disruptive effect on male zebrafish aggressive response. Moreover, the disruptive effect of Mix was more severe than that of EE2-high, which exerts a 2-fold estrogenic effect. The types and concentrations of EEs used in this study were based on those found in the Huai River Basin (Huang et al., 2019). Prolonged exposure of fish to such an environment could potentially reduce their aggression, thereby decreasing the breeding competitiveness and affecting the reproduction of fish. It could also damage the individual and population competitiveness, survival capabilities, and territorial defence abilities, thereby impacting their population number and ecosystem balance. It is

therefore strongly suggested to adopt regulatory and diverse measures to control the release of EEs into aquatic environments.

4.2 Effect of environmental relevant concentrations of EEs on plasma levels of 11-KT, E2 and expression of aggressive response-related genes

11-KT, the most potent androgen regulating spermatogenesis and reproduction in male fish, is known to modulate aggressive responses (Filby et al., 2012; De Almeida et al., 2015). Elevated plasma 11-KT levels are often associated with heightened aggression in fish (Ros et al., 2004; Filby et al., 2012; White et al., 2023). Furthermore, E2 typically mitigate aggression in male fish (Colman et al., 2009; Filby et al., 2012; White et al., 2023). In this study, the 11-KT/E2 ratio was significantly reduced in male zebrafish treated with EE2 and Mix, indicating that EE2 and Mix

may attenuate aggression by suppressing 11-KT synthesis and increasing E2 levels, which is consistent with previous research (Tan et al., 2024; Ros et al., 2004; Colman et al., 2009; Filby et al., 2012).

Androgens exert their effects via their receptors (AR), and 11-KT may regulate the aggressive response of fish by binding to AR in the brain (Filby et al., 2012; Wacker et al., 2016). Consistent with this, increased aggression in fish is often accompanied by higher *ar* mRNA expression in brain regions (Wacker et al., 2016; Liu et al., 2020b). The present study found that the expression of *ar* decreased after all treatments, aligning with the reduced 11-KT/E2 ratio. Notably, both EE2 and Mix treatments inhibited 11-KT synthesis and *ar* expression, yet only Mix significantly reduced aggression. This discrepancy may be explained by the ability of BPA and NP, components of Mix, to inhibit AR transcriptional and binding activities, thereby disrupting androgen function (Xu et al., 2006; Lerner et al., 2007a). Study has indicated that BPA or 4-NP, acting as androgen receptor antagonists, can bind to AR and thereby disrupt the regulatory function of endogenous androgens on androgen-dependent transcription (Xu et al., 2006). Alternatively, the Mix probably has a stronger estrogenic effect and toxicity than the theoretical value, an effect that is also reflected at the molecular level described as below. This mechanism may underlie the observed reduction in aggression in Mix-treated fish, which was not seen in EE2-treated fish.

A substantial body of research has identified various local factors within the brain that regulate aggressive responses, including *c-fos*, *Btg-2*, *Bdnf1*, and *Dusp1* (Chao et al., 2006; Haller et al., 2006; Malki et al., 2016). Androgens modulate the expression of *c-fos*, *Bdnf1*, and *Dusp1* via their receptors, thereby influencing aggressive responses (Allen et al., 2011; Ito et al., 2011; Vaarala et al., 2012). In this study, the mRNA levels of *btg-2* and *bdnf1* were significantly downregulated in the brains of zebrafish following all treatments, while *dusp1* expression was inhibited in EE2-high and Mix groups, consistent with decreased the 11-KT/E2 ratio. In contrast, *c-fos* mRNA levels were only suppressed in Mix-treated fish, correlating with reduced 11-KT levels and aggressive responses. Notably, *bdnf1* and *dusp1* expression was more sharply suppressed in Mix-treated fish than in EE2-treated fish, suggesting their involvement in regulating aggression. BTG-2, a key effector regulating aggressive responses, is negatively regulated by estrogen via estrogen receptors (Karmakar et al., 2010; Malki et al., 2016). In this study, *btg-2* expression in the brain was inhibited in all treatment groups (particularly in Mix-treated fish), indicating that EEs may elevate plasma E2 levels while suppressing *btg-2* expression, thereby mitigating aggressive response of zebrafish.

Interestingly, despite similar estrogenic potency, the Mix exerts a more pronounced effect on aggression than EE2. This may be due to BPA in the Mix activating non-genomic pathways like the PI3K/Akt and MAPK signalling pathways via membrane oestrogen receptors (mERs), as well as potential additive effects of the E2, BPA and 4-NP in the Mix (Jalal et al., 2018; Chen et al., 2019). In

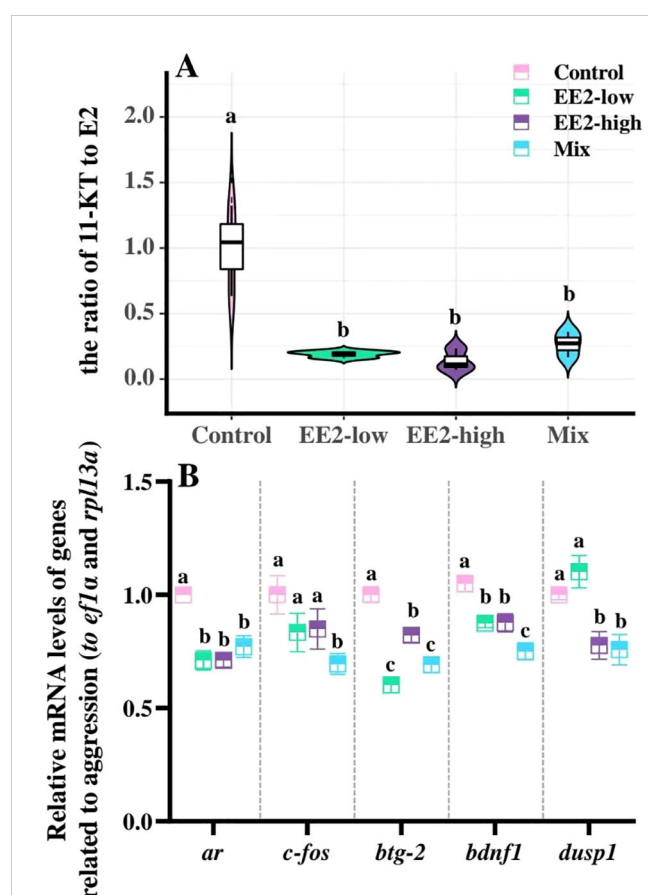


FIGURE 3
Effect of environmental relevant concentrations of EEs on the ratio of 11-KT to E2 (A) in plasma and expression of aggressive response-related genes (B). Different letters on boxplots (a–c) in each figure represent significant differences between groups ($P < 0.05$). Data are presented as mean $\pm$ SE, $N = 3$.

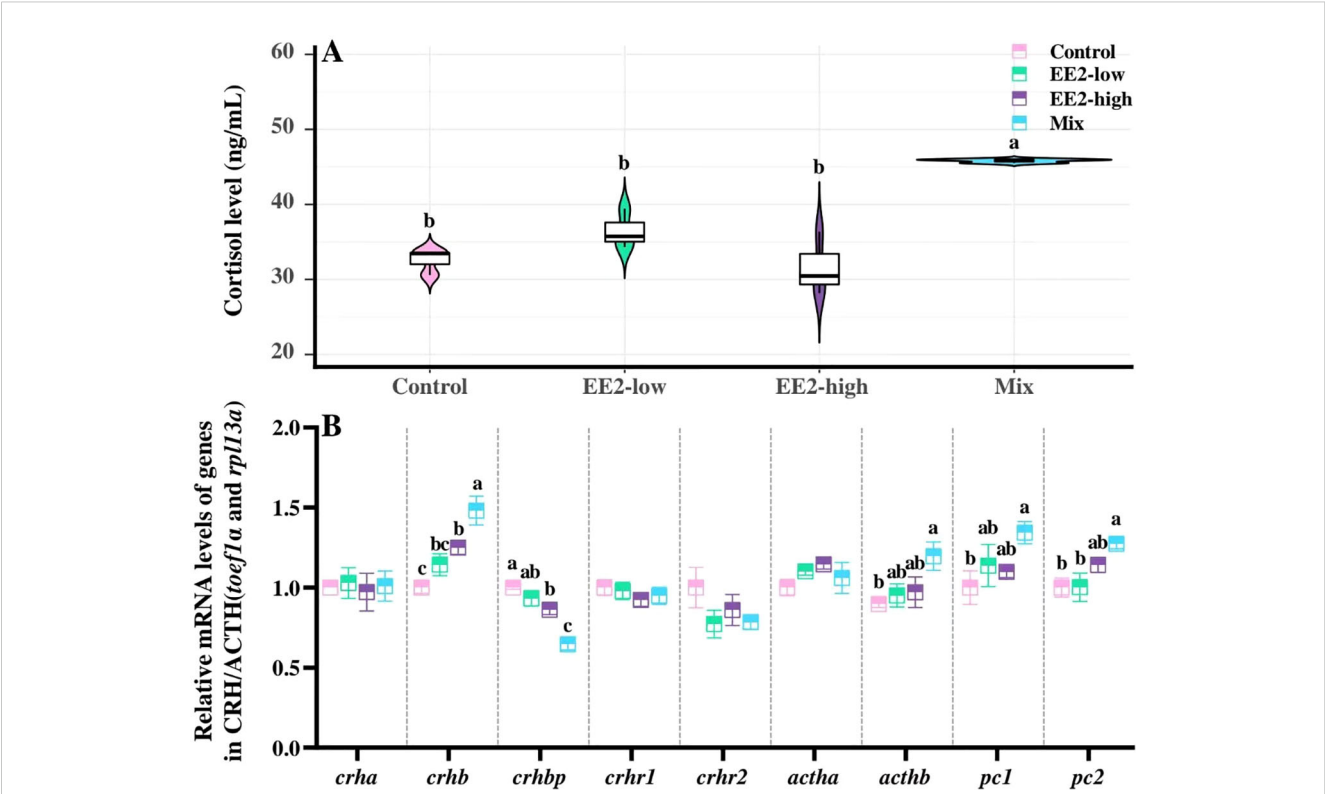


FIGURE 4
Effect of environmental relevant concentrations of EEs on plasma levels of cortisol (A) and expressions of genes in HPI axis (B). Different letters on violin plots and boxplots (a–c) in each figure represent significant differences between groups ($P<0.05$). Data are presented as mean $\pm$ SE, $N=3$.

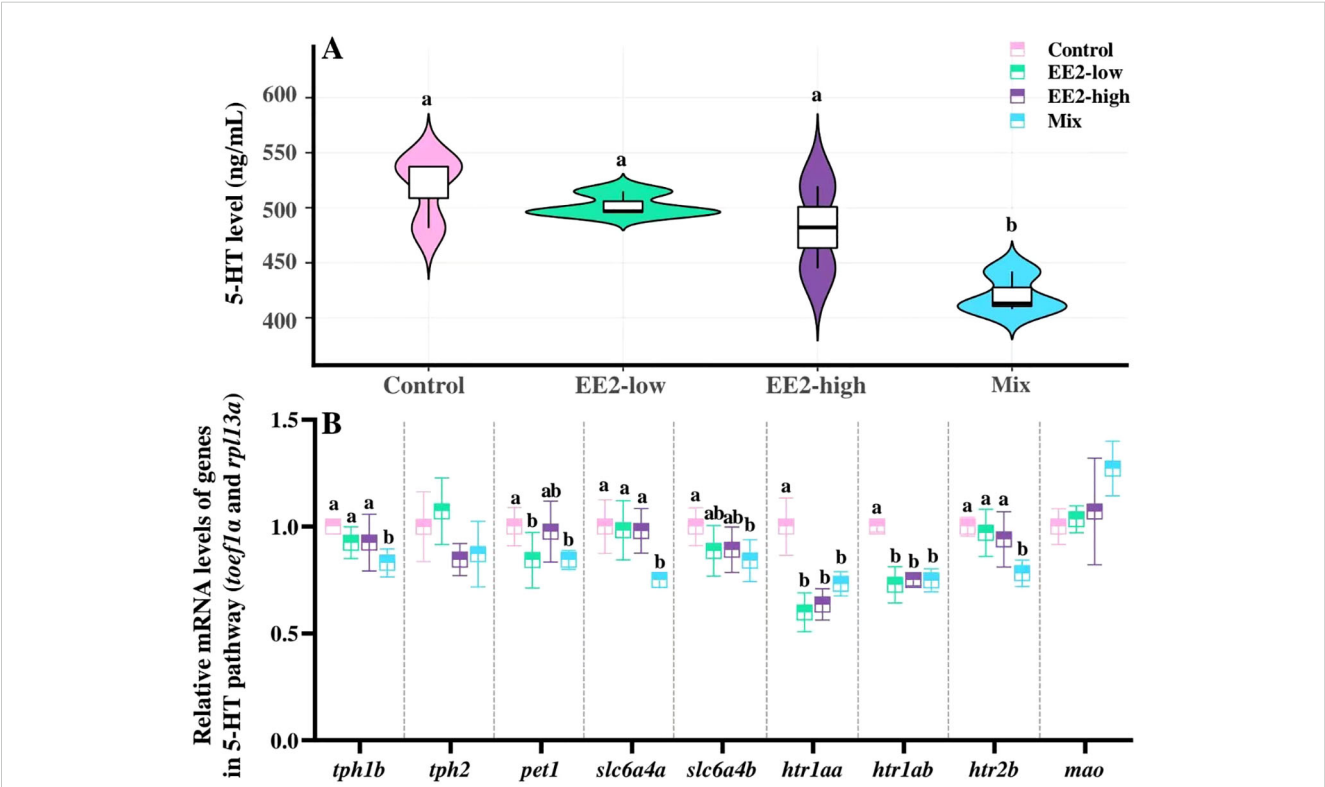


FIGURE 5
Effect of environmental relevant concentrations of EEs on brain levels of 5-HT (A) and expression of related genes (B). Different letters on violin plots and boxplots (a, b) in each figure represent significant differences between groups ($P<0.05$). Data are presented as mean $\pm$ SE, $N=3$.

summary, EEs may mitigate the aggressive response of zebrafish, probably by decreasing the 11-KT/E2 ratio, and altering the expression of effectors (especially *ar*, *c-fos*, *bdnf1* and *btg-2*) in the brain.

4.3 Effect of environmental relevant concentrations of EEs on plasma levels of cortisol and expressions of genes in HPI axis

Cortisol and corticosterone are key glucocorticoids in fish and tetrapods, known to moderate aggressive responses (Zabegalov et al., 2019; Mehta et al., 2008).

In this study, Mix-treated led to a significant increase in plasma cortisol levels in male zebrafish, concurrently with a reduction in aggressive responses, findings that are in accordance with prior studies (Mehta et al., 2008). This suggests that elevated cortisol levels may be one of the reasons for the diminished aggressive response in zebrafish exposed to Mix. Consistent with this, the mRNA levels of genes involved in cortisol synthesis and secretion, such as *crhb*, *acthb*, *pc1*, and *pc2*, were significantly upregulated in the brains of Mix-treated fish, while *crhbp* expression, which is negatively correlated with cortisol synthesis, was significantly downregulated. In contrast, neither plasma cortisol levels nor the expression of most genes in the HPI axis changed significantly in

EE2-treated fish, aligning with the unchanged aggressive response. These data suggest that long-term Mix exposure may alter the HPI axis function, elevate plasma cortisol levels, and presumably combine with a combination of other factors, including alterations in E2 levels and neurotransmitter levels and relative genes (as mentioned in the context), which thereby disrupt the aggressive response of zebrafish.

In vertebrates, sex steroids are pivotal in moderating the HPA axis: estrogens stimulate it, whereas androgens attenuate its effects (Pottinger et al., 1996; Fuzzen et al., 2011). In this study, the 11-KT/E2 ratio reduced in all treatment groups, while plasma cortisol levels only rose in Mix-treated fish. These data indicate that the increase in plasma cortisol levels is not attributable to direct regulation by sex steroids. Additionally, prior research has indicated that gradient EE2 treatment in roaches (*Rutilus rutilus*) can suppress 11-KT levels, with no significant alteration in cortisol levels (Flores-valverde et al., 2010). In contrast, direct exposure to BPA, NP, and E2, which are important components of the Mix, has been shown to elevate cortisol levels (Lerner et al., 2007b; Jamei and Sadeghi, 2016). This data suggests that the attenuation of aggressive responses in Mix-treated fish, which contain BPA, NP, and E2, may be achieved through modulation of cortisol levels.

In summary, our findings suggest that environmentally relevant concentrations of EEs could affect the HPI axis and plasma cortisol levels, thereby suppressing aggressive responses in zebrafish.

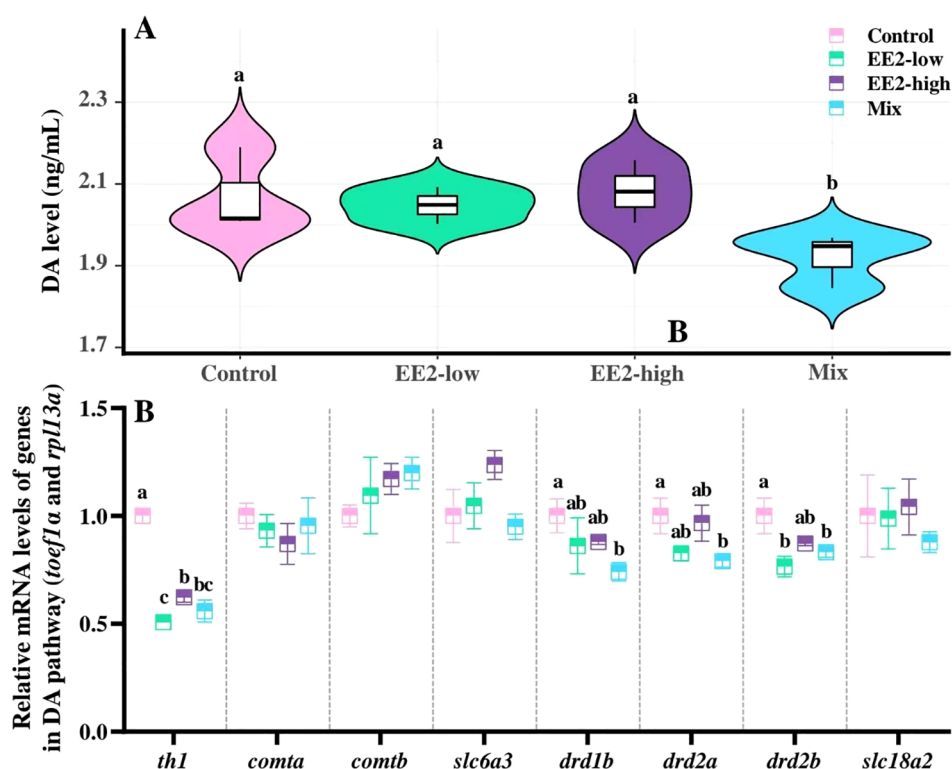


FIGURE 6

Effect of environmental relevant concentrations of EEs on brain levels of DA (A) and expression of related genes (B). Different letters on violin plots and boxplots (a–c) in each figure represent significant differences between groups ($P < 0.05$). Data are presented as mean $\pm$ SE, $N = 3$.

4.4 Effect of environmental relevant concentrations of EEs on brain levels of 5-HT and DA and expression of related genes

Neurotransmitters, particularly 5-HT and DA, are key modulators of mood and behaviour, and are implicated in the regulation of aggressive responses in vertebrates (Chen et al., 2005; Kulikov et al., 2012; Narvaes and Almeida, 2014).

In this study, a significant decrease in brain 5-HT levels was observed in Mix-fish. This aligns with previous findings that environmental estrogens reduce brain 5-HT levels and aggression (Kulikov et al., 2012). Additionally, expression of genes involved in 5-HT synthesis (*tph1b* and *pet1*) and action (*htr1aa*, *htr1ab*, and *htr2b*) were downregulated in Mix-fish, indicating a diminished 5-HTergic effect, aligning with the reduction in aggression. These suggest that Mix exposure may disrupt the aggressive response in zebrafish by disturbing the synthesis and 5-HTergic system. Besides, in this study, brain DA levels were significantly reduced only in Mix-fish, consistent with previous findings that exposure to EEs inhibited DA synthesis in zebrafish (Qi et al., 2024). Moreover, the mRNA expression of genes involved in DA synthesis (*th1*) and action (*drd1b*, *drd2a*, and *drd2b*) was significantly decreased, suggesting a further decrease in DAergic activity in Mix-exposed fish. These indicate that the DAergic system is also an indispensable effector involved in the modulation of Mix-inhibited aggressive responses in male zebrafish. Besides, previous research has indicated that the 5-HT/DA system in specific brain regions,

such as the raphe nuclei, is implicated in the regulation of aggressive behaviour (Mitsui and Takahashi, 2024). Therefore, whether gene changes are dominated by specific brain regions deserves more in-depth and detailed studies.

Prior research has shown that androgens positively influence the activation and effects of 5-HT and DA systems (Silva et al., 2008). However, in this study, both EE2 and Mix suppressed plasma 11-KT levels, whereas 5-HT and DA levels decreased only in Mix. Our previous work demonstrated that various environmental estrogens can act synergistically or additively (Tan et al., 2024). Concurrently, previous research further indicates that exposure to BPA or E2 can alter levels of 5-HT and DA (Jia and Pittman, 2015). Therefore, the present study suggests that the altered aggressive responses in mixed-exposed fish may be related to the presence of BPA and E2 in Mix, both of which affect the 5-HTergic and DAergic properties, rather than through 11-KT levels.

In fact, numerous studies have reported that the HPA axis negatively modulates 5-HT and DA levels and their activities (Porter et al., 2004; McArthur et al., 2010). In this study, plasma cortisol levels were significantly elevated in Mix-fish, while aggressive responses and brain levels of 5-HT and DA were reduced. This suggests that the attenuated aggressive responses in Mix-treated fish may result from alterations in the HPI/cortisol axis and 5-HT/DAergic systems, rather than changes in plasma 11-KT levels. Our findings align with previous studies highlighting the regulatory role of corticosteroids in the 5-HT and DA systems (Porter et al., 2004; Peng et al., 2021).

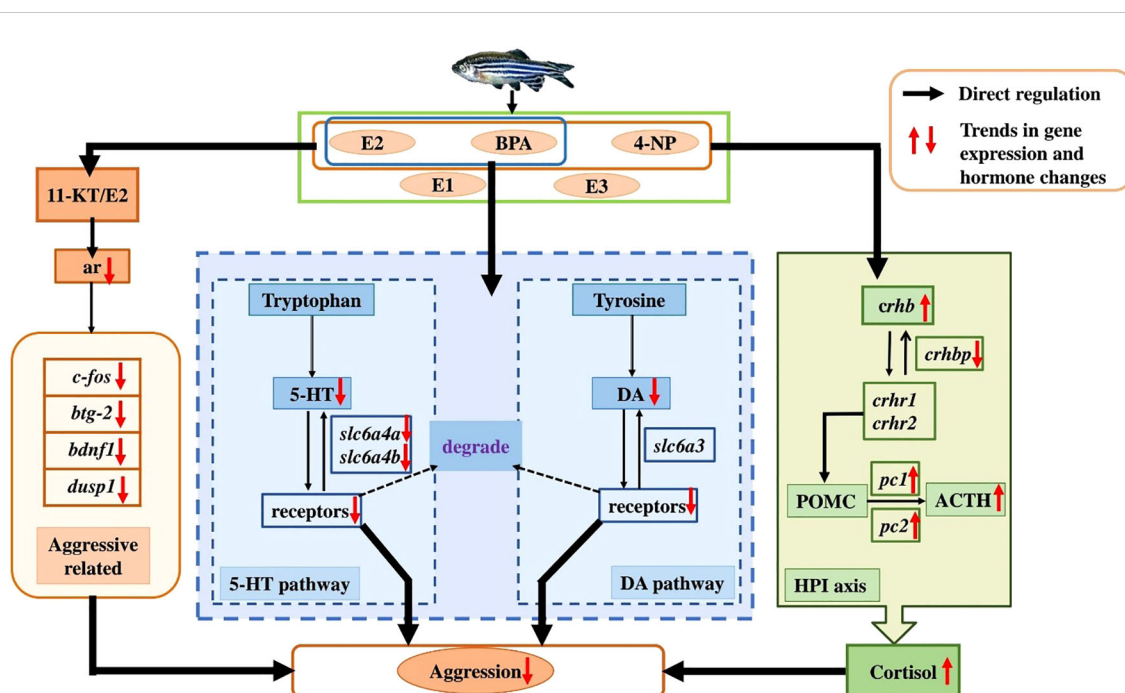


FIGURE 7

Schematic diagram of EES modulate the aggressive responses in male zebrafish through multiple pathways. Solid black arrows, regulatory pathways that EES directly regulate the HPI axis and the 5-HT/DA system, as well as the 11-KT/E2 ratio, AR and aggressive related pathways, thereby influencing aggressive responses in male zebrafish, as demonstrated in this study; Dashed black arrows, regulatory pathways that the 11-KT/E2 ratio may indirectly regulate the HPI axis and the 5-HT/DA system via AR, thereby modulating aggressive responses; Dashed blue arrows, regulatory pathways that the potential interactions between the HPI axis and the 5-HT/DA system, as well as between BDNF1 and the 5-HT/DA system, as revealed by previous studies.

Previous studies have shown distinct regulatory mechanisms between different types of estrogens (Flores-valverde et al., 2010). As previously mentioned, exposure to EE2 did not alter plasma cortisol levels, whereas exposure to E2, BPA, and NP individually induced significant changes in cortisol levels (Lerner et al., 2007b; Flores-valverde et al., 2010; Jamei and Sadeghi, 2016). In this study, the Mix included E2, BPA, and 4-NP but not EE2, which may explain the differential effects on aggressive responses. While E2, BPA and 4-NP can each independently influence hormone and neurotransmitter levels, in the Mix they may exert synergistic or additive effects, thereby producing more pronounced behavioural changes than EE2-low (Jalal et al., 2018; Chen et al., 2019). The distinct alterations in aggression between EE2 and Mix exposure may thus result from the complex composition of Mix, underscoring the need to investigate the ecological risks posed by EEs in natural waters.

5 Conclusion

The present study indicates that long-term exposure to environmentally relevant concentrations of EEs (Mix) suppresses the aggressive response in male zebrafish. Exposure to Mix altered the plasma levels of cortisol and E2/11-KT ratio, decreased the brain levels of 5-HT and DA, and disrupted the expression of genes involved in the HPI/cortisol axis, the 5-HT/DAergic system, and other response effectors, thereby affecting aggressive responses (Figure 7). In contrast, EE2 treatment did not significantly affect the aggressive response of male zebrafish, nor did plasma cortisol levels and 5-HT/DAergic systems. These results indicate that a mixture of EEs may have a potent impact on aggressive responses in fish, potentially posing significant risks to fish populations and generating potential ecological impacts. However, whether long-term exposure to environmentally relevant concentrations of EEs affect female zebrafish and other fish species remains to be further investigated. Additionally, further studies are required to corroborate the laboratory findings through *in situ* assessment of the effects of EEs on fish aggressive behaviour. To mitigate the pollution of EEs, it is imperative to reduce the emission of pollutants, actively seek out less toxic alternatives to the relevant pharmaceuticals and develop more effective degradation technologies to accelerate the breakdown of EEs in natural water bodies.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary Material. Further inquiries can be directed to the corresponding author.

Ethics statement

The animal study was approved by the Committee of Laboratory Animal Experimentation at Chongqing Normal University. The study was conducted in accordance with the local legislation and institutional requirements.

Author contributions

Y-YZ: Data curation, Investigation, Writing – original draft. HT: Data curation, Investigation, Writing – original draft. Y-JS: Methodology, Writing – review & editing. Q-LC: Formal Analysis, Software, Visualization, Writing – review & editing. Z-HL: Conceptualization, Funding acquisition, Supervision, Writing – review & editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fmars.2025.1578762/full#supplementary-material>

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