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# Endogenous 17 $\beta$ -estradiol regulates sexually dimorphic anxiety responses in zebrafish via the HPI axis and 5-HT/DA pathways

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Anxiety is a multifaceted emotional response exhibited by animals when confronted with potential threats. Among most vertebrates, including mammals and fish, there is a pronounced sexual dimorphism in anxiety responses, with females typically demonstrating higher anxiety levels than males. Concurrently, endogenous estrogen levels, specifically 17 $\beta$ -estradiol (E2), are significantly higher in females compared to males. This suggests a potential positive regulatory role of E2 on anxiety, contributing to sexually dimorphic anxiety in fish. To elucidate the role of E2 in mediating sexually dimorphic anxiety responses, male zebrafish (*Danio rerio*) were administered E2 (E2-M), while females were treated with letrozole (LET, an aromatase inhibitor that reduces E2 synthesis, LET-F) for 60 days, and plasma and brain levels of E2 were detected and anxiety response was evaluated by a novel tank diving test. Females (C-F) showed significantly higher anxiety responses, along with elevated E2 and cortisol levels in plasma and brain, and reduced brain serotonin (5-HT) and dopamine (DA) levels compared to males (C-M). Treatment with LET significantly decreased E2 levels in the plasma and brain of female zebrafish, which corresponded with reduced anxiety responses, lower plasma cortisol levels, and increased brain 5-HT and DA content. Additionally, the expression of genes associated with E2, cortisol, 5-HT, and DA pathways was relevantly altered. Conversely, E2 treatment in males (E2-M) increased E2 levels and anxiety responses, elevated plasma cortisol levels, and decreased brain 5-HT and DA content, with corresponding changes in gene expression. These findings strongly suggest that E2 positively regulates sexually dimorphic anxiety responses possibly by modulating plasma cortisol levels and the synthesis and action of 5-HT/DA in the brain.

## KEYWORDS

sexually dimorphic anxiety, zebrafish, 17 $\beta$ -estradiol, cortisol, serotonin, dopamine

## 1 Introduction

Anxiety is a physiological, psychological, and behavioral stress response elicited by actual or perceived distress or existential threats, typically triggered in individuals confronted with threatening stimuli or potential danger (Zarrindast and Khakpai, 2015; Russart and Nelson, 2018). Appropriate levels of anxiety enable animals to maintain vigilance, keenly perceive environmental changes, and evade predators, thereby reducing the risk of predation (Perrot-Minnot et al., 2017); however, altered anxiety may disturb feeding and social interactions in animals and affect their social status and reproductive competitiveness, ultimately being detrimental to the survival of individuals and perpetuation of the populations (Kavaliers and Choleris, 2001; Perrot-Minnot et al., 2017).

The endocrine system, with its array of hormones, plays a pivotal role in the modulation of stress responses in animals, and cortisol is central to the regulation of these stress reactions (Ghisleni et al., 2012; Duboué et al., 2017). In zebrafish (*Danio rerio*), the hypothalamus-pituitary-interrenal (HPI) axis governs cortisol production and secretion. Upon stimulation, the hypothalamus produces corticotropin-releasing hormone (CRH; encoded by *crha* and *crhb*), which stimulates the release of pro-opiomelanocortin through the receptors CRHR1 and CRHR2 (encoded by *crhr1* and *crhr2*) (Alderman and Bernier, 2009; Herman et al., 2016; Oh et al., 2020). Under the action of prohormone convertases 1 and 2 (encoded by *pc1* and *pc2*, respectively), adrenocorticotropic hormone (ACTH; encoded by *actha* and *acthb*) is produced and released, which leads to the synthesis and secretion of cortisol in the interrenal tissue (Alderman and Bernier, 2009; Herman et al., 2016; Oh et al., 2020). Moreover, CRH-binding protein (encoded by *crhbp*) inhibits ACTH secretion and reduces cortisol production by competitively binding to CRH (Shontz et al., 2018).

Additionally, extensive research has confirmed that the inhibitory neurotransmitter serotonin (5-hydroxytryptamine; 5-HT) and excitatory neurotransmitter dopamine (DA) exert anxiolytic effects in a range of vertebrate species, including fish (Kalueff et al., 2012; Jia and Pittman, 2015; Tu et al., 2020). In zebrafish, 5-HT in the brain is synthesized by tryptophan hydroxylase (encoded by *tph1* and *tph2*) and regulated by the transcription factor Pet-1 (encoded by *pet1*) (Bellipanni et al., 2002; Lillesaar, 2011). When transported into synaptic vesicles by the vesicular monoamine transporter (VMAT2; encoded by *slc18a2*), 5-HT is released into the synaptic cleft and is subsequently reabsorbed by the serotonin transporter (encoded by *slc6a4a* and *slc6a4b*), which is responsible for 5-HT recycling from the synaptic cleft (Norton et al., 2008). 5-HT exerts its effects by binding to specific receptors (such as 5-HT1A and 5-HT1B) on effector neurons and is subsequently degraded by monoamine oxidase (encoded by *mao*) (Norton et al., 2008; Albert et al., 2014; Maximino et al., 2015).

Similar to 5-HT, the synthesis of DA in the brain is mediated by tyrosine hydroxylase (encoded by *th1* and *th2*), transported by VMAT2, and released into the synaptic cleft, where it is reabsorbed by the DA transporter (encoded by *slc6a3*), which is responsible for the reuptake of free DA from the synaptic cleft back into the

DAergic neuron (Kaslin and Panula, 2001; Shontz et al., 2018; Tang et al., 2019). DA binds to Drd1 and Drd2 receptors to elicit excitatory neurotransmission and is subsequently metabolized and inactivated by catechol-O-methyltransferase (encoded by *comta* and *comtb*) (Kaslin and Panula, 2001; Shontz et al., 2018; Tang et al., 2019). Changes in the expression of these genes are believed to affect the levels of 5-HT and DA and their effects on the brain, thereby disrupting the anxiety response in zebrafish.

Among vertebrates, including higher mammals and more primitive fish, anxiety (a stress response) exhibits sexual dimorphism (Guo et al., 2004; Ossenkopp et al., 2005; Weil et al., 2007; de Abreu et al., 2021). In fish and rodents, females appear to be more prone to anxiety and exhibit heightened perceptivity and vigilance in response to various environmental stressors (Ossenkopp et al., 2005; Weil et al., 2007; Woodward et al., 2023).

Sex hormones, such as estrogen, which regulate a broad range of physiological functions, are considered the most important causes of various behaviors and physiological sexual dimorphisms (Gegenhuber et al., 2022; Zhang et al., 2022). Among these estrogens, 17 $\beta$ -estradiol (E2) is the primary estrogen during reproductive stages and a major regulator of sexual differentiation in vertebrates (Lagranha et al., 2018; Saito and Cui, 2018). An extensive body of evidence suggests that estrogen levels and functions exhibit sexual dimorphism, including differences within the neuroendocrine system (Che et al., 2019; Gegenhuber et al., 2022). E2 is considered to play a significant role in regulating physiological functions and cognitive abilities in female individuals (Hwang et al., 2022; Iqbal et al., 2024). Fluctuations and changes in the levels of endogenous estrogens, such as E2, are generally assumed to susceptibility to anxiety, particularly in humans (Borrow and Handa, 2017).

Based on the positive correlation between E2 levels and anxiety responses as well as research on sex-related differences in neurotransmitters and hormones, several studies have focused on the key role of E2 in regulating anxiety responses in rodents, alongside 5-HT, DA, and the hypothalamus-pituitary-adrenal (HPA) axes (Songtachalert et al., 2018; Yin et al., 2022). However, the research on fish remains limited. Interestingly, brain regions and related neurotransmitter systems associated with anxiety responses also exhibit sexual dimorphisms in vertebrates, including fish. Previous studies have shown that various neurotransmitters (such as 5-HT) in the hippocampus display distinct sexual dimorphisms (Jones and Lucki, 2005; Gressier et al., 2016). In the DAergic system, DA receptors exhibit different expression patterns in brain regions between the sexes, and the distribution and number of neurons also differ between male and female brain regions (Orendain-Jaime et al., 2016; Zachry et al., 2021). In mammals, E2 promotes neurogenesis and prevents neuronal damage, and on the other hand, regulates the neuronal activity and neurotransmitter release, thereby affecting synaptic function (Sherwin, 2003; Genazzani et al., 2007). Furthermore, the HPA/HPI axis is more active in females, with cortisol levels significantly higher than in males (Asher et al., 2017; Wang et al., 2024). This evidence suggests that E2 may play a role in regulating vertebrate mood by regulating the above-mentioned factors (Sharma et al., 2021; He et al., 2024).

Although preliminary studies have revealed the role of E2 in anxiety responses in rodents, whereas such research in fish is very limited (Sharma et al., 2021; He et al., 2024). In fact, the mechanisms by which E2 may regulate sexually dimorphic anxiety responses in animals remain largely unknown. The effects and sexually dimorphic regulatory mechanisms of E2 in anxiety in vertebrates, particularly in fish, remain to be explored (Ossenkopp et al., 2005; Ellis et al., 2023).

Zebrafish is a widely used model animal, findings in zebrafish are usually highly representative among fish species (Muralidharan et al., 2024). Consequently, the role of endogenous E2 in the regulation of anxiety responses in fish and the possible regulatory pattern of endogenous E2 in sexually dimorphic anxiety responses were studied using zebrafish as the model in this study.

To understand the role of endogenous E2 in the regulation of anxiety responses in fish and the possible regulatory pattern of endogenous E2 in sexually dimorphic anxiety responses, adult male and female zebrafish with artificially disturbed endogenous E2 levels were subjected to a novel tank diving test to assess their anxiety responses. Subsequently, plasma cortisol and E2 levels, as well as brain E2, 5-HT, and DA levels were measured using enzyme-linked immunosorbent assays (ELISA). The mRNA levels of key genes associated with E2 synthesis and action (*cyp19a1a*, *cyp19a1b*, *foxl2*, *esr1*, *esr2a*, *esr2b*, and *gper1*), HPI/cortisol axis (*crha*, *crhb*, *crhbp*, *crhr1*, *crhr2*, *actha*, *acthb*, *pc1*, and *pc2*), and 5-HT (*tph2*, *tph1b*, *mao*, *pet1*, *slc6a4a*, *slc6a4b*, *htr1aa*, *htr1ab*, and *htr1b*)/DA (*th1*, *comta*, *comtb*, *slc6a3*, *drd1b*, *drd2a*, *drd2b*, and *slc18a2*) pathways were further investigated by quantitative reverse-transcription PCR (qRT-PCR).

## 2 Materials and methods

### 2.1 Animals

Adult zebrafish (AB strain, 6 months of age) were purchased from the China Zebrafish Resource Center and were housed in a thermostatic recirculating aquaculture system before the experiment (temperature:  $28 \pm 0.5^\circ\text{C}$ ; lighting: 14 h; average illuminance:  $800 \pm 350$  lx; dissolved oxygen: 6.2–7.5 mg/L; pH: 7.3–7.6). The zebrafish were fed a microparticle-based compound diet (Shengsu, Co. Ltd., Shandong, China) three times per day, i.e., at 9:00 am, 3:00 pm, and 9:00 pm. To avoid intraspecific stress, the average rearing density of the zebrafish was maintained at 0.8 individuals/L. 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 of Chongqing Normal University (Approval No. CKLCUFF20240701-02).

### 2.2 Drug treatment

Ninety female and ninety male fish were randomly selected from the culture system and placed in 12 parallel tanks ( $50 \times 25 \times 30$  cm with 30 L water). Four groups with three replicates (15 fish per tank) were established: control-female (solvent, acetone; C-F), control-male

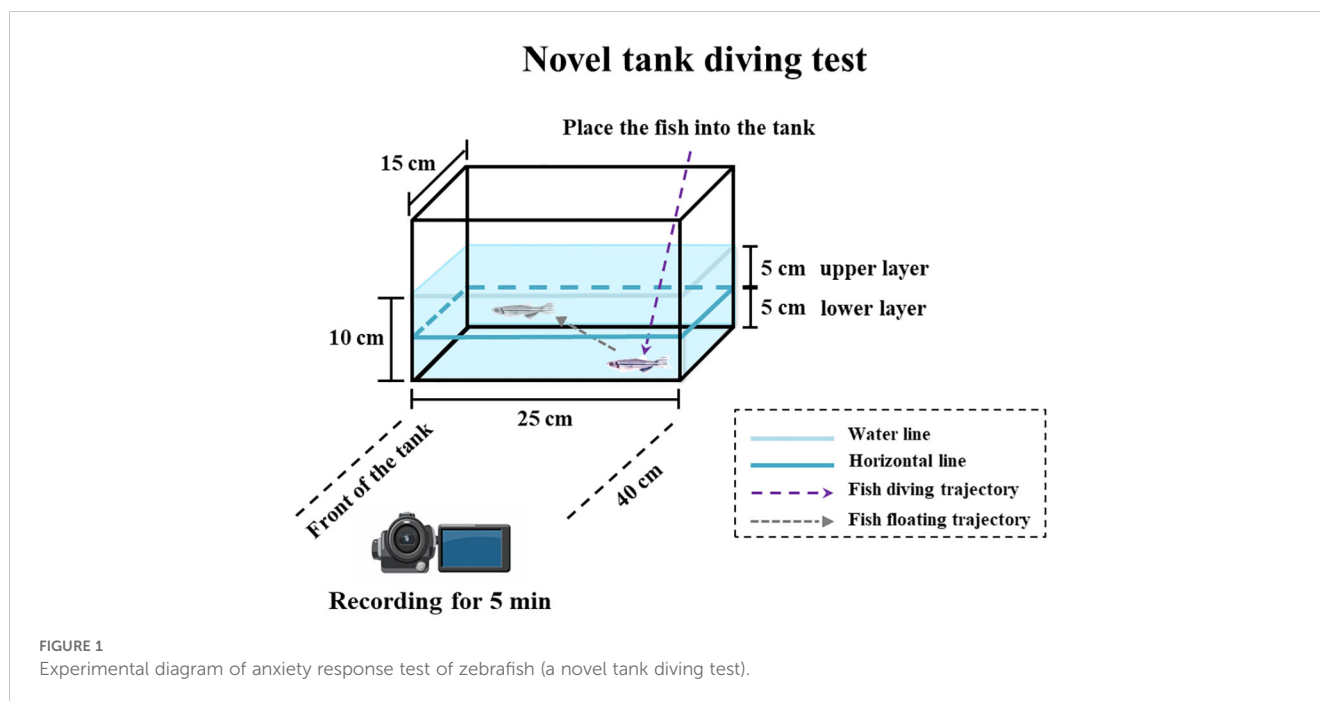
(solvent, acetone; C-M), letrozole-female (6 mg/g letrozole; LET-F), and E2-male (200 ng/g E2; E2-M). For pharmacological treatment, E2 (200 ng/g) and letrozole (LET; an aromatase inhibitor used to reduce E2 synthesis; 6 mg/g) were dissolved in acetone. The LET-F and E2-M diets were moistened with acetone containing the respective drugs, whereas the C-F and C-M diets were moistened with an equal volume of drug-free acetone. The treated diets were thoroughly dried at room temperature before use. Zebrafish of each group were fed the respective diet three times per day (at 9:00 am, 3:00 pm, and 9:00 pm) for 60 days. The daily amount of feed was 1.5%–2% of the fish body weight.

### 2.3 Novel tank diving test

To date, the novel tank diving test has proven to be effective in studying anxiety in zebrafish and is currently one of the most commonly used behavioral test models for fish (Levin et al., 2007; Kysil et al., 2017). The novel tank diving test was conducted according to the method described by Tu et al. (2020). Briefly, dechlorinated tap water was added to a novel tank ( $15 \times 20 \times 25$  cm) to a height of 10 cm, and a horizontal line was drawn 5 cm from the waterline, dividing the water evenly into upper and lower layers (Figure 1). A high-definition camera (SONY FDR-AX60) was placed approximately 40 cm in front of each novel tank to record the behavior of the experimental fish. After individually placing 45 fish from each group in the novel tank, behavioral parameters were recorded for 5 min ( $N = 45$ ). Generally, once a fish enters a new or strange environment (e.g., a novel tank) that triggers an anxiety response, it instinctively dives to the bottom of the waterbody to seek shelter. Consequently, when introduced to a novel tank, zebrafish immediately descend to the bottom of the tank in search of shelter and only begin to explore upward when they perceive the environment as safe. Based on previous research including our study (Egan et al., 2009; Tu et al., 2020), the following behavioral parameters were selected: 1) the latency to enter upper layer (the onset at which the zebrafish initially explored the upper strata of the water), and 2) the total duration spent in lower layer (the total duration that zebrafish spent in the lower water layer) (Cachat et al., 2011; Tu et al., 2020).

### 2.4 Sampling

After the novel tank diving test, fish were returned to their treatment tanks for recovery. After 24 h of recovery, MS-222 at a concentration of 0.1% was carefully administered to the corner of the tank using a pipette to anesthetize all experimental fish, which were subsequently dissected on ice. Post-anesthesia, blood was collected from 15 fish (4  $\mu\text{L}$  per fish) using heparinized capillary tubes to compile one sample ( $N = 1$ ), with each group having three biological replicates ( $N = 3$ ). These blood samples were used to determine plasma E2 and cortisol hormone levels. After blood collection, the fish were dissected to retrieve their brains, which were allocated as follows: six brains per biological sample ( $N = 1$ ), with three brain samples ( $N = 3$ ) per group for the detection of brain E2 levels; three



brains per biological sample ( $N = 1$ ), with three brain samples ( $N = 3$ ) per group for the detection of brain 5-HT levels; three brains per biological sample ( $N = 1$ ), with three brain samples ( $N = 3$ ) per group for the detection of brain DA levels; and three brains per biological sample ( $N = 1$ ), with three brain samples ( $N = 3$ ) per group for total RNA extraction. For the brains intended for total RNA extraction, each biological sample was placed in an RNA-free tube containing 1 mL TRIzol (RNAiso Plus, Takara, Dalian, China) and was homogenized. After thorough grinding in a high-throughput tissue grinder (Scientz-48, Ningbo, China), the homogenate was stored at  $-80^{\circ}\text{C}$  until total RNA extraction.

## 2.5 Measurements of E2, cortisol, 5-HT, and DA levels

Following the method described by Tu et al. (2020), the plasma E2 and cortisol levels were measured using specific E2 and cortisol ELISA kits (Cayman, Ann Arbor, MI, USA). Briefly, the blood samples were centrifuged at  $5000 \times g$  and  $4^{\circ}\text{C}$  for 10 min, and the supernatant was collected to obtain plasma. The obtained plasma was aliquoted and diluted 40-fold (for E2 of females), 20-fold (for E2 of males), and 150-fold (for cortisol) to determine the plasma E2 and cortisol levels according to the kit manufacturer's instructions (Cayman).

For the estimation of brain E2 levels, the brain samples were rinsed with physiological saline (0.86%) at  $4^{\circ}\text{C}$  and accurately weighed. Pre-chilled physiological saline was added to each sample at a ratio of 9:1 (mL/g). The brains were homogenized in a high-throughput tissue grinder and centrifuged at  $5000 \times g$  and  $4^{\circ}\text{C}$  for 5 min. The supernatant was collected, and E2 concentrations were measured using an E2 ELISA kit (Cayman) according to the kit manufacturer's instructions.

The levels of 5-HT and DA in the brain were quantified using 5-HT and DA ELISA kits (SINOBESTIO, Shanghai, China). The procedure involved rinsing the brain samples designated for the 5-HT and DA assays with ice-cold physiological saline, followed by precise recording of their weights. Subsequently, each sample was mixed with pre-chilled physiological saline at a ratio of 9:1 (mL/g). Following homogenization, the samples were centrifuged at  $5000 \times g$  and  $4^{\circ}\text{C}$  for 5 min. The resulting supernatant was used to determine the concentrations of 5-HT and DA, according to the manufacturer's instructions.

## 2.6 qRT-PCR

Total RNA extraction and qRT-PCR were performed as previously described (Wang et al., 2019). Briefly, brain tissues were homogenized in 1 mL of TRIzol reagent (Invitrogen, NY, USA) to isolate total RNA. RNA quality was verified by agarose gel electrophoresis, and RNA concentration was determined using a NanoDrop ND-2000 spectrophotometer (Thermo Electron Corporation, Waltham, MA, USA). RNA integrity was confirmed using agarose gel electrophoresis. DNase I was applied to eliminate genomic DNA contamination, and cDNA synthesis was performed from 1  $\mu\text{g}$  RNA using a TakaRa Reverse Transcription Kit (TaKaRa, Dalian, China). Gene-specific primers for each target gene and the internal reference genes (*rpl13a* and *ef1a*) was prepared as published previously (Table 1), and their specificity were confirmed by observing a single band of the expected size in the PCR products derived from the cDNA of C-F fish. The internal reference genes *rpl13a* and *ef1a* were selected because of their relatively constant expression levels across various tissues in zebrafish, and their expression levels remained unchanged after drug treatment. Following the method described previously (Wang

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>cyp19a1a</i> (cytochrome P450 family 19 subfamily A polypeptide 1a)	F: AGATGTCGAGTTAAAGATCCTGCA R: CGACCGGGTGAAAACGTAGA	NM131154	131	Wang et al., 2019
<i>cyp19a1b</i> (cytochrome P450 family 19 subfamily A polypeptide 1b)	F: ACTAAGCAAGTCTCCGCTGTGTACC R: TTAAACATACCGATGCATTGCAGACC	NM_131642	100	Wang et al., 2019
<i>foxl2</i> (forkhead box L2a)	F: CGAGAAGAACAAGAAGGGATGG R: TTGCCTGGCTGGAAATGG	BC116585.1	219	Wang et al., 2019
<i>gper1</i> (G protein-coupled estrogen receptor 1)	F: AACCTCGCTGCATTCTCCAA R: GACCAGCTTGCCTTCCTCTT	NM_001128723.1	110	Wang et al., 2019
<i>esr1</i> (estrogen receptor 1)	F: AACCTTCCTCGCCACCTGC R: CCACCGCAAGCGTCTGTA	XM_009299459	226	Wang et al., 2019
<i>esr2a</i> (estrogen receptor 2a)	F: CGACTTCAACAGAACCATGCTACTAG R: CTTACACGACCACACTCCATAATG	NM_180966.2	211	Wang et al., 2019
<i>esr2b</i> (estrogen receptor 2b)	F: CAGTCCCTCTCAGCACCTCTTTC R: TATCCAGCCAGCAGCATTCCAG	NM_174862.3	287	Wang et al., 2019
<i>thp1b</i> (tryptophan hydroxylase 1 b)	F: GTAAATCGAGACGGCGCAAC R: GGCACCTCAGTAAGATCGCT	NM_001001843	132	Liu et al., 2020
<i>thp2</i> (tryptophan hydroxylase 2)	F: CGTCTCATCCGCTTCCAG R: GAGGGTGAGGATTGTGGGTG	NM_214795	139	Tu et al., 2020
<i>pet1</i> (plasmacytoma expressed transcript 1)	F: CCATTCAAGTTTCAGGTATTTC 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: CTCGGATGATGTAGCCGACC	NM_001177459.1	132	Tu et al., 2020
<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: CTGTGTCGCTGCCTTTTC R: AGTTTGGTGTGGGCGAGA	NM_001145766.1	189	Tu et al., 2020
<i>htr1b</i> (5-hydroxytryptamine receptor 1 B)	F: CTGGGTCTCATCACTCTCGC R: GACGCGATCAGAAAAGTTCGC	NM_001128709.1	104	Tu et al., 2020
<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: CCTCCAAGCCATCCTTGGT	NM_131149.1	178	Li 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: TGCTCTGTGTGATTGCGA R: GCATGTGCGTTTGGTGTGA	NM_183068.1	151	Tu et al., 2020
<i>drd2b</i> (dopamine receptor D2b)	F: TGTGGGGATGGAAATGGTGG R: TGGACTGATATTCGGCGTGG	NM_197936.1	134	Tu et al., 2020
<i>slc6a3</i> (dopamine Transporter)	F: TGATCATGGTGCCGCTCTAC R: CAGTCTCAGGAGTATGGCG	NM_131755.1	93	Tu et al., 2020
<i>comta</i> (catechol-O-methyltransferase a)	F: GCGACGAGAAAGGCCTCATA R: GCCAGCGTAGGCGATTATCT	NM_001030157.2	194	Tu et al., 2020
<i>comtb</i> (catechol-O-methyltransferase b)	F: TGTTGGAGATGAGAAAGGCTGT 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

(Continued)

TABLE 1 Continued

Gene name (Protein name)	Primer sequences (5' to 3')	Accession NO.	Size(bp)	References
<i>crha</i> (corticotropin releasing hormone a)	F: ATTTAGTTCGAACCGCAGCCA R: ATCTCAGTCGGTGTCTCTCCA	XM_009298729.3	155	Tu et al., 2020
<i>crhb</i> (corticotropin releasing hormone b)	F: TTCCCGCCGTATGAATGT R: TACTGGGATGTCTCGGGAA	NM_001007379.1	187	Tu et al., 2020
<i>crhbp</i> (corticotropin releasing hormone binding protein)	F: TCATCGGGGAACCTACTGAC R: CCTTCATCACCAGCCATCA	NM_001003459.1	105	Fuzzen et al., 2010
<i>chr1</i> (corticotropin releasing hormone receptor 1)	F: CCACCGAATACCCGAAACA R: CACCCCGAGGATGATCAGTG	XM_691254.6	151	Tu et al., 2020
<i>chr2</i> (corticotropin releasing hormone receptor 2)	F: TGACGAGCCACCTGTTTCTC R: CACAGGTGAGGATTAGGCG	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: TATCGCATGACCCACTCCG R: GGGGTTTGTGGGATTCTGCT	NM_001083051.1	91	Tu et al., 2020
<i>pc1</i> (prohormone convertase 1)	F: GAGTGGAAACCACCGACAT R: TTGTTGCTGCTTGCATGGC	NM_001137662.1	158	Tu et al., 2020
<i>pc2</i> (prohormone convertase 2)	F: CATACCCTTACCCGCGCTAC R: CCCACCCACAGATGTTGTT	NM_001142266.1	103	Tu et al., 2020
<i>rpl13a</i> (ribosomal protein L13 $\alpha$ )	F: CCCGCGTGTCTTTCTTTCC R: CTTGCTTGGCCACAATAGCG	NM_212784	111	Tu et al., 2020
<i>ef1a</i> (eukaryotic translation elongation factor 1 alpha 1, like 1)	F: GATCACTGGTACTTCTCAGGCTGA R: GGTGAAAGCCAGGAGGGC	NM_131263	121	Tan et al., 2024

et al., 2019), primer fit and stability of the reference gene across all groups were validated. qRT-PCR was performed using a SYBR<sup>®</sup> Premix Ex Taq<sup>™</sup> kit (TaKaRa) on a CFX96<sup>™</sup> Real-time PCR Detection system (Bio-Rad, Hercules, CA, USA). All samples were assayed in triplicates in 96-well PCR plates (Axygen Biosciences, Union City, CA, USA). qRT-PCR thermal cycling conditions were as follows: 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 each primer pair produced a single PCR product and to avoid contamination, dissociation curve analysis and non-template controls were conducted for each PCR reaction. Using a series of cDNA dilutions and their corresponding Ct values, the amplification efficiencies of all genes were calculated to be between 94.8% and 103.8%. As previously described, the expression of each target gene was quantified relative to the average value of *rpl13a* and *ef1a* using the  $2^{-\Delta\Delta CT}$  method (Livak and Schmittgen, 2001).

## 2.7 Statistical analysis

The data are expressed as means  $\pm$  standard error of the mean. The normality and homogeneity of variance of the original data were analyzed using Shapiro–Wilk and Levene’s tests, respectively. Given that the behavioral data did not conform to a normal distribution, a generalized linear model was used to analyze the relationship between the different treatments and anxiety responses. In this analysis process, a 95% confidence interval level was established, and the “Wald” Chi-squared statistic was used, followed by pairwise comparisons of each treatment group. One-

way ANOVA was used to analyze other indicators (hormones, neurotransmitters, and gene expression), followed by *post-hoc* testing using Tukey’s test. Statistical analysis was performed using SPSS 27.0 (Chicago, IL, USA), and the statistics results were statistically significant when  $P < 0.05$ . GraphPad Prism 8.0 (San Diego, CA, USA) was used for graphing purposes, and R software (v.4.2.2) with the packages “ggpubr” (v0.4.0) and “ggplot2” (v3.4.2) through Hiplot Pro (<https://hiplot.com.cn/>, a comprehensive web service for biomedical data analysis and visualization) was utilized to generate figures (Villanueva and Chen, 2019; Kassambara, 2020).

## 3 Results

### 3.1 Plasma and brain E2 levels and expressions of genes for E2 synthesis and action

The plasma and brain E2 levels of C-F fish were significantly higher than those of C-M fish (Figure 2A,  $P_{(\text{plasma E2})} < 0.001$ ; Figure 2B,  $P_{(\text{brain E2})} < 0.001$ ). Moreover, the mRNA levels of *cyp19a1a*, *foxl2*, *esr1*, and *gper1* in the brains of C-F fish were significantly higher than those in C-M fish brains (Figure 2C,  $P_{(\text{cyp19a1a})} < 0.001$ ,  $P_{(\text{foxl2})} = 0.045$ ,  $P_{(\text{esr1})} = 0.008$ ,  $P_{(\text{gper1})} = 0.002$ ).

The plasma and brain E2 levels in LET-F fish were significantly lower than those in C-F fish (Figure 2A,  $P_{(\text{plasma E2})} < 0.001$ ; Figure 2B,  $P_{(\text{brain E2})} = 0.007$ ) but were still significantly higher than those in C-M fish (Figure 2A,  $P_{(\text{plasma E2})} < 0.001$ ; Figure 2B,  $P_{(\text{brain E2})} = 0.005$ ). Furthermore, compared with the C-F fish, the

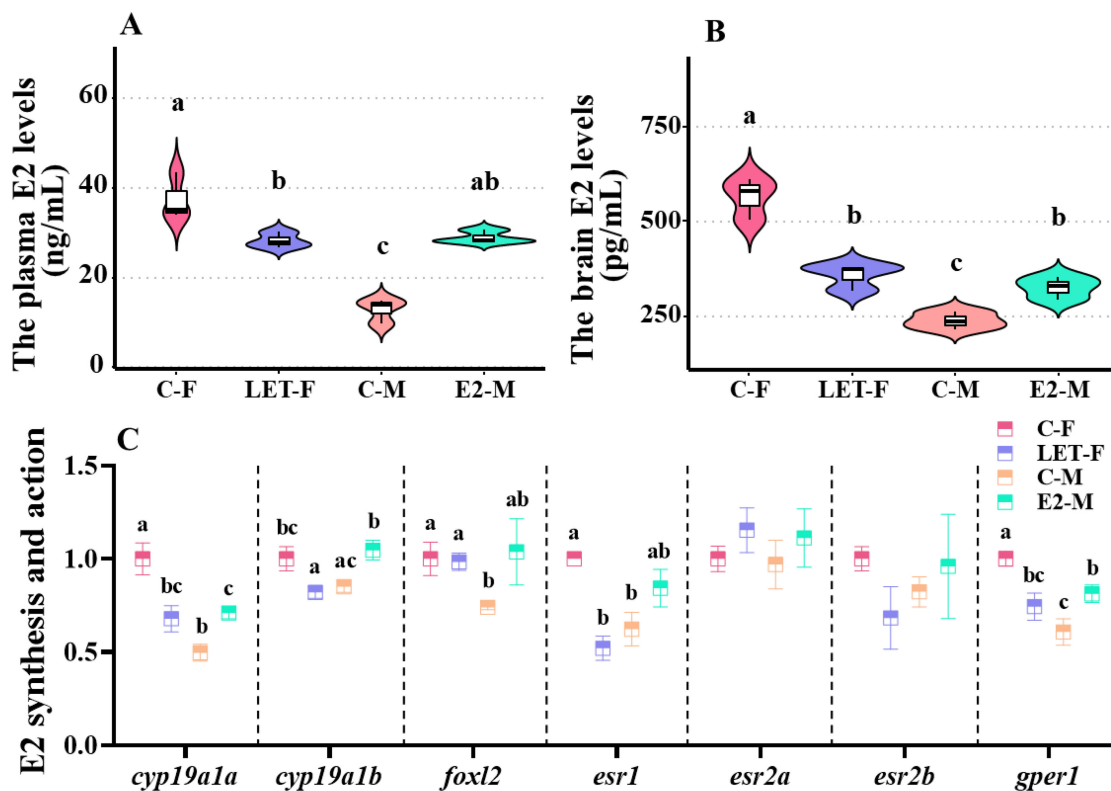


FIGURE 2

Plasma E2 levels and expressions of related genes in the brain. C-F, control-female (solvent, acetone); C-M, control-male (solvent, acetone); LET-F, letrozole-female (6 mg/g Letrozole); E2-M, E2-male (200 ng/g E2). (A) plasma E2 levels; (B) brain E2 levels; (C) expressions of genes responsible for E2 synthesis and action. Different letters on boxplots in each figure represent significant differences between groups (a, b, and c;  $P < 0.05$ ).  $N = 3$ .

expression levels of *cyp19a1a*, *cyp19a1b*, *esr1*, and *gper1* in the brain were significantly downregulated ( $P_{(cyp19a1a)} = 0.006$ ,  $P_{(cyp19a1b)} = 0.028$ ,  $P_{(esr1)} = 0.002$ ,  $P_{(gper1)} = 0.016$ ), whereas the expression levels of *foxl2*, *esr2a*, and *esr2b* did not show significant changes (Figure 2C,  $P > 0.05$ ).

The plasma and brain levels of E2 in E2-M fish were significantly higher than those in C-M fish (Figure 2A,  $P_{(plasma\ E2)} < 0.001$ ; Figure 2B,  $P_{(brain\ E2)} = 0.021$ ). Moreover, the plasma E2 levels in E2-M fish did not show significant changes compared with those in C-F fish (Figure 2A,  $P > 0.05$ ), whereas the brain E2 levels in E2-M fish were significantly lower than those in C-F fish (Figure 2B,  $P = 0.01$ ). In comparison to the C-M fish, apart from the significant upregulation of *cyp19a1a* ( $P = 0.041$ ), *cyp19a1b* ( $P = 0.02$ ) and *gper1* ( $P = 0.041$ ), the mRNA levels of the other genes investigated in the E2-M fish were not significantly different (Figure 2C,  $P > 0.05$ ).

### 3.2 Effects of drug treatments on anxiety responses in zebrafish

Compared with C-F fish, the latency to enter the upper layer (Figure 3A,  $P < 0.001$ ) and the total duration spent in the lower layer were significantly lower in C-M fish (Figure B,  $P < 0.001$ ).

Compared with C-F fish, LET-F fish showed significantly decreased latency before entering the upper layer ( $P = 0.003$ ), but this duration was still significantly higher than that of the C-M fish

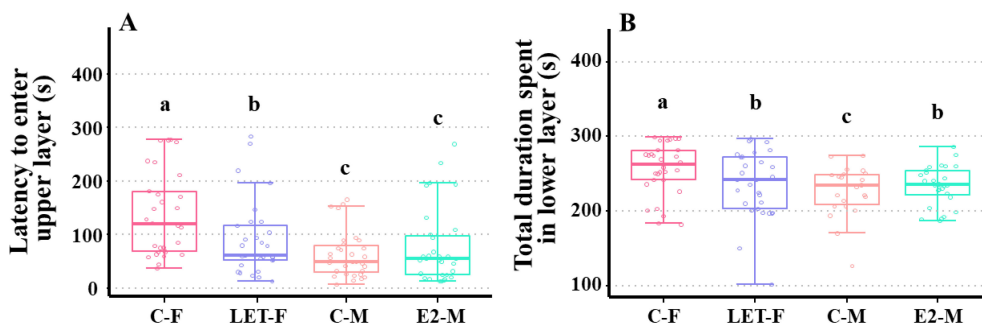
(Figure 3A,  $P = 0.012$ ). Similarly, the total duration spent in the lower layer was significantly reduced in LET-F fish ( $P = 0.026$ ) compared with that of C-F fish, and the duration time was significantly longer than that of C-M fish (Figure 3B,  $P = 0.002$ ).

After E2 treatment, the total duration spent in the lower layer was significantly increased in E2-M fish compared with C-M fish ( $P = 0.006$ ), and it was significantly shorter than that of C-F fish (Figure 3B,  $P = 0.012$ ). There was no significant change in the latency to enter the upper layer between E2-M and C-M fish (Figure 3A,  $P > 0.05$ ).

### 3.3 Plasma cortisol levels and expressions of genes of the HPI/cortisol pathway

Plasma cortisol levels were significantly higher in C-F fish than in C-M fish ( $P < 0.001$ ). Compared with C-F fish, the plasma cortisol levels were significantly lower in the LET-F fish ( $P < 0.001$ ), with no significant difference compared with those in C-M fish ( $P > 0.05$ ). In contrast, the plasma cortisol levels in E2-M fish were significantly higher than those in C-M fish ( $P = 0.02$ ) and remained significantly lower than those in C-F fish (Figure 4A,  $P = 0.005$ ).

The expression of *crha*, *crhr2*, and *actha* was significantly upregulated in the brains of C-F fish compared to that in C-M fish ( $P_{(crha)} = 0.006$ ,  $P_{(crhr2)} = 0.008$ ,  $P_{(actha)} = 0.017$ ), whereas the expression of *crhbp* was significantly downregulated ( $P = 0.023$ ).

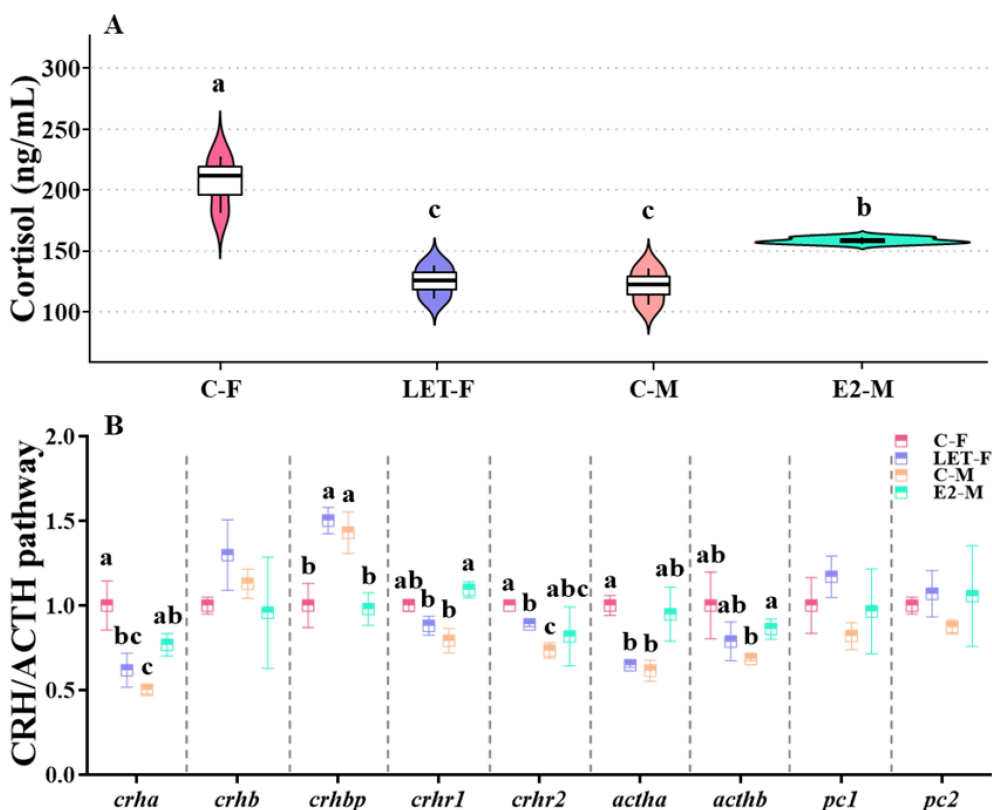


**FIGURE 3**  
Effects of drug treatments on anxiety response in zebrafish. C-F, control-female (solvent, acetone); C-M, control-male (solvent, acetone); LET-F, letrozole-female (6 mg/g Letrozole); E2-M, E2-male (200 ng/g E2). (A) latency to enter upper layer; (B) total duration spent in lower layer. Different letters on boxplots in each figure represent significant differences between groups (a, b, and c;  $P < 0.05$ ).  $N = 45$ .

Compared to C-F fish, the expression levels of *crha*, *crhr2*, and *actha* were significantly suppressed in LET-F fish ( $P_{(crha)} = 0.022$ ,  $P_{(crhr2)} = 0.027$ ,  $P_{(actha)} = 0.024$ ), whereas the expression of *crhbp* was significantly upregulated ( $P = 0.011$ ). In contrast, compared to the C-M fish, the mRNA levels of *crha*, *crhr1*, and *acthb* were significantly increased in E2-M fish ( $P_{(crha)} = 0.02$ ,  $P_{(crhr1)} = 0.004$ ,  $P_{(acthb)} = 0.043$ ), whereas the expression of *crhbp* was significantly decreased (Figure 4B,  $P = 0.019$ ).

### 3.4 Brain 5-HT levels and expressions of genes of the 5-HT pathway

Brain 5-HT levels in C-F fish were significantly lower than those in C-M fish ( $P = 0.004$ ). Compared with C-F fish, brain 5-HT levels in LET-F fish were significantly higher ( $P = 0.006$ ) and were not significantly different from those in C-M fish ( $P > 0.05$ ). In contrast, brain 5-HT levels in E2-M fish were significantly lower than those



**FIGURE 4**  
Effects of drug treatments on the plasma cortisol levels and relative mRNA levels of genes in CRH/ACTH pathway of zebrafish brain. C-F, control-female (solvent, acetone); C-M, control-male (solvent, acetone); LET-F, letrozole-female (6 mg/g Letrozole); E2-M, E2-male (200 ng/g E2). (A) plasma cortisol levels; (B) relative mRNA levels of genes in CRH/ACTH pathway. Different letters on boxplots in each figure represent significant differences between groups (a, b, and c;  $P < 0.05$ ).  $N = 3$ .



in C-M fish ( $P = 0.024$ ), with no significant difference compared with those in C-F fish (Figure 5A,  $P > 0.05$ ).

Moreover, the mRNA levels of *tph2*, *tph1b*, *slc6a4a*, and *htr1ab* in the brains of C-F fish were significantly lower than those in the brains of C-M fish ( $P_{(tph2)} = 0.002$ ,  $P_{(tph1b)} < 0.01$ ,  $P_{(slc6a4a)} = 0.002$ ,  $P_{(htr1ab)} = 0.009$ ). Compared with C-F fish, the mRNA levels of *tph2*, *tph1b*, *pet1*, *slc6a4a*, and *htr1ab* were significantly upregulated in LET-F fish ( $P_{(tph2)} = 0.005$ ,  $P_{(tph1b)} = 0.014$ ,  $P_{(pet1)} = 0.047$ ,  $P_{(slc6a4a)} < 0.001$ ,  $P_{(htr1ab)} = 0.019$ ). In contrast, compared with C-M fish, the expression levels of *pet1*, *slc6a4a*, *htr1aa*, *htr1ab*, and *htr1b* in the brains of E2-M fish were significantly down-regulated (Figure 5B,  $P_{(pet1)} = 0.013$ ,  $P_{(slc6a4a)} = 0.03$ ,  $P_{(htr1aa)} = 0.002$ ,  $P_{(htr1ab)} = 0.017$ ,  $P_{(htr1b)} = 0.022$ ).

### 3.5 Brain DA levels and expressions of genes of the DA axis pathway

Brain DA levels in C-F fish were significantly lower than those in C-M fish ( $P = 0.002$ ). Compared to C-M fish, brain DA levels in LET-F fish were significantly higher ( $P = 0.012$ ), but were not significantly different from those in C-M fish ( $P > 0.05$ ). In contrast,

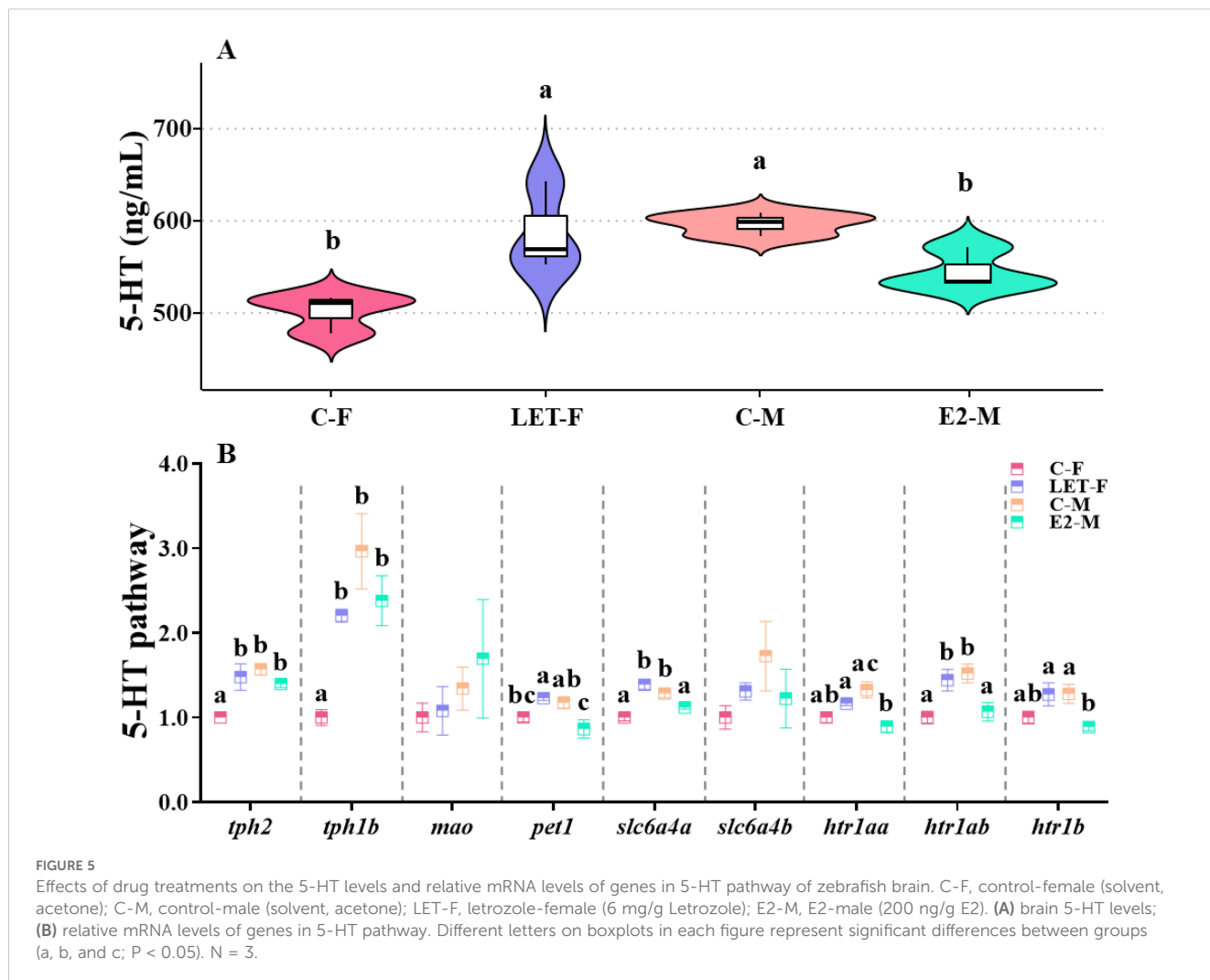
compared to C-M fish, the brain DA levels in E2-M fish were significantly inhibited ( $P = 0.045$ ), but remained significantly higher than those of the C-F fish (Figure 6A,  $P = 0.013$ ).

Furthermore, the mRNA levels of *th1*, *drd2a*, and *drd2b* were significantly lower in the C-F fish than in the C-M fish ( $P_{(th1)} < 0.001$ ,  $P_{(drd2a)} = 0.037$ ,  $P_{(drd2b)} = 0.04$ ). Compared to C-F fish, the mRNA levels of *th1*, *comta*, *comtb*, *drd1b*, *drd2a* and *drd2b* were significantly increased in LET-F fish ( $P_{(th1)} = 0.004$ ,  $P_{(comta)} = 0.046$ ,  $P_{(comtb)} = 0.008$ ,  $P_{(drd1b)} = 0.049$ ,  $P_{(drd2a)} = 0.033$ ,  $P_{(drd2b)} = 0.048$ ). In contrast, compared with C-M fish, the expression of *th1*, *comta*, and *drd2a* in the brains of E2-M fish was significantly lower (Figure 6B,  $P_{(th1)} = 0.03$ ,  $P_{(comta)} = 0.046$ ,  $P_{(drd2a)} = 0.005$ ).

## 4 Discussion

### 4.1 The role of endogenous E2 in anxiety responses of zebrafish

Based on our results, there was a significant sexual dimorphism in anxiety responses in zebrafish, with females exhibiting a more intense anxiety response than males. Furthermore, reducing



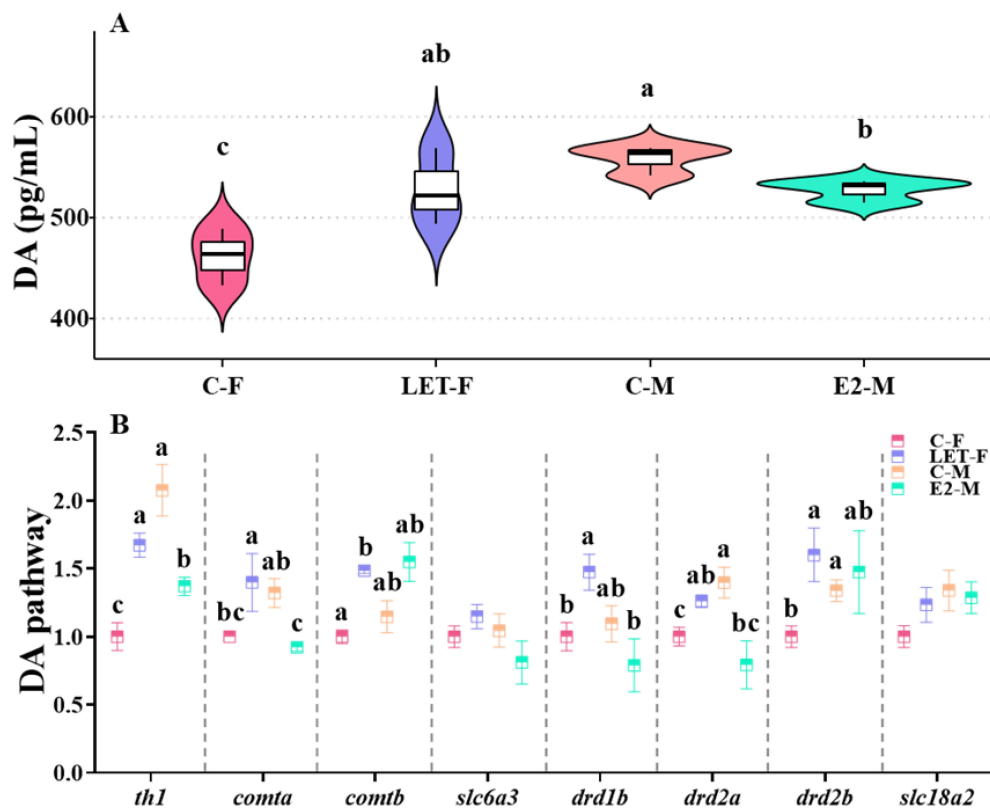


FIGURE 6

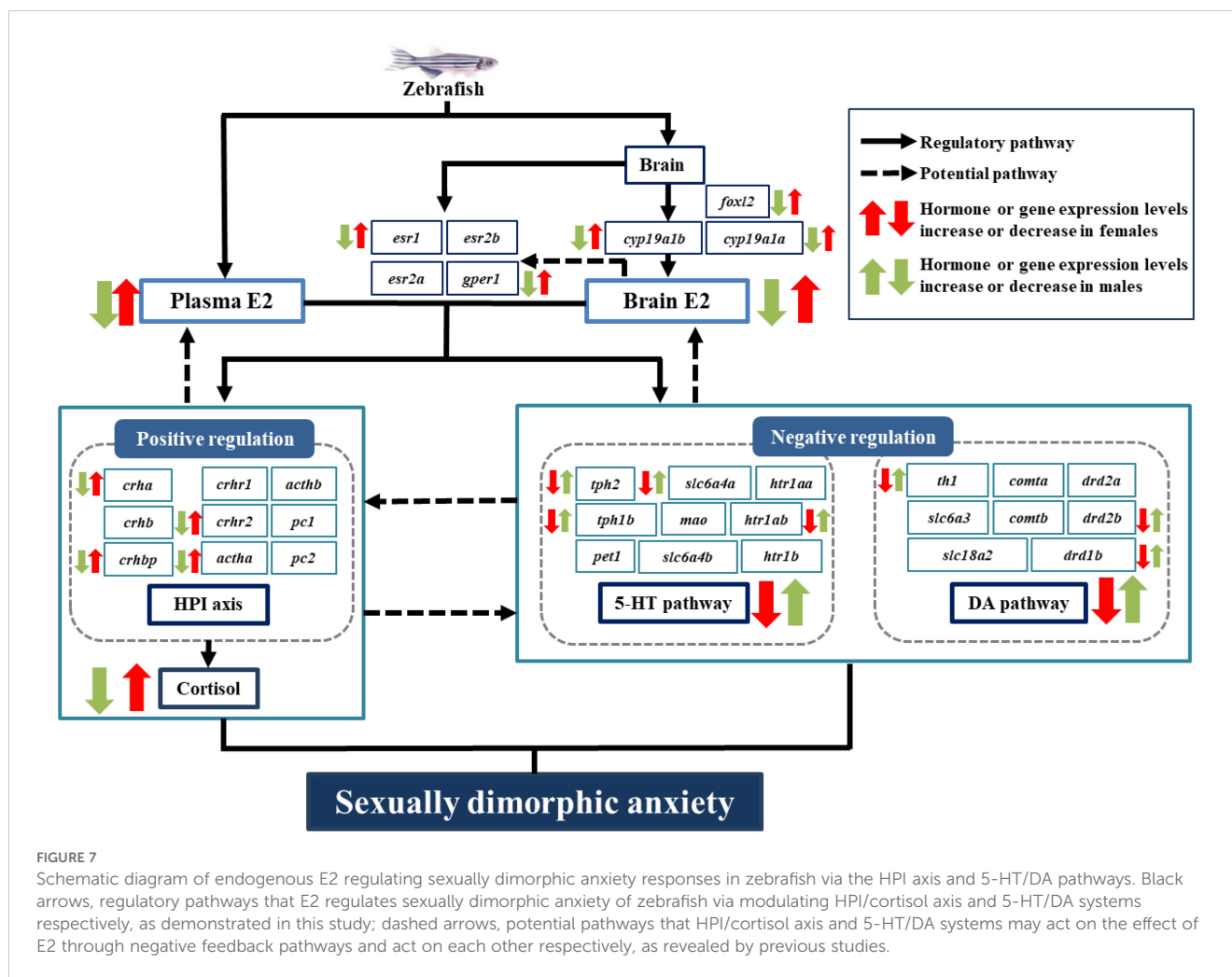
Effects of drug treatments on the DA levels and relative mRNA levels of genes in DA pathway of zebrafish brain. C-F, control-female (solvent, acetone); C-M, control-male (solvent, acetone); LET-F, letrozole-female (6 mg/g Letrozole); E2-M, E2-male (200 ng/g E2). (A) brain DA levels; (B) relative mRNA levels of genes in DA pathway. Different letters on boxplots in each figure represent significant differences between groups (a, b, and c;  $P < 0.05$ ).  $N = 3$ .

endogenous E2 levels in females decreased anxiety, whereas increasing endogenous E2 levels in males exacerbated anxiety. These findings suggest that endogenous E2 plays a pivotal role in the anxiety response in zebrafish.

Anxiety is a stress reaction regulated by the neuroendocrine system, which is important for animal survival (Berridge and Krangelbach, 2008; Russart and Nelson, 2018). Previous research has elucidated pronounced universal sexual dimorphic anxiety responses in vertebrates (Bland et al., 2005; Weiser et al., 2008; Ter Horst et al., 2012; Wallace et al., 2020; Loughery et al., 2021). This sexually dimorphic anxiety response typically manifests as more intense anxiety reactions or a greater susceptibility to anxiety in females than in males. In the present study, female zebrafish exhibited a more intense anxiety response than male zebrafish, which is consistent with previous study results (Amar and Ramachandran, 2023; Xiang et al., 2023). Accordingly, the neuroendocrine system that regulates anxiety responses also showed distinct sexual dimorphism, with higher plasma cortisol levels and lower brain 5-HT/DA levels in females, and the expression levels of related pathway genes followed a similar trend. These findings suggest that sexual dimorphic anxiety responses are highly conserved among vertebrates, indicating the pivotal ecological significance of this dimorphism in vertebrates.

In zebrafish, adult females are typically larger than males because of differences in gamete size and nutritional requirements (Yang et al., 2016), which increases their visibility to predators and leads to higher alertness to ensure survival rates (Moretz et al., 2007). In contrast, male zebrafish are smaller and dominate reproductive activities, often being more exploratory and aggressive in discovering, contesting, and defending spawning areas favored by females (Paull et al., 2010; Baatrup and Henriksen, 2015; Conradsen and McGuigan, 2015; Shontz et al., 2018). This may explain why male zebrafish exhibit lower levels of anxiety than females. Although wild-type zebrafish are highly domesticated laboratory strains, our results are consistent with wild-caught males being “bolder” than females (Dahlbom et al., 2011). More intense anxiety responses in female zebrafish can help them remain vigilant and enhance survival and reproductive success rates, whereas lower anxiety levels in male zebrafish can aid them in exploring new environments and improving reproductive success. This sexual dimorphism in the anxiety response of zebrafish is beneficial for the stability and continuation of the population.

Numerous studies have confirmed that sexual dimorphisms in animal phenotypes largely attributed to differences in sex hormones (such as estrogens and androgens) (Gegenhuber et al., 2022; Zhang et al., 2022). Thus, the sexual dimorphic anxiety response in



zebrafish is speculated to be closely related to differences in sex hormone levels in both the nervous and endocrine systems (Gegenhuber et al., 2022), especially with regard to E2 (Diotel et al., 2013). Endogenous E2 in zebrafish is regulated by aromatases and is associated with the expression of *cyp19a1a*, in gonads, and *cyp19a1b*, in radial glial cells of the brain (Pellegrini et al., 2005; Diotel et al., 2010). Our data showed that both the plasma and brain E2 levels were significantly higher in female than in male zebrafish. Compared to males, the expression of *cyp19a1a* and *foxl2* (positive regulatory factors of aromatase) was markedly higher in female fish, whereas the expression of *cyp19a1b* and other receptors did not differ. Additionally, the expression levels of estrogen receptors (*esr1* and *gper1*) were higher in the female brain, suggesting a greater estrogenic effect in females. Previous studies investigated sexual dimorphism in the expression of aromatase during the developmental and reproductive periods in zebrafish, and was suggested that this sexual dimorphism influences sex differentiation and a range of sexually dimorphic traits in zebrafish (Kallivretaki et al., 2007; Santos et al., 2008). In the present study, no significant differences in the expression of *cyp19a1b* were observed between the brains of adult male and female zebrafish, which may be related to their reproductive maturity. Additionally, an intriguing study suggested that

androgens may upregulate the expression of *cyp19a1b*, potentially explaining the lack of significant sexual dimorphism in *cyp19a1b* expression in the zebrafish brain (Mouriec et al., 2009). Based on these results, E2 levels may be closely associated with sexually dimorphic anxiety responses in zebrafish.

Previous studies have found that changes in endogenous E2 levels are closely associated with alterations in anxiety responses. However, these studies have primarily focused on rodents, with limited research on fish (Ossenkopp et al., 2005; Ellis et al., 2023). In the present study, LET treatment significantly reduced E2 levels in the plasma and brain of female zebrafish, concurrently downregulating the expression of genes associated with E2 synthesis (*cyp19a1a* and *cyp19a1b*) and estrogen receptor genes (*esr1* and *gper1*) in the brain. As an aromatase inhibitor, the inhibitory effect of LET on zebrafish E2 levels was highly consistent with previous findings (Adhikari et al., 2024; Zhang et al., 2024). In contrast, E2 treatment significantly increased E2 levels in the brain and plasma of male zebrafish, thereby upregulating the expression of *cyp19a1a*, *cyp19a1b*, and *gper1*. Interestingly, the anxiety response in LET-F fish significantly diminished, whereas that in E2-M fish was significantly enhanced. These results imply a pronounced positive correlation of E2 levels with anxiety in zebrafish, consistent with the findings of previous rodent studies (Flores et al., 2020). Fluctuations in E2 levels may

induce changes in the expression levels of estrogen receptors, thereby maintaining homeostasis. This effect is generally considered to be a form of negative regulatory mechanism (Christian et al., 2008; Shi et al., 2013). However, the expression of estrogen receptors (primarily *esr1* and *gper1*), has been shown to be stimulated by E2, suggesting a possible positive correlation between E2 and estrogen receptors (Menuet et al., 2004; Liu et al., 2017). Furthermore, E2 signaling occurs via both genomic and non-genomic pathways, with *esr1*, *esr2a*, and *esr2b* participating in the genomic pathway, while *gper1* is involved in the non-genomic pathways (Shi et al., 2013; Liu and Shi, 2015). Combining previous research with our results, endogenous E2 may participate in the regulation of anxiety responses in zebrafish through genomic and non-genomic pathways via a potential positive regulatory mechanism (Liu and Shi, 2015).

The close relationship observed between endogenous E2 levels and anxiety responses in zebrafish and mammals suggests that the role of E2 in anxiety responses may be conserved among vertebrates to some extent (Altemus et al., 2014; de Abreu et al., 2021; Ellis et al., 2023).

## 4.2 The pathways through which endogenous E2 regulates anxiety responses in zebrafish

A substantial body of previous research indicates that anxiety responses in fish are regulated by a range of neuroendocrine systems, including the 5-HT and DA systems, as well as the HPI axis (Wommack and Delville, 2007; Berridge and Kringelbach, 2008; Kalueff et al., 2012; Jia and Pittman, 2015; Oh et al., 2020; Tu et al., 2020). Concurrently, E2 is synthesized primarily through two pathways in vertebrates: release by the hypothalamic-pituitary-gonadal axis and local release in the brain, indicating that E2 signaling is regulated by both neuronal and endocrine systems (Hamilton et al., 2017). A number of studies have been focusing on the interactions between E2 and the neuroendocrine system, such as its effects on the HPA axis and the serotonergic system (Weiser and Handa, 2009; Ulhaq and Kishida, 2018). Some evidence suggests that E2 may cause changes in animal emotional activities by affecting or modulating the neuroendocrine system (Ter Horst et al., 2009; Hernández-Hernández et al., 2019).

Cortisol, synthesized under the influence of the HPA/HPI axis, plays a crucial role in the modulation of stress responses in animals (Berridge and Kringelbach, 2008; Ghisleni et al., 2012; Duboué et al., 2017). Interestingly, the plasma cortisol levels in many animal species exhibit significant sexual dimorphism, typically characterized by higher levels in females compared to males (Bangasser and Valentino, 2014; Culbert et al., 2021). This sexual dimorphism in cortisol levels corresponds with the sexual dimorphism observed in anxiety and E2 levels. Numerous studies have revealed the positive regulatory effect of E2 on cortisol production in animals (Weiser et al., 2008; Weiser and Handa, 2009). In the current study, compared with C-F fish, LET-F fish showed a significant decrease in plasma cortisol levels, corresponding to a reduction in anxiety levels. In contrast, E2-M fish exhibited a sharp increase in plasma cortisol levels compared with C-M fish, which corresponded to an exacerbation of anxiety responses. Changes in plasma cortisol levels positively correlated with changes in E2 levels in

the plasma and brain. In addition, the expression of a series of genes involved in the regulation of cortisol synthesis and metabolism in the HPI axis of male and female fish treated with the drugs exhibited corresponding changes. These changes were consistent with alterations in plasma cortisol levels and anxiety responses observed in male and female fish after treatment. Gonadal steroid hormones play an essential role in regulating the function of the HPI/HPA axis (Suzuki et al., 2001; Zhang et al., 2009). In rodents, E2 supplementation can counteract the decrease in corticosterone and ACTH levels induced by stress in ovariectomized females (Heck and Handa, 2019). Additionally, E2 therapy in gonadectomized male rats increased stress-induced levels of *c-fos* mRNA, CRH hnRNA, and corticosterone, suggesting that E2 regulates anxiety by stimulating the HPA axis (Weiser and Handa, 2009). Our results, combined with these previous findings, strongly suggest that E2 regulates anxiety responses in zebrafish via the HPI/cortisol axis and that this action may be conserved in vertebrates (Weiser and Handa, 2009; Heck and Handa, 2019).

As an important inhibitory neurotransmitter, 5-HT alleviates anxiety responses in fish (Kalueff et al., 2012; Tu et al., 2020). Notably, the 5-HTergic system exhibits a distinct sexual dimorphism in vertebrates, characterized by a higher rate of 5-HTergic synthesis in males compared to females (Jones and Lucki, 2005; Gressier et al., 2016). In our results, compared with C-F fish, the brain 5-HT levels of LET-F fish were significantly higher, whereas those of E2-M fish were significantly lower than those of C-M fish. Furthermore, the expression of genes related to the 5-HT pathway (including its synthesis, action, and degradation) also changed. These alterations corresponded with the changes in anxiety responses observed in both female and male fish post-treatment and were negatively correlated with changes in E2 levels (in the plasma and/or brain). Similarly, a significant negative correlation between E2 and 5-HT levels has been observed in tilapia (*Oreochromis mossambicus*) and zebrafish larvae (Tsai et al., 2000; Ulhaq and Kishida, 2018). In the present study, the expression levels of the receptors for the two pathways of E2 action (*esr1* and *gper1*) exhibited a significant inverse correlation with the levels of 5-HT. A close association between estrogen receptors and 5-HTergic neurons in zebrafish has been postulated (Lillesaar, 2011). E2 may regulate the 5-HT system in the brain by interacting with estrogen response elements or other transcription factors, thereby modulating the mood (Hernández-Hernández et al., 2019). These findings imply that higher levels of anxiety in females are closely associated with the interaction between endogenous E2 and 5-HT, with estrogen receptors potentially playing a key role in modulating this interaction, thereby suppressing 5-HT levels in the brain and stimulating a stronger anxiety response.

Similarly, DA, a neurotransmitter associated with the reward mechanism, is also closely related to the regulation of anxiety responses in fish (Jia and Pittman, 2015), and the DAergic system in males tends to be more active than that in females (Orendain-Jaime et al., 2016; Zachry et al., 2021), which is negatively correlated with sexual dimorphism in anxiety levels and E2 concentrations.

Consistent with 5-HT, DA exhibits a complex inter-regulatory relationship with E2 in mammals (Dluzen, 2005; Petersen et al., 2021; Bendis et al., 2024). In the present study, the brain DA levels

of LET-F fish increased dramatically compared to C-F fish, whereas the brain DA levels in E2-M fish decreased significantly compared with those in C-M fish. These changes were also negatively correlated with changes in E2 (in the plasma and/or brain), suggesting that endogenous E2 may negatively regulate DA levels in the zebrafish brain. Moreover, the expression of genes related to the DA pathway (including synthesis, action, and degradation) in male and female fish treated with the drugs showed corresponding changes. These changes were consistent with the alterations in plasma DA levels and anxiety responses observed in male and female fish after treatment. Similar to our results, an inhibitory effect of E2 on the DA pathway has been observed in rainbow trout (*Oncorhynchus mykiss*), where a decrease in the expression of the DA receptor DR2A was correlated with elevated plasma E2 levels (Crago and Schlenk, 2015; Magnuson et al., 2021). These results complicate the mode of action of E2 on DA pathways. Nonetheless, our results provide a new perspective on the action of E2 on DAergic neurons, suggesting that E2 elicits anxiety responses by inhibiting the DAergic pathway.

Interestingly, the activation of the HPI axis has been demonstrated to be closely associated with the activity of 5-HT and DA neurons, and the 5-HTergic and DAergic systems also play significant roles in the function of the HPI/HPA axis (Contesse et al., 2000; Porter et al., 2004; McArthur et al., 2005; Fink and Göthert, 2007). These findings illustrate the complex relationship between the HPI axis and 5-HTergic/DAergic systems. Endogenous E2 may participate in the regulation of anxiety responses in zebrafish via complex interactions between the HPI axis and the 5-HT and DA systems.

In summary, this study confirmed that zebrafish exhibit a sexually dimorphic anxiety response, with females showing higher levels of anxiety than males. The anxiety responses elicited by artificially altering endogenous E2 levels, along with the associated changes in the HPI/cortisol axis and the 5-HT/DA system, suggest that endogenous E2 influences the anxiety of zebrafish by positively modulating the HPI/cortisol axis and negatively modulating the 5-HT/DA pathways (Figure 7). The present study revealed how E2 modulates anxiety and the associated neurochemical pathways in fish, offering new insights into the impact of sex hormones on anxiety in animals.

## 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 ethics committee of Chongqing Normal University (Approval No. CKLCUFF20240701-

02). The study was conducted in accordance with the local legislation and institutional requirements.

## Author contributions

HT: Data curation, Investigation, Methodology, Writing – original draft. YZ: Data curation, Investigation, Methodology, Writing – original draft. YS: Data curation, Writing – review & editing. QC: Conceptualization, Methodology, Writing – review & editing. ZL: Conceptualization, Funding acquisition, Project administration, 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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