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Impact of environmentally relevant concentrations of fluoxetine on zebrafish larvae: From gene to behavior

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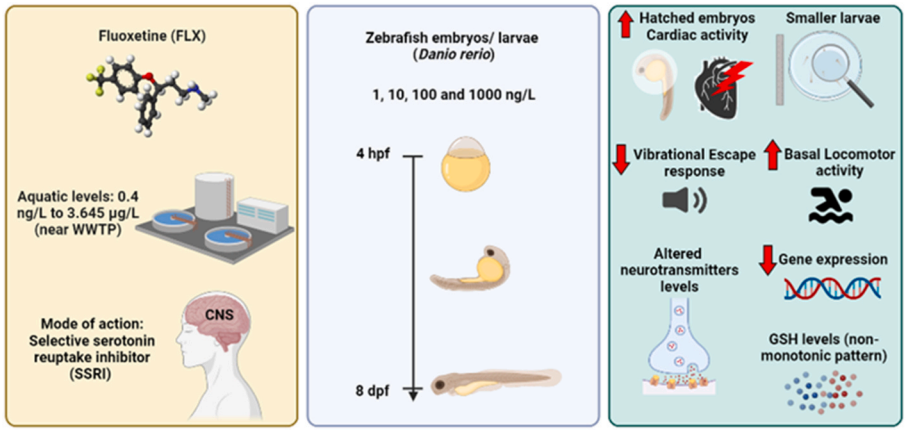
HIGHLIGHTS

- Low concentrations of fluoxetine (FLX) altered zebrafish development.
- FLX promoted early hatching, growth inhibition and increased heart rate.
- FLX exposure decreased startle response and increased basal locomotor activity.
- FLX decreased the expression of monoaminergic genes.
- FLX affected the levels of monoaminergic neurotransmitters.

GRAPHICAL ABSTRACT



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ABSTRACT

Fluoxetine is widely prescribed for the treatment of depressive states, acting at the level of the central nervous system, consequently affecting non-target organisms. This study aimed to investigate the influence of environmentally relevant fluoxetine concentrations (1–1000 ng/L) on *Danio rerio* development, assessing both embryotoxicity and behavior, antioxidant defense, gene expression and neurotransmitter levels at larval stage. Exposure to fluoxetine during early development was found to be able to accelerate embryo hatching in embryos exposed to 1, 10 and 100 ng/L, reduce larval size in 1000 ng/L, and increase heart rate in 10, 100 and 1000 ng/L exposed larvae. Behavioral impairments (decreased startle response and increased larvae locomotor activity) were associated with effects on monoaminergic systems, detected through the downregulation of key genes (*vmat2*, *mao*, *tph1a* and *th2*). In addition, altered levels of neurochemicals belonging to the serotonergic and dopaminergic systems (increased levels of tryptophan and norepinephrine) highlighted the sensitivity of early

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life stages of zebrafish to low concentrations of fluoxetine, inducing effects that may compromise larval survival. The obtained data support the necessity to test low concentrations of SSRIs in environmental risk assessment and the use of biomarkers at different levels of biological organization for a better understanding of modes of action.

1. Introduction

Fluoxetine is commonly prescribed for the treatment of symptoms related to depression and anxiety (Stewart et al., 2014), being a selective serotonin reuptake inhibitor (SSRI) antidepressant. Its mechanism of action consists of the inhibition of the serotonin receptor carrier in the presynaptic terminal, resulting in increased levels of 5-hydroxytryptamine (5-HT or serotonin) in the synaptic cleft (Chai et al., 2021; McDonald, 2017). Once taken, fluoxetine is metabolized into norfluoxetine and subsequently excreted in the urine (with less than 10% excreted unchanged) ending up in wastewater treatment plants (WWTP) (Hiemke and Härtter, 2000). Contamination of aquatic environments with fluoxetine is mainly associated with WWTPs effluent release from domestic, hospital and pharmaceutical industry sources, due to the inefficient removal of these compounds during water treatment. Consequently, fluoxetine and its derivate norfluoxetine are commonly identified in the water ecosystem (Hiemke and Härtter, 2000), registering levels ranging from 0.4 ng/L to 3.645 μg/L in proximity to WWTPs (Metcalfe et al., 2010; Salgado et al., 2011; Sousa et al., 2011) for fluoxetine. For its metabolite, concentrations reaching up to 10.4 and 11.7 μg/L have been recorded in WWTP influents and effluents, respectively (Shraim et al., 2012).

Several studies have shown that fluoxetine can accumulate in different fish tissues (e.g., brain and liver) (Dorelle et al., 2020; Pan et al., 2018; Yan et al., 2020) and exert effects at different biological levels (e.g., gene expression (Craig et al., 2014; Evsiukova et al., 2021; Parolini et al., 2019; Theodoridi et al., 2017; Yan et al., 2020), biochemical and metabolic profiles (Blahova et al., 2021; Ding et al., 2016; Duarte et al., 2020; Mishra et al., 2017), reproductive processes (Bertram et al., 2018; Prasad et al., 2015; Thoré et al., 2020), and behavior (e.g., locomotion, sociability, feeding and predator avoidance behaviors (Farias et al., 2019a,b,c; Greaney et al., 2015; Martin et al., 2017, 2019; Meijide et al., 2018; Sehonova et al., 2018; Vera-Chang et al., 2018; Zindler et al., 2020)). However, the available information about the effects induced by fluoxetine on the levels of neurotransmitters in fish is limited, especially during the early life stages. This information is particularly important as a developing central nervous system is more vulnerable to contaminants than an adult nervous system, interfering with normal functioning and compromising adaptation to the environment (Giordano and Costa, 2012). Furthermore, in a developing nervous system, neurotransmitters may have additional roles, including modulating cell proliferation and differentiation (Nguyen et al., 2001). Thus, exposure to neuroactive compounds during this sensitive phase may result in permanent changes in the brain.

The zebrafish (*Danio rerio*) is a model organism commonly used in ecotoxicology and neurotoxicological studies (Scholz et al., 2008), since it shares common neurotransmitter systems with other vertebrates, and has a neuroanatomical organization that shares similarities with humans (Horzmann and Freeman, 2016). In this study, the effects of fluoxetine on zebrafish development were studied, by exposing 4 h post-fertilization (hpf) organisms for 8 days to environmentally relevant concentrations (1, 10, 100 and 1000 ng/L). The potential embryotoxicity as well as the effects on locomotor behavior (startle and visual motor response), changes in the expression of key genes associated with the monoaminergic system and neurochemical profiles, antioxidant status and neurotransmitter levels at the end of the exposure period were studied.

2. Materials and methods

2.1. Fish husbandry and larvae production

Adult wild-type zebrafish were purchased from Pisciber BSF, Terrassa, Barcelona. All procedures were approved by the Institutional Animal Care and Use Committees at the CID-CSIC and conducted in accordance with the institutional guidelines under a license from the local government (No 11336) (Faria et al., 2022a,b).

2.2. Chemical stability of fluoxetine using LC-MS/MS analysis

The stability of fluoxetine in the water in which embryos were exposed was studied for 48 h using ultra-high performance liquid chromatography coupled to a hybrid triple quadrupole detector with ion trap spectrometer (Shimadzu, Sciex QTrap 7500). Fluoxetine solutions (1, 10, 100 and 1000 ng/L) were freshly prepared in fish culture water, and aliquots taken at times 0 and 48 h, using analytical methodology previously optimized for the determination of fluoxetine, among other pharmaceuticals (Bellot et al., 2021a; Gómez-Canela et al., 2021).

2.3. Embryo exposure

Fluoxetine hydrochloride (CAS number: 56296-78-7, molecular weight 345.79 g/mol, purity: >98%) was purchased from ChemCruz (Netherlands). The stock solution and the test concentrations were prepared in fish culture water on the experiment day. The test concentrations (1, 10, 100 and 1000 ng/L) were prepared in water by successive dilutions of the stock (4 μg/L), making a total of 150 mL of each concentration tested. The zebrafish embryos were exposed from 4 h post-fertilization (hpf) until 8 days post-fertilization (dpf), in 6-well plates with 10 embryos per well containing 10 mL of medium, and a total of 15 wells per treatment. A complete renewal of control and fluoxetine concentrations tested was performed every 2 days. Animals were checked daily, and several endpoints evaluated throughout the exposure: mortality, malformations, pericardial and yolk sac edema, heartbeat, hatching and swim bladder inflation. At the end of the exposure period, organisms presenting delayed swimming bladder inflation, or any malformation were not used for the following analysis. Larvae fulfilling the quality criteria were submitted to behavior analysis and then collected into 1.5 mL tubes, frozen in liquid nitrogen, and stored at −80 °C for biochemical (n = 8 pools), gene expression (n = 8 pools) and neurochemical analysis (n = 6 pools).

2.4. Cardiac activity

To evaluate the effects of fluoxetine on zebrafish larvae cardiac function, 8 dpf organisms from the control and treatments (14 larvae per treatment) were immobilized on a slide, in 4% methylcellulose, placed in ventral or lateral view. Before the video recording the heart movement, 8 dpf zebrafish were not under anesthetic. Cardiac activity analysis was performed in isolated behavior rooms at 27–28 °C. The cardiac activity of each larva was recorded for 30 s (AVI format at 30 fps) with a GigE camera (UI–5240CP-NIR-GL, Imaging Development Systems, Germany), attached on a Motic SMZ-171 stereomicroscope, as described by Faria et al. (2022a).

2.5. Behavior analysis

All behavioral analysis were performed using a DanioVision system

associated with the Ethovision XT 11 software (Noldus, Wageningen, the Netherlands), as previously reported by other authors (Faria et al., 2015, 2020), using 96 zebrafish larvae (8 dpf) per treatment, from two independent experiments. The trials were performed in 48-well plates, and four types of behavior were evaluated: vibrational startle response, habituation or non-associative learning, basal locomotor activity (BLM) and visual motor response (VMR). Briefly, fish larvae were acclimated in the DanioVision Observation Chamber (DVOC) for 20 min, in dark, before the vibratory and visual stimuli constituting the behavioral test were initiated. 51 tapping stimuli (intensity: 8; interstimulus interval: 1 s) were applied in the dark, followed by a 10 min recovery time. Then, a light cycle (intensity: 100%, duration: 10 min) and a dark cycle (intensity: 0%, duration: 10 min) were initiated. Videos were recorded at 30 frames per second.

2.6. Biomarkers analysis

All biochemical determinations were conducted in whole zebrafish larvae (8 dpf), collected from three independent experiments (96 larvae per treatment). The antioxidant enzyme activities of catalase (CAT) and superoxide dismutase (SOD), were determined according to Faria et al. (2019a; 2019b), while reduced glutathione levels (GSH) were measured following the method described by White et al. (2003). In total, per experimental condition, 8 pools (12 larvae per pool) were analyzed for CAT and SOD activities and GSH levels. All biochemical analysis were normalized by total protein concentration in the samples, measured using the Bradford method (Bradford, 1976) with bovine serum albumin (BSA) as standard.

2.7. RNA preparation and qRT-PCR analysis

Analysis of changes in larvae gene expression was performed as previously described by Prats et al. (2017). Total RNA was extracted from 8 pools (4 larvae with 8 dpf per pool, 32 larvae per treatment), collected from three independent experiments, using the Trizol Reagent (Invitrogen Life Technologies, Carlsbad, CA). The qRT-PCR was subsequently performed and data analysis was executed using the ΔΔCp method (Livak and Schmittgen, 2001). Primer sequences (Sigma-Aldrich, Steinheim, Germany) of the seven selected genes (*sert*, *vmat2*, *dat*, *mao*, *dbh*, *th2* and *tph1a*) are reported in Supplementary Table S1.

2.8. Monoaminergic compounds extraction and LC-MS/MS analysis

The extraction procedure and the chromatographic conditions to analyze the targeted monoaminergic compounds were performed as described in previous studies addressing neurotransmitters characterization in zebrafish (Bellot et al., 2021a,b; Mayol-Cabré et al., 2020). Briefly, stock solutions (1000 ng/L) of the target compounds were prepared in methanol (MeOH), water or dimethylsulfoxide (DMSO) depending on their solubility. An internal standard mixture (ISM) was prepared in a mixture of acetonitrile (ACN): H_2O 90:10 + 1% formic acid (FA). Pools of 15 heads of zebrafish larvae (n = 6 pools, per treatment), collected from two independent experiments were analyzed by chromatographic and mass spectrometry conditions described in previous studies (Mayol-Cabré et al., 2020).

2.9. Statistical analysis

Data were analyzed with IBM SPSS v25 (Statistical Package, 2010; Chicago, IL), and plotted with Microsoft Excel 2016. Normality was assessed using Kolmogorov-Smirnov and Shapiro-Wilk tests. One-way ANOVA followed by Tukey's post hoc test was applied for groups meeting parametric requirements, whereas data that failed normality were then transformed with logarithmic function and reprocessed. The Kruskal–Wallis test followed by Dunn's multiple comparison test was used for those groups that did not meet parametric assumptions, even after the log transformed. The results for hatching, startle response, biomarkers, and gene expression passed normality, while the results obtained for survival, larval length, heart rate, habituation, BLM and neurotransmitters profiles did not. The data related to the percentage of accumulative malformations and delayed swimming bladder inflation were analyzed by chi-square test. Fluoxetine stability samples were analyzed by pairwise Students' t-test. Significance was set at $p < 0.05$.

3. Results

3.1. Fluoxetine stability

The concentrations of fluoxetine at time 0 and 48 h are presented in Table 1. Overall, fluoxetine levels remained stable at the highest concentrations (100 and 1000 ng/L), while at the lowest concentrations, 1 and 10 ng/L, a decrease corresponding to approximately 45 and 59% was observed after 48 h (Student's t-test, 1 ng/L: $p = 0.192$; 10 ng/L: $p = 0.002$; 100 ng/L: $p = 0.470$; 1000 ng/L: $p = 0.918$) (Table 1).

3.2. Embryonic development

In this study, to assess the effects of fluoxetine on embryonic development, five endpoints were analyzed: (A) Survival; (B) Hatching; (C) Malformations; (D) Swimming bladder inflation; (E) Larvae length; (F) Cardiac activity (Fig. 1). The larvae exposure to fluoxetine did not significantly affect survival over time (48 h: $H(4) = 3.020$, $p = 0.554$; 72 h: $H(4) = 5.324$, $p = 0.256$; 96 h: $H(4) = 5.315$, $p = 0.256$; 168 h: $H(4) = 6.080$, $p = 0.193$) (Fig. 1A), occurrence of malformations ($\chi2 = 8.082$; $df = 4$; $p = 0.089$) (Fig. 1C) and time of delayed swimming bladder inflation ($\chi2 = 8.527$; $df = 4$; $p = 0.074$) (Fig. 1D). Statistical differences in terms of hatching rate were observed between concentrations ($F_{(4,69)} = 9.467$, $p < 0.001$), with a significant increase in the percentage of hatched embryos at 1 ($p < 0.01$), 10 ($p < 0.001$) and 100 ng/L ($p < 0.001$), at 48 hpf (Fig. 1B). Significant differences were also found in the larval length ($H(4) = 48.192$, $p < 0.001$), with a decrease occurring at the highest concentration of fluoxetine ($p < 0.001$) (Fig. 1E). Fluoxetine exposure promoted increased the heart rate ($H(4) = 25.246$, $p < 0.001$), although significant effects were only observed for 10 ($p < 0.001$), 100 ($p < 0.001$) and 1000 ng/L ($p < 0.001$) (Fig. 1F).

3.3. Behavior response

Four types of behaviors were analyzed in this study: A) escape response evoked by a vibrational acoustic stimulus (startle response), B) habituation to repetitive acoustic stimulation (which addresses non-associative learning), C) basal locomotor activity (BLM), and D) response to visual stimuli (VMR) (Fig. 2). The startle response was affected by fluoxetine exposure ($F_{(4,363)} = 2.692$, $p = 0.031$), with a significant decrease of startle movement, compared with the control, registered for 1000 ng/L ($p = 0.029$; Fig. 2A). No significant effects on larvae habituation ($H(4) = 1.009$, $p = 0.908$) (Fig. 2B) was observed, while BLM was significantly affected by fluoxetine exposure ($H(4) = 12.341$, $p = 0.015$), increasing, compared to the control, in organisms

Table 1
Stability of experimental fluoxetine concentrations at 0 and 48 h. Values are represented as mean ± SE of three replicates. The results obtained after 48 h were compared with those obtained at 0 h using Student's t-test, with significance set to $p < 0.05$ and represented by * when $p < 0.05$.

Nominal concentration (ng/L)	Exposure concentration (0 h) (mean ± SE) [a]	Concentration detected at 48 h (mean ± SE) [a]
1	0.9 ± 0.22	0.5 ± 0.06
10	11.7 ± 0.85	4.8 ± 0.43 *
100	117.5 ± 8.55	108.3 ± 1.16
1000	976.3 ± 84.79	963.2 ± 84.20

[a] n = 3.

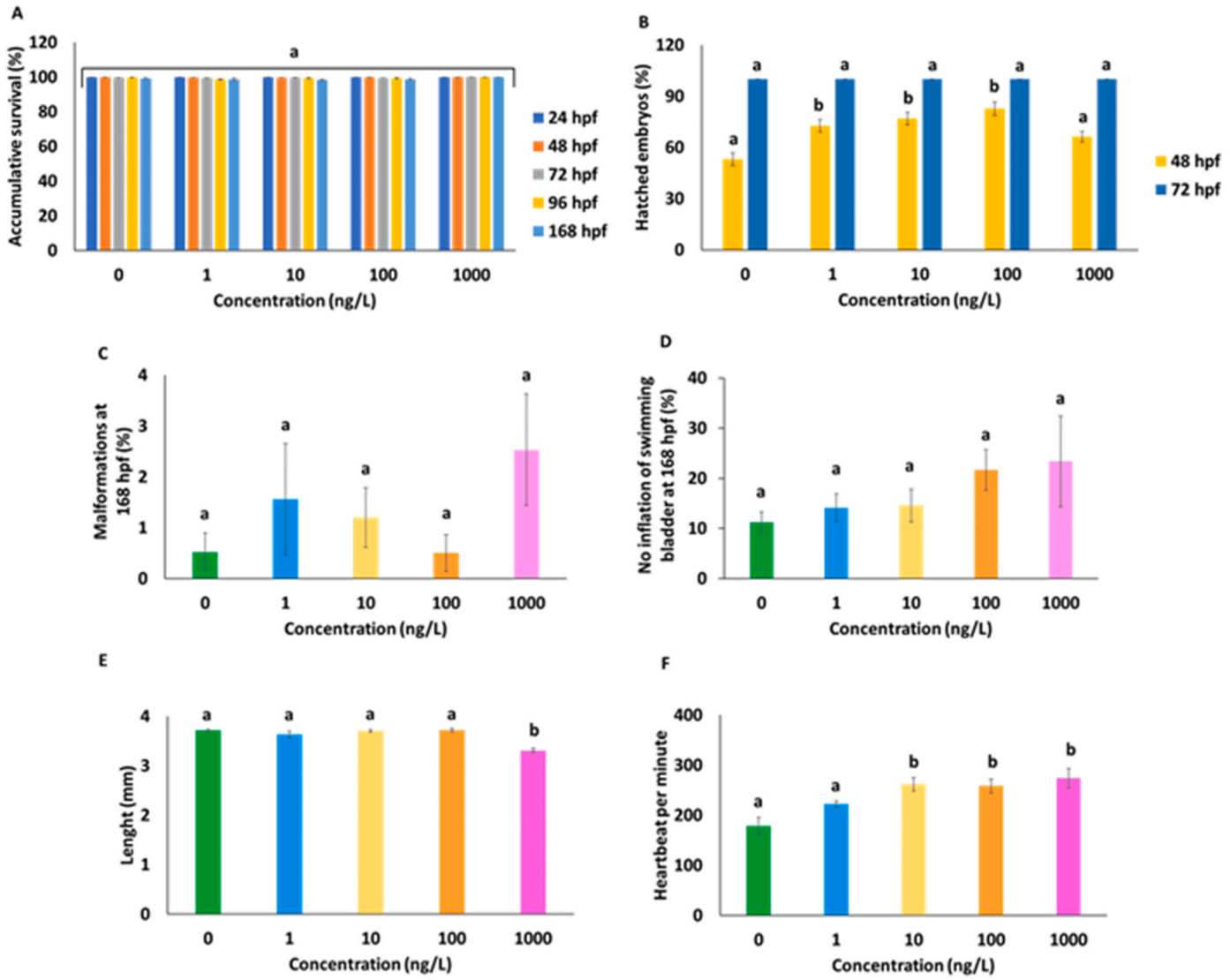

Fig. 1. Effect of fluoxetine on zebrafish embryonic development: (A) Accumulative survival (%) (n = 800); (B) Hatched embryos (%) (n = 300–340); (C) Malformations at 168 hpf (%) (n = 760–765; (D) No swimming bladder inflation at 168 hpf (%) (n = 550–560); (E) Zebrafish larvae length (n = 105); (F) Zebrafish larvae cardiac activity (n = 65–70). All data are reported as mean values ± standard error. Different letters indicate statistical different subset groups following either Kruskal-Wallis test with Dunn's multiple comparison test (Cumulative survival (%), zebrafish larvae length and cardiac activity) or one-way ANOVA with Tukey post hoc (Hatched embryos (%)). The data of accumulative observed malformations and delayed swimming bladder inflation were analyzed using the chi-square test.

exposed to 1 (p = 0.01) and 1000 ng/L (p = 0.023) (Fig. 2C).

3.4. Antioxidant cellular response

Three biomarkers related to antioxidant defense were analyzed to assess the effects of fluoxetine exposure: SOD activity, CAT activity, and GSH levels (Fig. 3). Organisms exposed to fluoxetine displayed no significant differences to control in terms of SOD ($F_{(4,34)}$ = 0.254, p = 0.905) (Fig. 3A) and CAT activities (Fig. 3B) ($F_{(4,34)}$ = 0.726, p = 0.580). However, GSH levels were significantly affected by fluoxetine exposure ($F_{(4,33)}$ = 4.421, p = 0.006). A trend towards a non-monotonic (reversed "U") pattern of effect was observed for GSH levels, with levels significantly higher than control at 10 ng/L (p = 0.016), which then decrease to control levels at 1000 ng/L (Fig. 3C).

3.5. Expression of monoaminergic genes

In order to study possible changes in zebrafish larvae serotonergic and dopaminergic system, the expression of the genes involved in the serotonin transport *slc6a4a* (*sert*), *scl18a2* (*vmat2*) and dopamine - *scl6a3* (*dat*), oxidation of serotonin (*mao*), synthesis of norepinephrine (*dbh*) and synthesis of serotonin and dopamine (*tph1a and th2*, respectively) were analyzed following developmental exposed to fluoxetine (Fig. 4). The *dbh* mRNA expression levels were not significantly affected by developmental exposure to fluoxetine ($F_{(4,28)}$ = 0.245, p = 0.910) (Fig. 4E), while a downregulation was in general observed for the remaining genes. In terms of *vmat2* (Fig. 4B), expression levels were in general affected by the treatments ($F_{(4,27)}$ = 6.217, p = 0.001), being significant for 10 (p = 0.002) and 100 ng/L (p = 0.015). In the case of *mao* ($F_{(4,28)}$ = 4.026, p = 0.011) and *tph1a* ($F_{(4,28)}$ = 5.406, p = 0.002) (Fig. 4D and G), mRNA expression levels was affected by 10 (*mao*: p = 0.034; *tph1a*: p = 0.008) and 1000 ng/L (*mao*: p = 0.039; *tph1a*: p = 0.016) and *th2* expression ($F_{(4,27)}$ = 2.953, p = 0.038) (Fig. 4F) was only affected by the highest concentration (1000 ng/L) (p = 0.032).

3.6. Monoaminergic neurotransmitters

The effect of fluoxetine on neurotransmitters associated with the

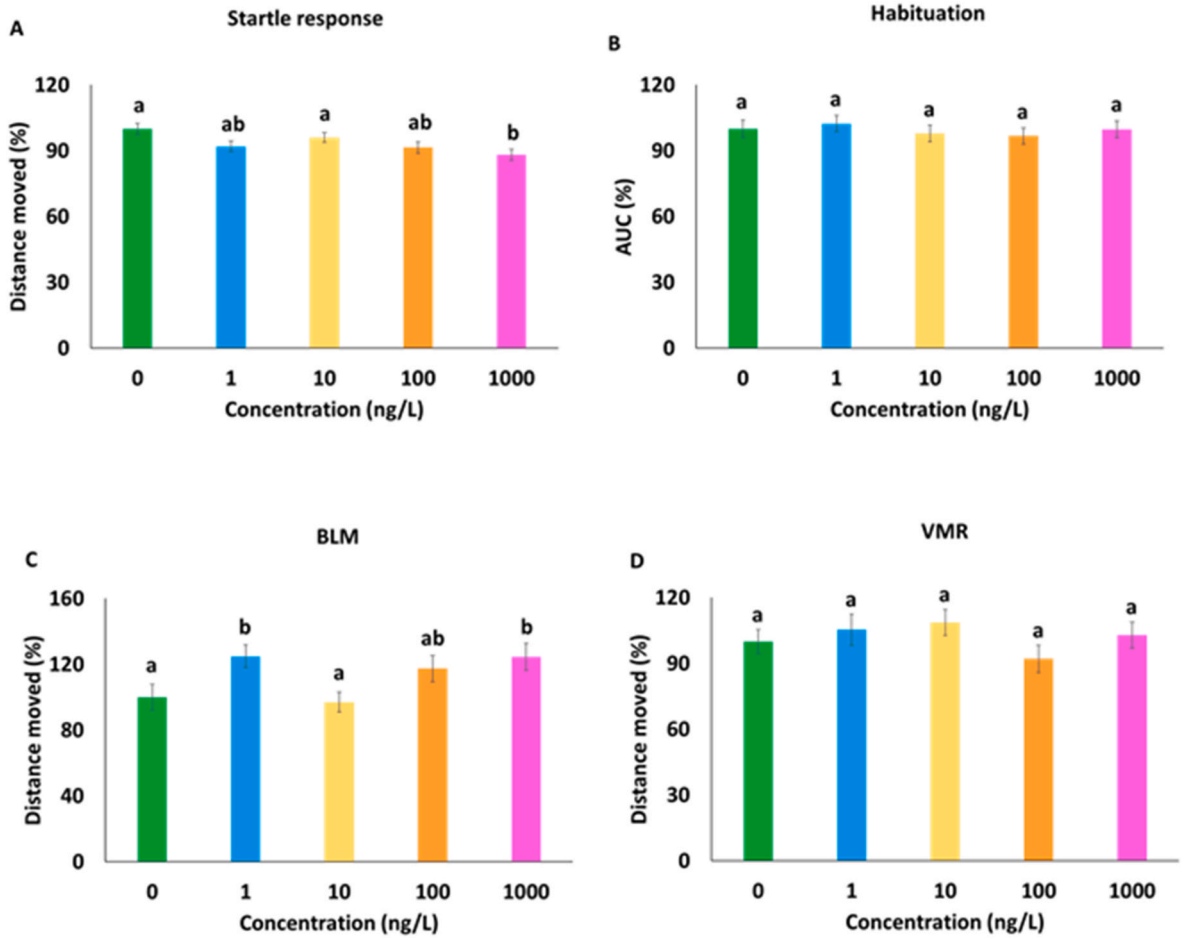

Fig. 2. Behavioral responses of 8 dpf zebrafish larvae, following developmental exposure to fluoxetine. Results are reported in terms of percentage to the control. (**A**) **Startle response** (n = 96); (**B**) **Habituation** of the acoustic/vibrational escape response (n = 96). (**C**) **Basal Locomotor (BLM)** activity (n = 96); (**D**) **Visual-motor response (VMR)** (n = 96). Data are reported as mean values ± standard error. Different letters indicate statistical different subset groups following either one-way ANOVA with Tukey post hoc (startle response and VMR) or Kruskal Wallis with Dunn's multiple comparison test (habituation and BLM).

serotonergic (Fig. 5) and dopaminergic (Fig. 6) systems was also assessed. Of the neurotransmitters associated with the serotonergic system assessed, serotonin (5-HT), tryptophan and 5-hydroxyindoleacetic acid (5-HIAA) (a metabolite of serotonin) neurotransmitters (Fig. 5A, B and C respectively), fluoxetine exposure only induced significant effects on tryptophan levels ($H(4) = 10.855$, $p = 0.028$). Thus, a significant increase in tryptophan levels was found in organisms exposed to fluoxetine (all concentrations tested) (1 ng/L: $p = 0.037$; 10 ng/L: $p = 0.027$; 100 ng/L: $p = 0.002$; 1000 ng/L: $p = 0.013$) (Fig. 5B).

In terms of dopaminergic system, the levels of dopamine and its metabolites (3-methoxytyramine (3-MT), homovanillic acid (HVA) and 3,4 dihydroxyphenylacetic acid (DOPAC)) (Fig. 6A, B, C and D respectively), tyrosine (Fig. 6E) and norepinephrine (synthesized from dopamine) (Fig. 6F) were analyzed. Dopamine levels remained unchanged ($H(4) = 0.582$, $p = 0.965$) (Fig. 6A) while the levels of DOPAC were reduced across all fluoxetine treatments ($H(4) = 10.234$, $p = 0.037$; 1 ng/L: $p = 0.019$; 10 ng/L: $p = 0.021$; 100 ng/L: $p = 0.002$; 1000 ng/L: $p = 0.024$) (Fig. 6D). An opposite trend was observed for norepinephrine levels ($H(4) = 11.158$, $p = 0.025$), where three of the four tested concentrations of fluoxetine prompted increases of its levels (1 ng/L: $p = 0.009$; 100 ng/L: $p = 0.002$; 1000 ng/L: $p = 0.016$) (Fig. 6F).

4. Discussion

Overall, the data from the present study show that exposure to environmentally relevant concentrations of fluoxetine, during embryonic development, can result in changes in parameters at different levels crucial for organismal fitness.

Fluoxetine tested concentrations had no effect on embryo survival, supporting the data of Cunha et al. (2016), that also found no lethal effects on zebrafish embryos exposed, between 32 and 80 h, to concentrations between 0.519 and 276.6 μg/L fluoxetine. Lack of effects on zebrafish larvae survival was also reported for the metabolite norfluoxetine (0.64–400 ng/L), after an 80-h exposure period (Rodrigues et al., 2020).

In addition, no teratogenic effects were observed in this study. However, previous studies have detected teratogenic effects in fish larvae due to fluoxetine exposure. Cunha et al. (2016) reported increased cumulative malformations in zebrafish embryos exposed for 32 and 80 h, to different concentrations of fluoxetine ranging from 0.519 to 276.6 μg/L. As the bioavailability of fluoxetine is pH-dependent, differences in the pH of the fish water used between both studies could justify the observed differences (Mishra et al., 2019). Despite the absence of teratogenicity, in this study, 1000 ng/L fluoxetine significantly decreased the larvae total length. An ability of fluoxetine to modulate fish larvae size after 120 h-exposure was previously reported by Kalichak et al. (2016), for 99 μg/L.

Fluoxetine promoted earlier hatching in organisms exposed to 3 of the four concentrations tested (1, 10 and 100 ng/L). A similar effect was also reported by Kalichak et al. (2016), but at a higher concentration (0.99 μg/L).

In the present work, fluoxetine tested concentrations increased heart

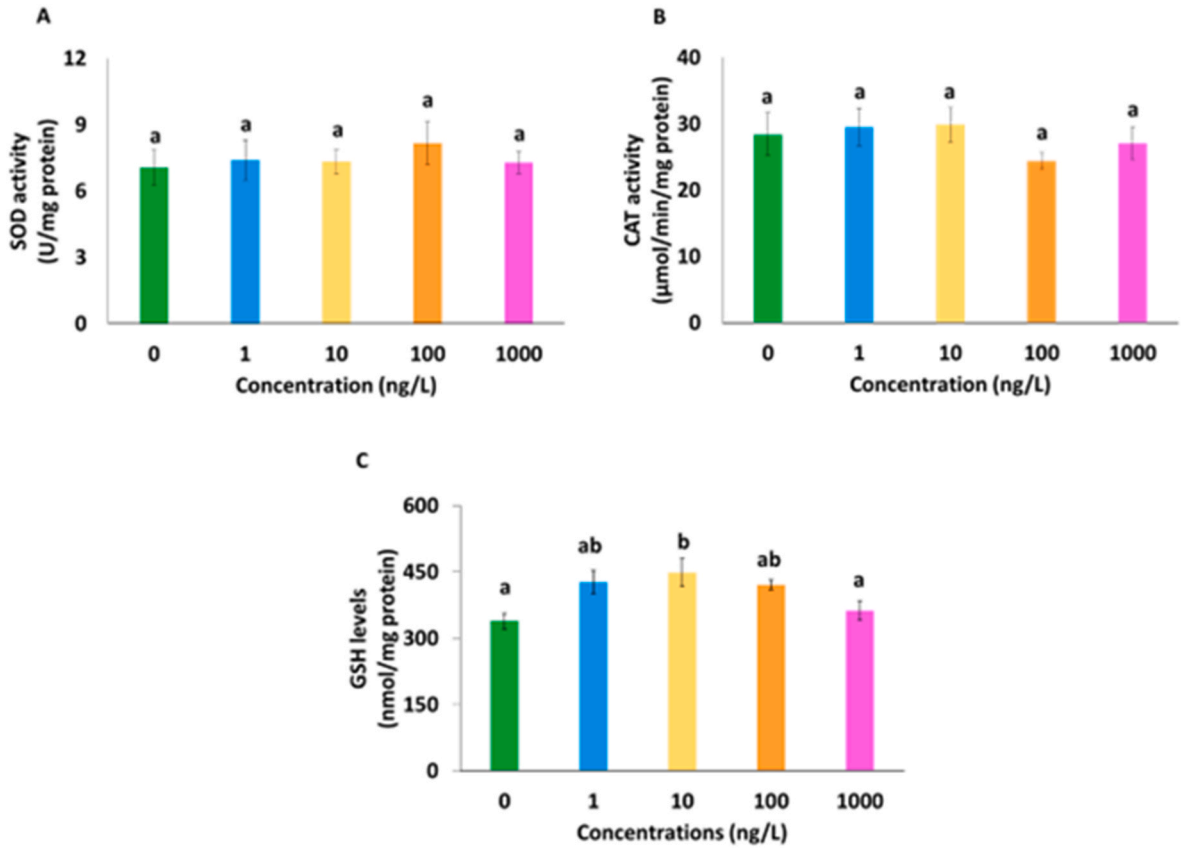

Fig. 3. Effect of fluoxetine on 8 dpf zebrafish larvae antioxidant status (n = 8 pools): (A) Superoxide dismutase (SOD) activity; (B) Catalase (CAT) activity; (C) Reduced glutathione (GSH) levels. Data reported as mean values ± standard error. Different letters indicate statistical different subset groups following one-way ANOVA with Tukey post hoc test.

rate in the organisms exposed to 10, 100 and 1000 ng/L. Other studies have reported the opposite effect of fluoxetine, but at higher concentrations. Kalichak et al. (2016) reported that 99 µg/L fluoxetine decreased the heart rate of zebrafish embryos at 49 hpf.

Altered behavior in zebrafish may be a useful tool to assess neurotoxicity during their development (Bertram et al., 2022). The influence of fluoxetine on behavior has been widely studied (reviewed by Correia et al. (2023)). In this study, zebrafish larvae were submitted to a several behavioral tests, associated with key essential behaviors, to assess potential neurological impairment resulting from developmental exposure to fluoxetine. One of the tests involved the rapid escape response evoked by a vibrational stimulus, crucial behavior for larvae survival, as they must be able to respond quickly and swim away from any threat (Faria et al., 2022b). The larvae exposed to the highest concentration of fluoxetine (1000 ng/L) showed a decreased startle response. A similar response was observed by Faria et al. (2021), in 8 day-old zebrafish larvae exposed to fluoxetine for 24 h to 154.67 µg/L. Environmentally relevant concentrations of sertraline (100 ng/L), another SSRI, have also been shown to weaken 8 day-old zebrafish larvae escape response, after a 24-h exposure (Faria et al., 2022b).

Habituation allows us to evaluate non-associative learning, resulting in a rapid decrease in the repeated response to a new stimulus or environment (Faria et al., 2022b). In the present study, fluoxetine exposure had no effect on this parameter. However, a higher exposure concentration (154.67 µg/L, 24 h) was reported to impair habituation in 8 day-old zebrafish larvae (Faria et al., 2021). These different results are likely related to differences in concentrations used, age of exposed organisms and exposure duration. The available data on the effects of fluoxetine on this parameter are scarce, and thus more research should be performed to understand the consequences of fluoxetine exposure on this behavior. Changes in spatial cognitive abilities (such as memory and learning) can result in greater difficulty in collecting, processing and memorizing important information for the organism, making it vulnerable to predators, increasing the difficulty in feeding and looking for partners (Jacquin et al., 2020).

Basal locomotor activity (BLM) is determined by the fish's optimal navigation in the dark environments (Farias et al., 2020). In the present study, fluoxetine exposure led to an increase in total distance swam, mainly for the concentrations 1 and 1000 ng/L. Interestingly, the profile of the effect of fluoxetine on BLM parallels the one of norepinephrine. Therefore, the observed increase in BLM might be mediated by the increase in the levels of the non-selective agonist of adrenergic receptors norepinephrine, since this neurotransmitter has been shown to increase locomotor activity in zebrafish larvae through the activation of the β-adrenergic receptors (Basnet et al., 2019).

In this study, visual motor response (VMR) allowed the assessment of the impact of fluoxetine exposure on larvae's response to changes in light conditions. Under normal conditions, the sudden transition from a light to dark environment trigger an increase in larvae swimming activity, reflecting anxiety-like behavior (Burton et al., 2017). Fluoxetine did not affect larvae VMR. However, higher concentrations have been shown to reduce the VMR response in zebrafish larvae following a short exposure period (e.g. Faria et al. (2021)).

Antioxidant enzymes (SOD and CAT) are considered a first line of defense against oxidative stress inducers and serve as early indicators of exposure to pollutants, while GSH, a non-protein thiol, plays a key role as a reactive oxygen species scavenger, in addition to its role on enzyme such as glutathione peroxidase and glutathione S-transferase (Banaee et al., 2023a; Banaee et al., 2023b; Cahova et al., 2021; Plhalova et al., 2018; Rashidian et al., 2023). Some studies with fish embryos/larvae

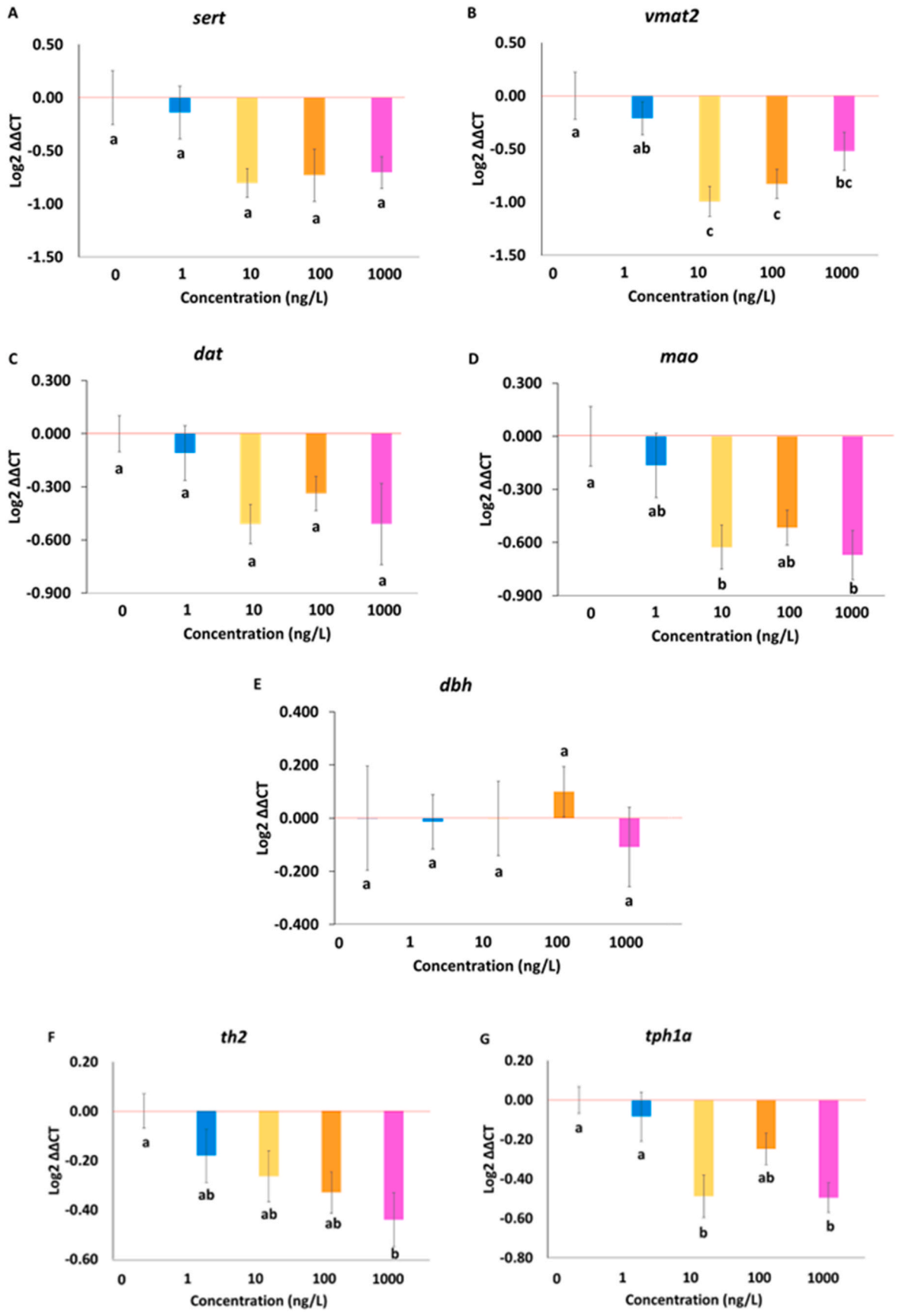

Fig. 4. Effect of fluoxetine on 8 dpf zebrafish larvae mRNA expression of genes associated with the serotonergic and dopaminergic system (A - *slc6a4a (sert)*; B - *scl18a2 (vmat2)*; C - *scl6a3 (dat)*; D - *mao*; E – *dbh*; F – *th2*; G – *tph1a*) (n = 8 pools). Data reported as mean values ± standard error. Different letters indicate statistical different subset groups following one-way ANOVA with Tukey post hoc test.

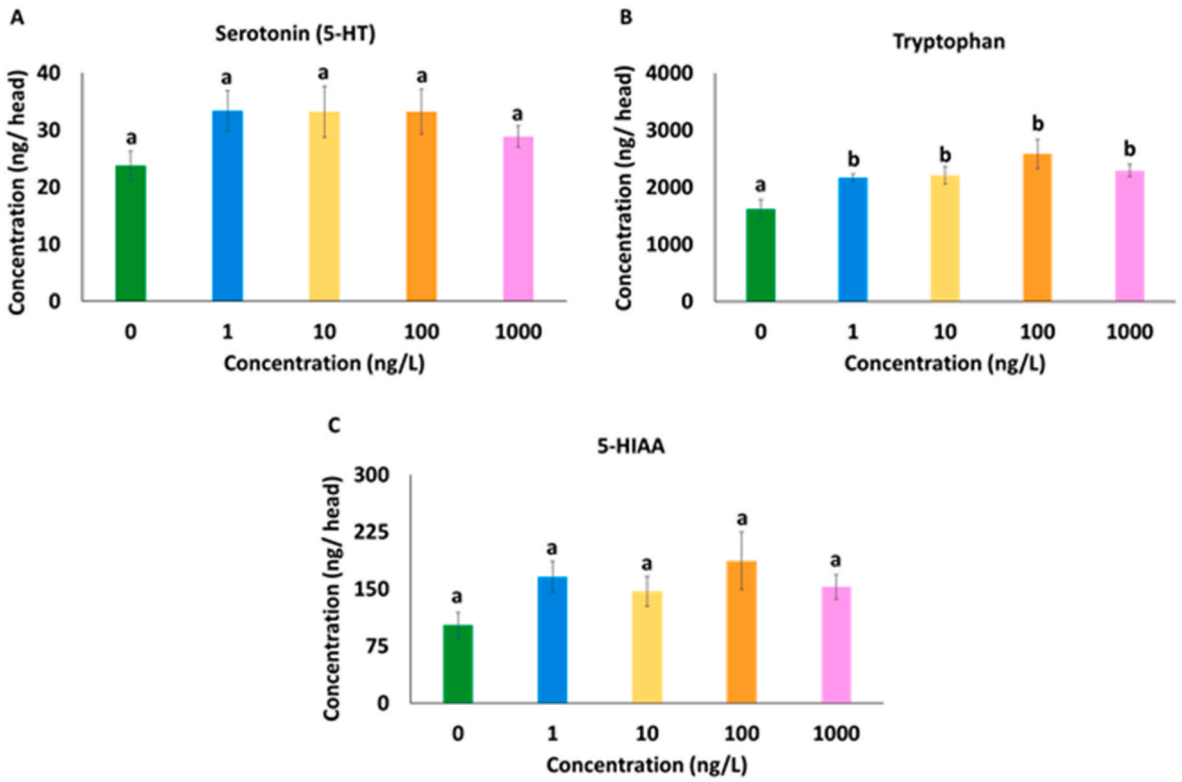

Fig. 5. Effect of fluoxetine on 8 dpf zebrafish larvae serotonergic neurotransmitters profiles (n = 6 pools): (A) Serotonin (5-HT); (B) Tryptophan; (C) 5-Hydroxyindoleacetic acid (5-HIAA). Data reported as mean values ± standard error. Different letters represent different subset groups following Kruskal Wallis with Dunn's multiple comparison test.

have reported the ability of fluoxetine to alter the activity of these antioxidant enzymes (Orozco-Hernández et al., 2022; Cunha et al., 2016). Orozco-Hernández et al. (2022) reported a dose-response increase in SOD and CAT activity in zebrafish embryos following 72- and 96-h exposure to environmentally relevant concentrations of fluoxetine ranging from 5 to 40 ng/L. SOD inhibition was also reported, in the same species and life stage as mentioned above, after 80-h exposure to 34.6, 172.9 and 276.6 μg/L fluoxetine (Cunha et al., 2016), with CAT activity induction observed at 0.46 and 154.7 μg/L. Yet, in the present study, no significant effects on the activity of these enzymes were found.

Regarding GSH levels, a trend towards a non-monotonic response pattern was observed. Such response patterns are not uncommon in ecotoxicity studies (Hill et al., 2018; Lagarde et al., 2015). Nonetheless, the mechanisms underlying this phenomenon are still unclear. The ability of fluoxetine to cause fluctuations in GSH levels in fish species has been previously reported. In *Pseudorasbora parva* juveniles exposed for 4 h to 50 μg/L fluoxetine, an increase (153%) in hepatic GSH was shown at 200 μg/L, which then decrease (28.5%) after 42 days exposure (Chen et al., 2018). In gills, acute exposure to 200 μg/L fluoxetine depleted GSH levels, whereas in chronic exposure, the levels were unchanged (Chen et al., 2018).

SSRIs antidepressants have been reported to be able to alter the gene expression in exposed organisms. 5-HT performs an important function in regulating neurotransmission processes, participating in numerous physiological and behavioral systems (McDonald, 2017). Alterations at several critical points of monoamine metabolism (e.g. synthesis, release, reuptake, catabolism, and serotonin receptors) may be a consequence of dysfunctions of 5-HT neurotransmission (Morilak and Frazer, 2004). In the present study, altered mRNA expression was found at three key points in serotonergic pathway. Fluoxetine exposure led to the downregulations of *vmat2* (important in the storage and synaptic release of all monoamines, including 5-HT (Eiden and Weihe, 2011)), *mao* (involved in the degradation of serotonin) and *tph1a* (involved in the synthesis serotonin). Downregulation of *vmat2* and *mao* by fluoxetine have been reported in other studies. Cunha et al. (2018) and Rodrigues et al. (2018) found decreased expression levels of *vmat2* in zebrafish embryos, after 80 h exposure to 0.519 μg/L and 0.43–0.46 μg/L, respectively. Additionally, Rodrigues et al. (2018, 2022) also found downregulation of *mao* expression in zebrafish larvae exposed for 80 h to 0.43, 0.46 and 15.5 μg/L fluoxetine. Finally, Faria et al. (2021) reported downregulation of *mao* expression, but not of MAO activity, in 8 day-old zebrafish larvae exposed for 24 h to 154.67 μg/L fluoxetine.

Dopaminergic neuronal activity is involved in different physiological processes such as cognitive system, locomotor behavior, memory, and learning (Beaulieu & Gainetdinov, 2011). The *dat* gene (also known as SLC6A3), is a transmembrane protein responsible for the reabsorption of dopamine from the synapse into the cytosol of dopaminergic neurons (Lamothe and Zhang, 2016). Despite the lack of effect observed in the present study, a 80-h exposure to 0.519 and 34.6 μg/L fluoxetine has been reported to induce a decrease in zebrafish embryos dopamine transporter transcription levels (Cunha et al., 2018). This downregulation was also found in zebrafish larvae, after 80-h exposure to 0.46 and 15.5 μg/L fluoxetine (Rodrigues et al., 2022) and 0.43–0.46 μg/L fluoxetine (Rodrigues et al., 2018).

In the present study, fluoxetine also altered the mRNA expression of *th2*, which encodes for the enzyme tyrosine hydroxylase 2, catalyzing the rate-limiting step in the synthesis of catecholamines. A decrease in the expression levels of this gene was observed, although only significant at the highest concentration (1000 ng/L). However, opposite findings have also been reported. On a 10-day chronic exposure to 100 μg/L fluoxetine *Oryzias latipes* adults showed increased mRNA expression levels of *th2* gene (Otsuka et al., 2022). These different results are certainly related to the organism species used.

In the present study, exposure to fluoxetine resulted in altered levels of brain neurotransmitters, endogenous chemicals that enable communication between neurons, providing a variety of functions of the brain

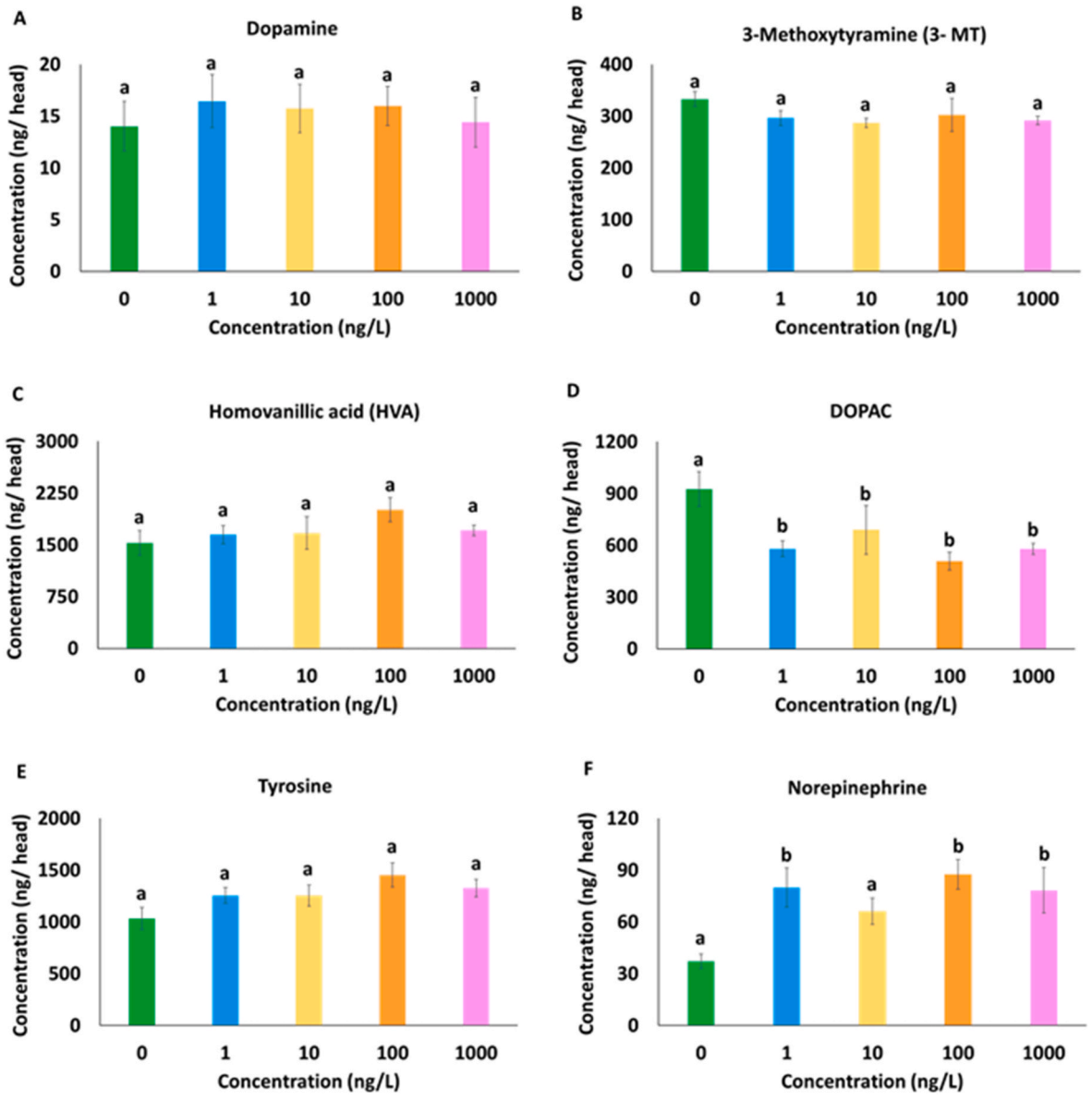

Fig. 6. Effect of fluoxetine on 8 dpf zebrafish larvae dopaminergic neurotransmitters profiles (n = 6 pools): (A) Dopamine; (B) 3-Methoxytyramine (3-MT); (C) Homovanillic acid (HVA); (D) 3,4 Dihydroxyphenylacetic acid (DOPAC); (E) Tyrosine; (F) Norepinephrine. Data reported as mean values ± standard error. Different letters represent different subset groups following Kruskal Wallis with Dunn's multiple comparison test.

through the process of chemical synaptic transmission (Rizo, 2018). The continuous firing of serotonergic signaling may be involved in the decreased expression of genes related to synthesis, transport, and metabolism. This is reflected by downregulation of *tph1a* buildup of tryptophan in the CNS. Tryptophan is an amino acid precursor for the synthesis of serotonin in the brain (Höglund et al., 2019). This increase in the levels of this amino acid was not reflected in the levels of serotonin, which remained unchanged. These mechanistic shifts of the serotonergic pathway may have led to larvae poor response to a stress stimulus. Some studies with zebrafish larvae have reported the fluoxetine effects on the neurotransmitters levels of the serotonergic system. In 8 day-old zebrafish larvae exposed to 154.67 μg/L fluoxetine for 24 h, no effects on serotonin levels were observed but 5-HIAA levels were significantly decreased (Faria et al., 2021). Additionally, 8 day-old zebrafish larvae 24-h exposed to 1 and 10 μg/L sertraline, displayed an increase in serotonin and 5-HIAA levels (increase in 5-HIAA levels), with the highest level recorded at 10 μg/L sertraline (Faria et al., 2022b).

Fluoxetine tested concentrations also altered catecholamine neurotransmission. Dopamine levels in the exposed organisms did not change, despite downregulation in the *th2* gene. However, the levels of DOPAC, one of dopamine metabolites, were decreased possibly due to the downregulation of *mao*, responsible for the conversion of dopamine into this metabolite. Norepinephrine, synthesized from dopamine, through dopamine β-monooxygenase (DBH), and responsible for preparing the organism for the larval start/leak response had its levels significantly increased at 1, 100 and 1000 ng/L, although its gene expression encoding for *dbh* were inconclusive. With the lack of effect on *dbh* expression, there is a possibility of changes in its protein expression, which should be considered and analyzed in future studies. The increase in norepinephrine levels may explain the effects observed in basal locomotor activity, as exposed larvae showed an increase in this behavior. However, the increase in norepinephrine levels was not exhibited in the larvae's startle behavior, as this response was opposite of what was expected, given the higher norepinephrine levels. The increased larvae heart rate observed in the present study may also be related to this

increase in norepinephrine levels. Fluoxetine can interact with the 5-HT_{2C} receptors, and through this mechanism, it may to increase norepinephrine (Bymaster et al., 2002). Norepinephrine is known to promote a contracting action on blood vessels, resulting in an increase in blood pressure and heart rate (Hamzaoui et al., 2010).

The effect of fluoxetine on catecholamine neurotransmission levels have also been observed in other studies. Faria et al. (2021) in 8-day-old zebrafish larvae exposed to 154.67 μg/L fluoxetine for 24 h, dopamine and 3-MT levels were also unchanged, but DOPAC levels were higher after exposure to fluoxetine. Furthermore, in a study conducted with sertraline in 8-day-old zebrafish larvae supports our results, in which dopamine levels were unchanged and norepinephrine levels increased after exposure to 1 and 10 μg/L sertraline for 24 h (Faria et al., 2022b). Nevertheless, higher levels of 3-MT were observed at 0.1 μg/L fluoxetine and for DOPAC and HVA higher levels were also observed at 1 and 10 μg/L fluoxetine (Faria et al., 2022b). The available data on the effects of fluoxetine on brain neurotransmitter levels are scarce, so there is a need for increased research on this parameter for a better understand how this SSRI might affect this component in fish.

5. Conclusion

The present study demonstrated that environmentally relevant concentrations of fluoxetine can induce effects on fish early life stages. Exposure during embryonic development affected larvae heart rate and growth and affected behavioral responses important for survival (startle response and basal locomotor activity), potentially impairing important behavioral responses like escaping from predators and obtaining food. Furthermore, low concentrations of fluoxetine are capable to cause a decrease in the expression levels of genes of the monoaminergic system, also affecting the neurotransmitter system, increasing tryptophan and norepinephrine levels, and decreasing DOPAC levels. The fact that the tested concentrations can disrupt systems important for the development and survival of organisms supports the importance of monitoring the presence of these psychotropic drugs in the aquatic environment, and the inclusion of ecologically relevant behaviors, as well as study the enzymes activity involved in neurochemical pathways in environmental risk analyses.

CRediT authorship contribution statement

Daniela Correia: Conceptualization, Investigation, Writing – original draft, Writing – review & editing, Visualization, Funding acquisition. **Marina Bellot:** Writing – original draft, Formal analysis. **Eva Prats:** Investigation. **Cristian Gómez-Canela:** Investigation. **Hugo Moro:** Investigation. **Demetrio Raldúa:** Investigation. **Inês Domingues:** Conceptualization, Investigation, Writing – review & editing, Visualization, Supervision. **Miguel Oliveira:** Conceptualization, Investigation, Writing – review & editing, Visualization, Supervision, Funding acquisition. **Melissa Faria:** Conceptualization, Investigation, Writing – review & editing, Visualization, Supervision, Funding acquisition.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Daniela Correia reports financial support was provided by FCT - Fundação para a Ciência e Tecnologia. Miguel Oliveira reports financial support was provided by FCT - Fundação para a Ciência e Tecnologia. Melissa Faria reports financial support was provided by IDAEA-CSIC, Severo Ochoa Centre of Excellence.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.chemosphere.2023.140468.

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