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Parental exposure to antidepressants has lasting effects on offspring? A case study with zebrafish

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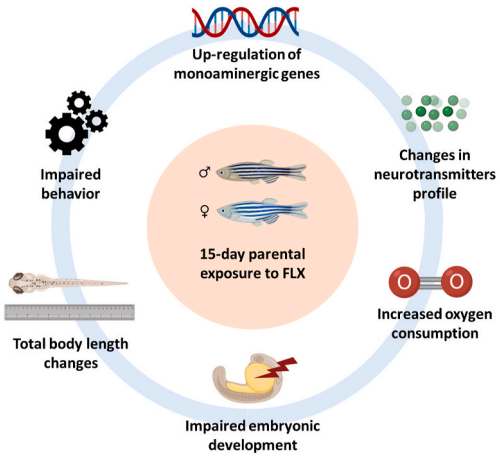
HIGHLIGHTS

- Parental exposure to fluoxetine affected the offspring;
- Parental exposure impaired the embryonic development of the offspring;
- Offspring early age behavior was affected;
- The expression of monoaminergic genes and neurochemical profiles were altered;
- Behavioral, genetic, and neurochemical alterations persisted in offspring adulthood.

GRAPHICAL ABSTRACT



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ABSTRACT

Fish have common neurotransmitter pathways with humans, exhibiting a significant degree of conservation and homology. Thus, exposure to fluoxetine makes fish potentially susceptible to biochemical and physiological changes, similarly to what is observed in humans. Over the years, several studies demonstrated the potential effects of fluoxetine on different fish species and at different levels of biological organization. However, the

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Monoaminergic system
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effects of parental exposure to unexposed offspring remain largely unknown. The consequences of 15-day parental exposure to relevant concentrations of fluoxetine (100 and 1000 ng/L) were assessed on offspring using zebrafish as a model organism. Parental exposure resulted in offspring early hatching, non-inflation of the swimming bladder, increased malformation frequency, decreased heart rate and blood flow, and reduced growth. Additionally, a significant behavioral impairment was also found (reduced startle response, basal locomotor activity, and altered non-associative learning during early stages and a negative geotaxis and scototaxis, reduced thigmotaxis, and anti-social behavior at later life stages). These behavior alterations are consistent with decreased anxiety, a significant increase in the expression of the monoaminergic genes *slc6a4a* (*sert*), *slc6a3* (*dat*), *slc18a2* (*vmat2*), *mao*, *tph1a,* and *th2*, and altered levels of monoaminergic neurotransmitters. Alterations in behavior, expression of monoaminergic genes, and neurotransmitter levels persisted until offspring adulthood. Given the high conservation of neuronal pathways between fish and humans, data show the possibility of potential transgenerational and multigenerational effects of pharmaceuticals' exposure. These results reinforce the need for transgenerational and multigenerational studies in fish, under realistic scenarios, to provide realistic insights into the impact of these pharmaceuticals.

1. Introduction

Growing aquatic pollution resulting from the production and consumption of pharmaceutical products has been an environmental concern since these ecosystems receive wastewater containing these chemicals and/or its metabolites, with unknown ecological impacts (Orive et al., 2022). Selective serotonin reuptake inhibitors (SSRIs) are the most prescribed and consumed antidepressant drugs (Castillo-Zacarías et al., 2021; Estrela et al., 2020), becoming a growing concern as emerging environmental contaminants (Desbiolles et al., 2018; Moreira et al., 2022). These antidepressants are used to treat depression and other psychiatric disorders (Mole and Brooks, 2019; Stewart et al., 2014) by blocking the serotonin reuptake transporter in the presynaptic neuron, resulting in an extracellular increase of serotonin levels (5-hydroxytryptamine or 5-HT) in the central nervous system (CNS) (Chai et al., 2021; Salahinejad et al., 2022; Sumpter and Margiotta-Casaluci, 2022; Chai et al., 2021; McDonald, 2017; Salahinejad et al., 2022; John P. Sumpter and Margiotta-Casaluci, 2022). Fluoxetine is one of the most frequently detected SSRIs in surface waters, with an estimated levels ranging from 0.012 to 1.4 μg/L (Christensen et al., 2009; Kolpin et al., 2002; Weinberger and Klaper, 2014). Additionally, fluoxetine has the ability to affect the synthesis, reuptake and metabolism of neurotransmitters of the monoaminergic system in the CNS of organisms (Horzmann and Freeman, 2016; Lin et al., 2023; Ng et al., 2015; Rizo, 2018).

The monoaminergic system is evolutionarily conserved in most vertebrates including fish species (Ford and Fong, 2016; Mezzelani et al., 2018; Prasad et al., 2015) and plays an important role in brain development, being involved in different physiological processes such as cell proliferation, migration, differentiation, synaptogenesis, and neurogenesis (Lambe et al., 2011; Thompson and Vijayan, 2022). In teleost fish, little studies have been done to understand whether exposure to fluoxetine in one generation can have effects on the health and behavior of future generations. Some findings suggest potential transgenerational and multigenerational effects of other contaminants, on behavior, neurodevelopment and reproductive outcomes in fish and other animal species (e.g., *Daphnia magna*). Zebrafish paternal 6 weeks exposure to carbamazepine (10 μg/L) resulted in a decrease in 11-ketotestosterone levels, reproductive performance, altered courtship, aggressiveness and sperm morphology in adult stage when compared to organisms from unexposed parents (Fraz et al., 2019); an exposure of adult zebrafish for 6 weeks to 10 μg/L of carbamazepine and gemfibrozil reduced reproductive success and fecundity, altered courtship behavior, morphology and sperm velocity in F1 offspring raised in clean water (Galus et al., 2014). Parental exposure to arsenic in zebrafish altered motor activity, increased anxiety behaviors until the F2 generation and induced epigenetic changes (Valles et al., 2020). Hao et al. (2022) reported that zebrafish exposed to 1 and 100 μg/L of bisphenol S, from 3 hpf to 120 dpf, increased DNA methylation in F1 testis and DNA methylation in F2 ovaries, leading to decreased expression of steroidogenic enzymes, elevated plasma 17β-estradiol and decreased testosterone levels. Minguez et al. (2015), that investigated the transgenerational effects of 21 days exposure to sertraline and venlafaxine (0.3, 30 and 100 μg/L) on *Daphnia magna* reported that sertraline exposure increased the fecundity of F0 daphnids and decreased the number of offspring F1, while exposure to venlafaxine decreased the number of offspring F0 and promoted tolerance to the drug in the F1 generation.

Several studies have shown that fluoxetine may be bioaccumulated and can cause a wide variety of effects in aquatic ecosystems, affecting gene expression, biochemical and metabolic parameters, histology, reproduction and behavior in several organisms (Correia et al., 2023b; McDonald, 2017; Mennigen et al., 2011; Salahinejad et al., 2022; Stewart et al., 2014; Sumpter et al., 2014). However, the available research regarding the transgenerational effects of this SSRI remains scarce. To our knowledge, only 5 studies addressed parental and transgenerational effects of fluoxetine in fish species (Al Shuraiqi et al., 2021; Martinez et al., 2019; Polverino et al., 2021; Vera-Chang et al., 2018, 2019). These studies mainly focused on behavioral changes in the offspring and neglected long-term consequences. There is thus a need to explore long-term effects in a wider range of endpoints.

The current study assessed if adult zebrafish exposure to relevant concentrations of fluoxetine for 15 days influences offspring's fitness. Effects of parental exposure were studied on offspring embryonic development, behavior (8 days post-fertilization (dpf)), genetic and neurochemical profiles, as well as the persistence of potential effects on the offspring behavior (1, 2- and 3-month-old), gene expression and neurotransmitters profile in adulthood (8 dpf vs 3-month-old).

2. Materials and methods

2.1. Fish husbandry and larvae production

Adult wild-type zebrafish (AB strain, 3.1–3.5 cm) were obtained from Pisciber BSF (Terrassa, Barcelona) at the CID-CSIC zebrafish facilities (Supplementary Material for additional details). All procedures were approved by Institutional Animal Care and Use Committees, and conducted in accordance with the institutional guidelines under a license from the local government (agreement number 9027).

2.2. Adult zebrafish exposure (parental generation)

Fluoxetine hydrochloride (CAS number: 56296-78-7, molecular weight 345.79 g/mol, purity: >98%) was purchased from ChemCruz (Netherlands). The test solutions (100 and 1000 ng/L fluoxetine hydrochloride) were prepared on the day of the experiment, from a 4 μg/L stock solution freshly prepared in fish water. Adult zebrafish (18 females: 15 males) were randomly selected from the CID-CSIC facilities and exposed for 15 days to 100 and 1000 ng/L fluoxetine hydrochloride, at 28.5 °C and 12L:12D photoperiod, in semi-static test conditions. The range of concentrations tested covered natural concentrations recorded

in surface waters, while the duration of exposure adopted was based in the fact that the anxiolytic effects of this SSRI manifest themselves after two weeks of exposure (Martin et al., 2017). Control fish were maintained in fish system water under identical conditions. Experiments were conducted in triplicate for each treatment, in glass tanks containing 1.5 L of water each, with the males and females in separate tanks (5 males and 6 females). A total of 33 fish were used per experimental treatment. Experimental solutions were renewed every 48 h, based in the concentration stability of fluoxetine discussed in a previous work (Correia et al., 2023a), 30 min after the first feeding of the day. Animals were daily checked for mortality and fed twice a day. At the end of the exposure period (day 15), the animals were rinsed twice in clean medium and transferred to breeding tanks with fish system water. In the next morning the eggs were collected, cleaned with fish water and checked under a stereomicroscope (Leica Microsystems - Leica eEZ4 16x, 4.4:1), where embryos presenting a similar developmental stage (stage 2: Blastula (Kimmel et al., 1995)) were selected and divided into two groups: **Group A** – for embryonic developmental evaluation and **Group B** – for evaluation at later life stages.

2.3. Offspring generation

2.3.1. Embryonic development evaluation (group A)

Zebrafish, 4 h post-fertilization (hpf) embryos, were placed in 6-well plates (10 embryos per well) containing 10 mL of clean fish water until 8 days post fertilization (dpf). A total of 540 embryos per condition were assessed. Complete water renewal was performed every 48h. The animals were daily checked, and endpoints like mortality and malformations (at 192 hpf), early hatching (at 48 and 72 hpf), swimming bladder inflation (at 96 and 120 hpf), cardiac activity analysis, total body length, and behavior were assessed throughout this period.

2.3.2. Cardiac activity analysis (group A)

Heart rate and blood flow activity were measured in 8 dpf zebrafish larvae (n = 15 organisms/condition) using the DanioScope software (Noldus Wageningen, the Netherlands). The larvae were not under anesthetic, being immobilized and positioned in lateral or ventral view, on a slide, in 4% methylcellulose. This procedure was performed in an isolated behavior room, at 27°–28 °C. The heartbeat and blood flow were video recorded for 30 s (s) with a GigE camera (AVI format at 30 fps; UI-5240CP-NIR-GL, Imaging Development Systems, Germany) (Bedrossiantz et al., 2023; Faria et al., 2022a). Videos were analyzed with the DanioScope software, as described by Cunha et al. (2020).

2.3.3. Total body length measurements (group A)

To assess the effects of parental exposure to fluoxetine on zebrafish offspring total body length, the individuals were measured at 8 dpf and then every 30 days (≈1, 2- and 3-month-old) (n = 20 organisms/condition). Total body length measurements were performed from the anterior part of the snout to the most posterior point of the tail. The 8 dpf larvae were measured using the Noldus EthoVision©XT 15.0 software. For 1-, 2- and 3-month-old organisms, they were photographed using a microscope (Nikon SMZ1500, NIKON Instruments INC. New York, USA) equipped with a Nikon Digital Sight DSRi1 camera (Fig. S1A), and the measurements taken with the GIMP 2.10.12 software (more details in Supplementary Material).

2.3.4. Behavior analysis

All the behavioral tests were carried out in an isolated behavioral room at 27–28 °C. The fish were transferred into the behavioral room 1 h before the tests to allow acclimation to the room. The behavioral tests were performed between 10:00 and 14:00. The zebrafish used were all of similar total body length.

2.3.5. 8 dpf (group A) and 1 month evaluation (group B)

All behavioral analysis of the offspring (8 dpf larvae and 1 month old juveniles) were performed as previously described (Faria et al., 2021b) using the DanioVision system controlled by Noldus EthoVision©XT 11 software (Noldus, Wageningen, the Netherlands) (Supplementary Material for additional details). Four distinct behaviors were assessed: vibrational startle response (VSR), habituation (to vibrational stimuli), basal locomotor activity (BLA), and visual motor response (VMR) (Faria et al., 2021b).

2.3.5.1. 2-month-old evaluation (group B). For 2-month-old zebrafish, novel tank test (NTT) and shoaling test (Figs. S2A and B) were performed to assess anxiety and social behavioral effects, respectively, using the experimental setup described by Faria et al. (2018) and testing 24–27 zebrafish from each experimental group. For each test, videos of 6 min were recorded (AVI format, 30 fps) using a GigE camera (UI–5240CP-NIR-GL, Imaging Development Systems, Germany) placed in front of the experimental tank, controlled by the uEye Cockpit software (Imaging Development Systems, Germany). After recording, the videos were processed using Noldus EthoVision©XT 15.0 (Noldus, Wageningen, the Netherlands). For the NTT, anxiety behavior was assessed individually by analyzing the geotaxis, a "diving" behavior exhibited by the fish when placed in a novel environment (e.g. new tank) (Cachat et al., 2010; Kysil et al., 2017; Nguyen et al., 2014) (Fig. S2A). The distance traveled in the top and in the bottom layers (%), total distance moved (cm), transitions to the top, time spent in the top and bottom layers (%), and freezing time (%) were determined. The shoaling test allows to assess changes in social cohesion (Gerlai, 2014; Miller and Gerlai, 2007). Groups of 9 fish from each experimental condition (n = 24–27 zebrafish) were used and the average interfish distance (cm) and the farthest fish distance (cm) determined (Faria et al., 2018) (Fig. S2B). After the tests, fish were returned to the Zebrafish housing rack to continue growing for a further 30 days.

2.3.5.2. 3-Month-old evaluation (group B). The 3-month-old zebrafish were submitted to behavioral tests: NTT, shoaling test, social preference test (SPT), light-dark test (LDT) and novel object test (NOT). The video recording setups, hardware and software used for recordings and video processing were the same as used in the previous section. At this stage of life, it was not possible to differentiate the gender of the offspring organisms.

The SPT consists of using social stimuli to investigate the behavioral response to a shoal of conspecifics (Engeszer et al., 2004; Gerlai et al., 2000; Ogi et al., 2021). The SPT was carried out using one experimental rectangular tank (20 cm long, 20 cm wide and 25 cm high) containing 5 L of fish water at 28 °C. On the left side, a shoal of 10 zebrafish (similar in total body length to the fish tested) was placed and, on the right side, a tank with water (no fish inside – neutral stimulus) (Fig. S2C). The SPT was assessed individually in 24 zebrafish from each treatment. For video analysis, tanks were divided into 3 equal virtual zones: near – closest to the shoal, intermediate, and far zone, and the distance traveled, and the time spent in each zone was measured (Fig. S7).

The LDT was performed according to Faria et al. (2019) and used to evaluate anxiety behavior by assessing zebrafish scototaxis, the preference of the fish for a dark background when a mild aversive stimulus (novelty) is presented (Maximino et al., 2011, 2013). The behavior of 24 zebrafish from each experimental group was studied individually, with the camera placed on top of the testing tanks (Fig. S2D). The time spent in the white and dark area was analyzed.

The NOT, used to evaluate anxiety and boldness in the fish, was conducted based on the experimental design of Hamilton et al. (2017), using a LEGO® figure as the novel object, fixed with Velcro in the center of the arena (Faria et al., 2021a) (Fig. S2E). The behavior of 24 zebrafish was recorded individually, with the camera placed on top of the testing tanks, and the distance and time spent in 3 virtual zones (far, middle and center) was analyzed (Fig. S8).

After the behavioral tests, fish were euthanized by thermal shock and

brains were collected individually frozen in liquid nitrogen and stored at −80 °C for gene expression (n = 8 brains per treatment) and neurotransmitters analysis (n = 6 brains per treatment).

2.3.6. Respirometry assays (group B)

Respirometry assay was performed as described by Agra et al. (2011), adapted for zebrafish. Oxygen consumption was measured by quantifying the levels of dissolved oxygen in the water with 2- and 3-month-old offspring (10 fish per condition and age). Briefly, each fish was introduced in a gastight 50 mL syringe (Hamilton, USA) filled with 50 mL of clean fish water saturated with O_2 (Oxygenating for 24h) and dissolved oxygen levels were measured using an oxygen meter (Strathkelvin Instruments, Glasgow), fitted with an oxygen electrode (Strathkelvin Instruments, Glasgow). Three blank controls were tested for each treatment to correct for ambient oxygen depletion and used as correction factor (Agra et al., 2011; Campos et al., 2012) (Supplementary Material for additional details).

2.4. RNA preparation and qRT-PCR analysis

Expression levels of monoaminergic genes were evaluated at two different time points: 8 dpf (Group A) and 3-month-old (Group B). For the gene expression analysis of the 8 dpf larvae, 8 pools were made with 10 larvae (from each treatment) and for the 3-month organisms, 8 adult zebrafish brains (from each treatment) were used. Briefly, extraction of total RNA from all samples was performed with Trizol reagent (Invitrogen Life Technologies, Carlsbad, CA), and all procedures were performed as previously described (Prats et al., 2017). The efficiency and specificity of the primer sequences (Sigma-Aldrich, Steinheim, Germany) of the six selected genes related to the monoaminergic system (*slc6a4a*, *slc6a3*, *slc18a2*, *mao*, *th2* and *tph1a*) (Supplementary Table S1) were verified before the analyses.

2.5. Extraction of monoaminergic neurotransmitters and LC-MS/MS analysis

The levels of monoaminergic neurotransmitters in the offspring were evaluated at two different time points: 8 dpf (Group A) and 3-month-old (Group B). For the neurotransmitters analysis of the 8 dpf larvae, 6 pools were made with 15 heads of zebrafish larvae (from each treatment) and for the 3-month organisms, 6 adult zebrafish brains (from each treatment) were used. Levels of neurotransmitters were analyzed by chromatographic and mass spectrometry conditions described in previous studies (Bellot et al., 2021; Mayol-Cabré et al., 2020). Data were processed using MassLynx v4.1 software package.

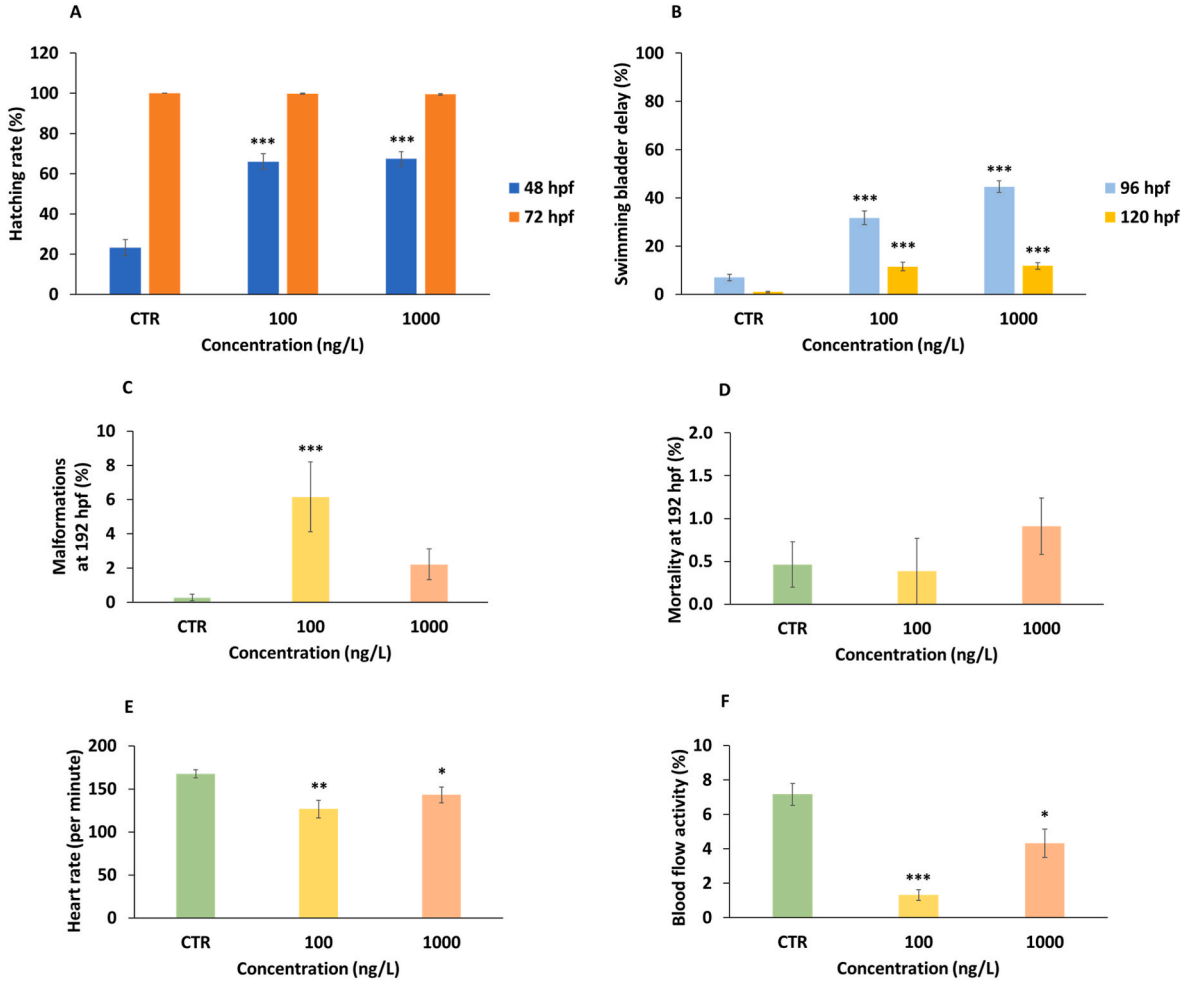

Fig. 1. Effects of zebrafish parental exposure to fluoxetine on offspring embryonic development: **(A)** Hatching rate (%) (n = 540); **(B)** No inflation of swimming bladder (%) (n = 540); **(C)** Malformations at 192 hpf (%) (n = 540); **(D)** Mortality at 192 hpf (%) (n = 540); **(E)** Heart rate (n = 45); **(F)** Blood flow activity (%) (n = 45). All data are reported as mean values ± standard error. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, compared with the control offspring; One-way ANOVA with Dunnett's multiple comparison test (heart rate) or Kruskal Wallis test with Dunn's multiple comparison test (hatching rate (%), no inflation of swimming bladder (%), malformations (%), mortality (%) and heart flow activity (%)). Data from 3 independent assays.

2.6. Statistical analysis

Data was analyzed with IBM SPSS v28 (Statistical Package 2010; Chicago, IL). Normality was assessed using Kolmogorov-Smirnov and Shapiro-Wilk tests. On normally distributed groups, a One-way ANOVA followed by a Dunnett's multiple comparison test was performed. The Kruskal-Wallis test, followed by Dunn's multiple comparisons test, was used for groups that did not meet normality. Data from 3 independent experiments was plotted with Microsoft Excel 2016 and presented as mean ± standard error and a $p < 0.05$ value was set to indicate statistical significance.

3. Results

3.1. Embryonic development

Parental exposure to fluoxetine had no significant effects on offspring mortality ($H(2) = 3.261$, $p = 0.196$) (Fig. 1D). However, effects on embryonic development were found. Statistical significant differences between concentrations were observed in the hatching rate ($H(2) = 44.20$, $p < 0.001$), where the hatching rate of offspring from exposed parents at 48 hpf was significantly higher than those from the control group (100 ng/L: $p < 0.001$; 1000 ng/L: $p < 0.001$) (Fig. 1A). Inflation of the swimming bladder of the fluoxetine offspring was significantly delayed at both 96 hpf ($H(2) = 100.77$, $p < 0.001$; 100 ng/L: $p < 0.001$; 1000 ng/L: $p < 0.001$) and 120 hpf ($H(2) = 47.578$, $p < 0.001$; 100 ng/L: $p < 0.001$; 1000 ng/L: $p < 0.001$), (Fig. 1B). Additionally, occurrence of malformations (head, eyes and tail malformations and pericardial and yolk sac edema) were observed ($H(2) = 10.139$, $p = 0.006$), with a significant increase observed in the offspring of fish exposed to 100 ng/L fluoxetine ($p = 0.001$) (Fig. 1C). Fluoxetine also affected heart rate ($F_{(2,41)} = 5.842$, $p = 0.006$) and blood flow activity ($H(2) = 20.883$, $p < 0.001$) of offspring, with both significantly decreasing at 100 and 1000 ng/L (heart rate: 100 ng/L: $p = 0.004$; 1000 ng/L: $p = 0.029$; blood flow activity: 100 ng/L: $p < 0.001$; 1000 ng/L: $p = 0.035$) (Fig. 1E and F).

The larvae total body length was affected at 8 dpf ($F_{(2,177)} = 40.48$, $p < 0.001$) and 1 month old offspring ($F_{(2,150)} = 19.92$, $p < 0.001$). At 8 dpf, larvae significant decreases in total body length were observed at both fluoxetine concentrations ($p < 0.001$) (Fig. 2), while 1 month old offspring were significantly bigger, at both concentrations (100 ng/L: $p = 0.008$ and 1000 ng/L: $p < 0.001$), than control (Fig. 2). No differences to control were observed in offspring at 2- and 3-month-old ($F_{(2,69)} = 3.01$, $p = 0.056$ and $F_{(2,105)} = 1.84$, $p = 0.164$, respectively).

3.2. Behavioral responses in offspring early life stages

Results of the vibrational startle response (VSR), habituation, basal locomotor activity (BLA), and visual motor response (VMR) are represented in a heat map (Fig. 3). All behavioral responses were significantly impaired in 8 dpf larvae (VSR: $F_{(2,340)} = 3.94$, $p = 0.02$; habituation: $H(2) = 26.19$, $p < 0.001$; BLA: $H(2) = 10.71$, $p = 0.005$; VMR: $H(2) = 36.85$, $p < 0.001$), whereas in 1 month old offspring only BLA ($H(2) = 8.92$, $p = 0.012$) and VMR ($H(2) = 22.32$, $p < 0.001$) were affected.

In 8 dpf larvae, the VSR significantly decreased at 1000 ng/L ($p = 0.01$), while the time required for habituation of vibrational stimuli increased significantly ($p < 0.001$). Larvae BLA decreased at both concentrations ($p < 0.01$), whereas the VMR showed biphasic responses, with the escape response evoked by the sudden transition from a light to a dark environment decreasing for 100 ng/L ($p < 0.001$) and increasing for 1000 ng/L fluoxetine offspring ($p = 0.034$).

In 1 month old zebrafish, a significant increase of BLA was observed at 1000 ng/L ($p = 0.021$) and of VMR at both concentrations (100 ($p = 0.035$) and 1000 ng/L fluoxetine ($p < 0.001$).

3.3. Behavioral evaluation in the offspring at 2-month-old

NTT in offspring of 100 and 1000 ng/L showed that fish have a negative geotaxis, with a significant increase in the distance moved in the top of the tank ($H(2) = 9.41$, $p = 0.009$; $p < 0.01$ for 100 and 1000 ng/L), and reduction of bottom layer exploring ($H(2) = 11.22$, $p = 0.004$; $p < 0.01$ for 100 and 1000 ng/L) (Fig. 4A). A similar response pattern was observed in terms of time spent on each layer, with fish spending more time in the top of the tank ($H(2) = 12.81$, $p = 0.002$; $p < 0.01$ for 100 and 1000 ng/L) and less time in the bottom ($H(2) = 13.66$, $p < 0.001$; $p < 0.01$ for 100 and 1000 ng/L) (Fig. 4A). Regarding the total distance moved in the whole tank, a significant decrease for both fluoxetine concentrations (100 ng/L: $p = 0.04$; 1000 ng/L: $p = 0.03$) (Fig. S5A) was observed. Additionally, a decreasing trend in the number of transitions to the top were also observed in 1000 ng/L fluoxetine offspring (Fig. 4B), as well as in the freezing behavior, but non were statistically significant (Fig. 4A). The shoal of offspring of 100 ng/L fluoxetine exposed organisms presented a significant increase in the average of interfish distance ($p < 0.001$) and the farthest distance ($p = 0.036$) (Fig. 4C) (Please see Supplementary Material for additional

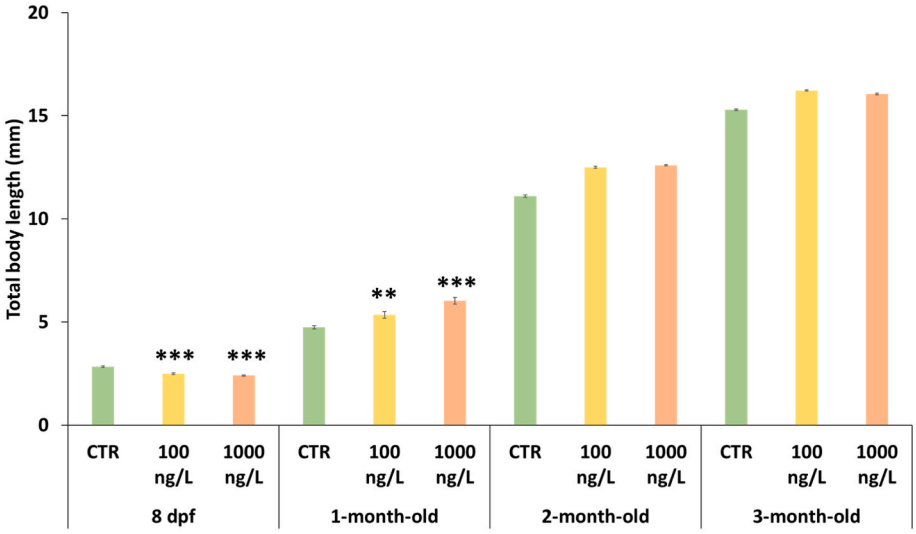

Fig. 2. Effects of zebrafish parental exposure to fluoxetine on offspring total body length at different developmental stages, following parental exposure: Total body length at 8 dpf (n = 180), 1 (n = 153), 2- (n = 72) and 3-month-old (n = 108). All data are reported as mean values ± standard error. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, compared with the control offspring; One-way ANOVA with Dunnett's multiple comparison test. Data from 3 independent assays.

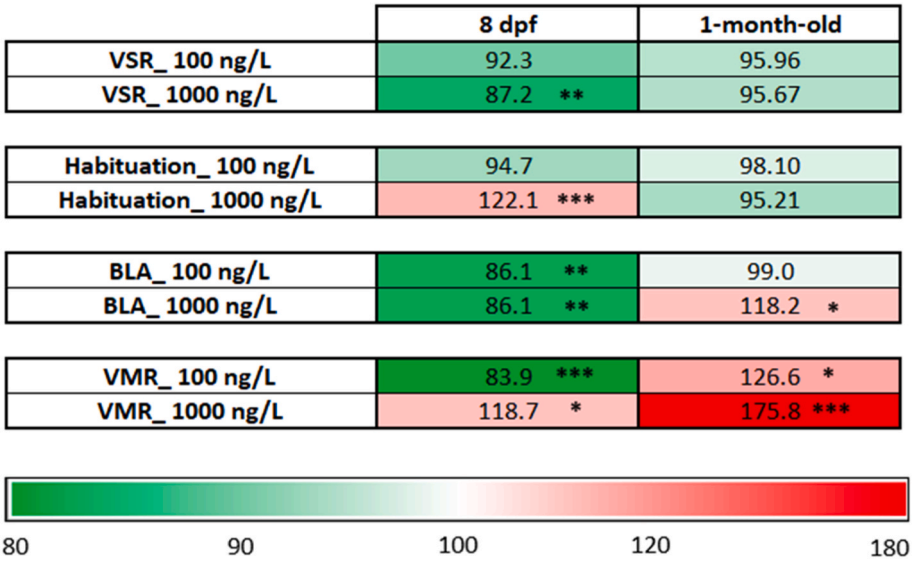

Fig. 3. Heat map diagram showing the effects of parental exposure to fluoxetine on vibrational startle response (VSR), habituation, basal locomotor activity (BLA), and visual motor response (VMR) of offspring at 8 dpf and 1-month-old. The colors in the heat map represent the deviation from the control offspring (white color), with a gradient of green or red for values below or above the controls, respectively. The number within each cell corresponds to the mean of the results for each parameter normalized as percentage of their respective controls. [8 dpf: (1) VSR, n = 144; (2) Habituation, n = 144; (3) BLA, n = 144; (4) VMR, n = 144 | 1-month-old: (1) VSR, n = 96; (2) Habituation, n = 96; (3) BLA, n = 96; (4) VMR, n = 96]. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$; [8 dpf: One-way ANOVA with Dunnett's multiple comparison test (VSR) or Kruskal Wallis test with Dunn's multiple comparison test (habituation, BLA and VMR); 1-month-old: Kruskal Wallis test with Dunn's multiple comparison test (VSR, habituation, BLA and VMR)]. Data from 3 independent assays.

detailed Figures (Fig. S3)).

3.4. Behavioral evaluation in the offspring at 3-month-old

NTT in 3-month-old offspring revealed similar response patterns as those observed at 2-months. Three month-old offspring from the 1000 ng/L condition, showed a negative geotaxis, with a significant increase in the distance moved on the top layer of the tank ($H(2) = 4.19$, $p = 0.123$; 1000 ng/L: $p = 0.04$) and a decrease on the bottom layer ($H(2) = 5.19$, $p = 0.075$; 1000 ng/L: $p = 0.024$) (Fig. 4A). This response pattern was also observed in the time spent in the two layers (top layer: $F_{(1,24)} = 4.86$, $p = 0.037$, 1000 ng/L: $p < 0.05$; bottom layer: $H(2) = 6.06$, $p = 0.048$; 1000 ng/L: $p < 0.05$) (Fig. 4A). In the total distance moved in the whole tank, a significant decrease for the offspring of 1000 ng/L fluoxetine ($p = 0.03$) was observed (Fig. S5B). Furthermore, a decreasing trend in the number of transitions to the top were also observed in 1000 ng/L fluoxetine offspring (Fig. 4B), as well as a downward trend in freezing behavior was observed in offspring from fluoxetine exposed fish (Fig. 4A).

The effect of the exposure of the parents to fluoxetine on the anxiety-like behavior in the offspring was analyzed with a second assay, the LDT, at the 3-month. A negative scototaxis was observed in the offspring of organisms from parents exposed to both doses of fluoxetine, with a significant increase in the time spent in the white arena ($H(2) = 10.70$, $p = 0.005$; Fig. 4A).

In addition to the geotaxis and scototaxis, boldness can also provide information on anxiety levels in an animal. A significant increase of the boldness was found in the 3-month offspring in the NOT. As Fig. 4A shows, offspring from exposed parents increased the distance moved ($H(2) = 20.34$, $p < 0.001$; 100 ng/L: $p < 0.001$; 1000 ng/L: $p < 0.001$) and the time spent ($H(2) = 20.84$, $p < 0.001$; 100 ng/L: $p < 0.001$; 1000 ng/L: $p < 0.001$) in the center of the area, where the novel objected was located.

When social behavior was analyzed in 3-month-old fish, significant changes were found in the size of the shoal. The average interfish distance significantly decreased in the 1000 ng/L shoal ($H(2) = 46.19$, $p < 0.001$; 1000 ng/L: $p < 0.001$), while the farthest fish distance ($H(2) = 46.62$, $p < 0.001$) decreased at both concentrations (100 ng/L: $p < 0.01$; 1000 ng/L: $p < 0.001$) (Fig. 4C), compared with the control. Significant differences in the behavior of the 3-month-old offspring were also found in the SPT ($H(2) = 11.59$, $p = 0.003$). A significant decrease in the total distance moved in the area closest to the conspecifics was found for both fluoxetine concentrations (100 ng/L: $p < 0.001$; 1000 ng/L: $p < 0.05$) (Fig. 4A). In terms of time, zebrafish offspring from 1000 ng/L fluoxetine spent significantly less time in the zone near the conspecifics ($H(2) = 5.28$, $p = 0.071$; 1000 ng/L: $p = 0.036$) than control offspring (Fig. 4A) (Supplementary Material for additional detailed Figures (Figs. S4 and S6)).

3.5. Oxygen consumption

Oxygen consumption in the 2- and 3-month-old offspring was significantly affected by parental exposure to fluoxetine (2-month-old: $H(2) = 16.76$, $p < 0.001$; 3-month-old: $H(2) = 21.39$, $p < 0.001$), with the offspring of both concentrations showing a significant increase in the rate of oxygen consumption at the two times points studied (2-month-old: 100 and 1000 ng/L: $p < 0.001$; 3-month-old: 100 ng/L: $p < 0.001$; 1000 ng/L: $p = 0.002$) (Fig. 5).

3.6. Expression of genes involved in monoaminergic system

The expression of genes involved in the serotonin reuptake (*slc6a4a* or *sert*), vesicular transport of monoaminergic neurotransmitters (*scl18a2* or *vmat2*), dopamine reuptake (*scl6a3* or *dat*), serotonin oxidation (*mao*) and synthesis of serotonin and dopamine (*tph1a and th2*, respectively) were analyzed in zebrafish offspring at 8 dpf (Group A) and in brains of 3-month-old fish (Group B). As Fig. 6 shows, in 8 dpf larvae the expression of *sert* and *dat* was significantly affected by the parental exposure to fluoxetine (*sert*: $F_{(2,18)} = 3.59$, $p = 0.049$; *dat*: $F_{(2,19)} = 4.77$, $p = 0.021$), being up-regulated in the 1000 ng/L fluoxetine offspring (*sert*: 1000 ng/L: $p = 0.037$; *dat*: 1000 ng/L: $p = 0.026$). An increase in the expression of *mao* ($F_{(2,19)} = 4.38$, $p = 0.027$) was also observed in 1000 ng/L fluoxetine offspring ($p = 0.023$). Regarding the genes involved in serotonin and dopamine synthesis, the expression of *tph1a*

A

		2-month-old		3-month-old	
		100 ng/L	1000 ng/L	100 ng/L	1000 ng/L
NTT	Distance moved on top (%)	↑ **	↑ **	↑	↑ *
	Distance moved on bottom (%)	↓ **	↓ **	↓	↓ *
	Cumulative duration on top (%)	↑ ***	↑ **	↑	↑ *
	Cumulative duration on bottom (%)	↓ ***	↓ **	↓	↓ *
	Freezing time (%)	↓	↓	↓	↓
LDT	Time DARK ARENA (%)	Not applicable		↓ **	↓ **
	Time WHITE ARENA (%)			↑ **	↑ **
SPT	Distance moved NEAR (%)	Not applicable		↓ ***	↓ *
	Cumulative duration NEAR (%)			↓	↓ *
NOT	Distance moved in Center (%)	Not applicable		↑ ***	↑ ***
	Cumulative duration in Center (%)			↑ ***	↑ ***

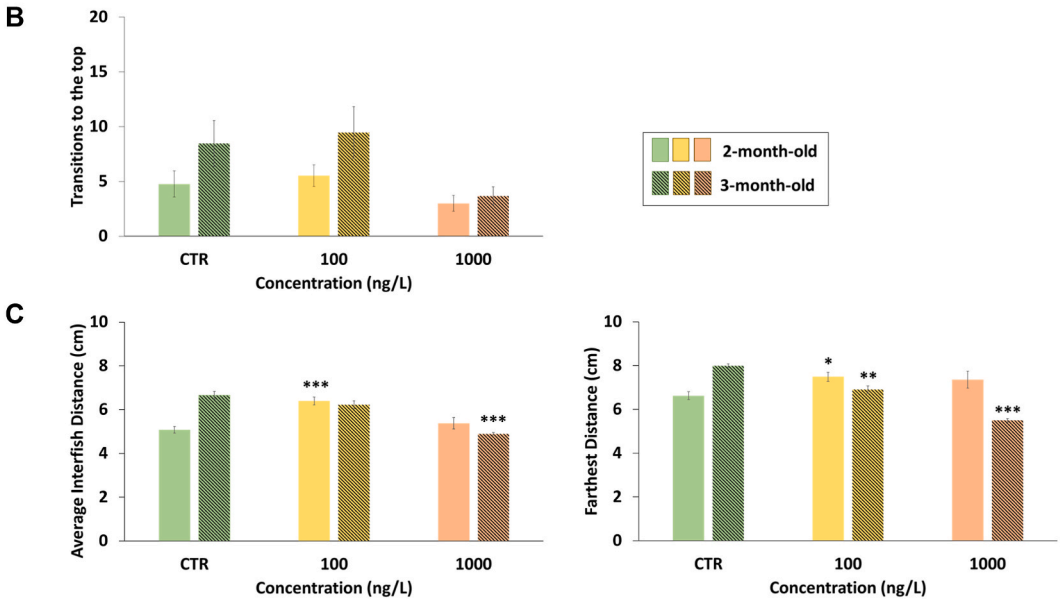

Fig. 4. Effects of zebrafish parental exposure to fluoxetine on offspring at 2- and 3-month-old offspring. **(A)** Table with the changes in the behavioral parameters evaluated in NTT, LDT, SPT and NOT. **(B)** NTT: Transitions to the top at 2- (n = 24) and 3-month-old offspring (n = 24); **(C)** Behavioral parameters evaluated in the shoaling test: Average interfish distance (cm) at 2- (n = 24) and 3-month-old offspring; Farthest fish distance at 2- (n = 24) and 3-month-old offspring (n = 24). Data are reported as mean values ± standard error. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, compared with the control offspring; [NTT: Kruskal Wallis test with Dunn's multiple comparison test (transitions to top at 2- and 3-month-old offspring); Shoaling test: Kruskal Wallis test with Dunn's multiple comparison test (average interfish distance and farthest fish distance at 2- and 3-month-old offspring)]. Data from 3 independent assays.

($F_{(2,19)} = 5.25$, $p = 0.015$) and *th2* ($F_{(2,19)} = 3.94$, $p = 0.037$) was significantly up-regulated in the 1000 ng/L offspring (*tph1a*: 1000 ng/L: $p = 0.009$; *th2*: 1000 ng/L: $p = 0.032$), when compared to control offspring. The expression of *vmat2* was not significantly affected by parental exposure to fluoxetine.

In 3-month-old fish brains (Group B), the expression levels of monoaminergic genes remained altered in fluoxetine offspring. Expression of *dat* was significantly altered ($F_{(2,14)} = 5.35$, $p = 0.019$), showing a significant upregulation in both concentrations ($p < 0.05$). The expression of *vmat2 and th2* was also affected ($F_{(2,15)} = 4.11$, $p = 0.038$; $F_{(2,14)} = 4.63$, $p = 0.029$, respectively), augmented at 1000 ng/L fluoxetine offspring ($p < 0.05$). Regarding the expression of *mao* ($F_{(2,14)} = 6.76$, $p = 0.009$), offspring from parents exposed to fluoxetine, especially to 100 ng/L, upregulated its expression (100 ng/L: $p = 0.007$; 1000 ng/L: $p = 0.042$). Regarding *sert* and *tph1a* expression, despite an upregulation trend, results no significant effects were found (*sert*: $F_{(2,13)} = 3.46$, $p = 0.063$; *tph1a*: H(2) = 2.47, $p = 0.291$). Overall, the expression of *dat* showed a fold-change increase of 47.1 and 2.0 for the 100 and 1000 ng/L fluoxetine offspring, respectively. In the 1000 ng/L fluoxetine offspring, a 7.1- and 3.3-fold change increase was observed in the expression of *vmat2* and *th2*, respectively. The gene expression of *mao* in 100 and 1000 ng/L offspring was also 7.5- and 1.1-fold change increased (Supplementary Material for additional detailed figures (Fig. S9)).

3.7. Neurotransmitters involved in monoaminergic system

Results of the levels of monoaminergic neurotransmitters at 8 dpf (Group A) and 3-month-old (Group B) are represented in a heat map (Fig. 7). In terms of serotonergic system of 8 dpf larvae, parental exposure significantly affected the levels of serotonin (5-HT) ($F_{(2,12)} = 4.785$, $p = 0.03$) and tryptophan ($F_{(2,12)} = 9.14$, $p = 0.004$). The serotonin levels decreased in 100 ng/L offspring ($p = 0.023$), while tryptophan levels significantly increased in the same organisms ($p = 0.004$).

Regarding the dopaminergic system, both concentration promoted increased levels of dopamine (100 ng/L: $p = 0.027$; 1000 ng/L: $p = 0.005$) whereas homovanillic acid (HVA) were significantly decreased in the offspring of 1000 ng/L fluoxetine ($p = 0.04$). Concerning the

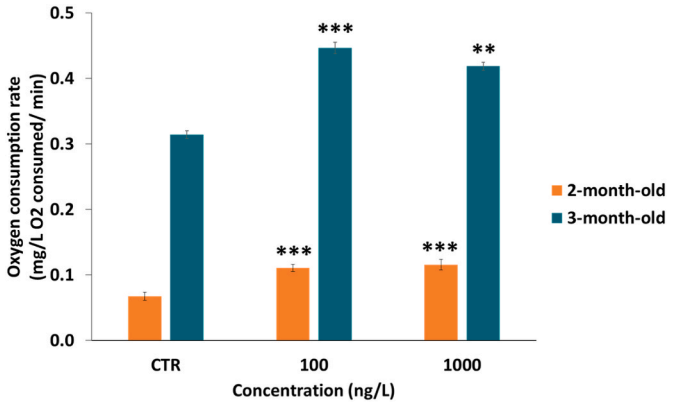

Fig. 5. Effects of zebrafish parental exposure to fluoxetine on offspring oxygen consumption at 2- (n = 30) and 3-month-old (n = 30). All data are reported as mean values ± standard error. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, compared with the control offspring; Kruskal Wallis test with Dunn's multiple comparison test. Data from 3 independent assays.

neurotransmitter tyrosine, a non-monotonic response was found (H(2) = 6.62, $p = 0.036$), with 100 ng/L fluoxetine offspring displaying significantly higher levels ($p = 0.022$) than the control and 1000 ng/L fluoxetine offspring. Parental exposure also affected the levels of norepinephrine ($F_{(2,10)} = 3.96$, $p = 0.05$), promoting a significant increase in 1000 ng/L offspring ($p = 0.04$).

In 3-month-old fish brains (Group B), the levels of monoaminergic neurotransmitters remained different than control in the offspring of fluoxetine exposed organisms. The levels of dopamine, tyrosine, HVA and tryptophan were significantly altered (dopamine: $F_{(2,9)} = 11.15$, $p = 0.004$; tyrosine: $F_{(2,13)} = 13.68$, $p < 0.001$; HVA: $F_{(2,8)} = 20.97$, $p < 0.001$; tryptophan: $F_{(2,11)} = 14.87$, $p < 0.001$), showing significant decreases in 1000 ng/L offspring (dopamine: $p = 0.004$; tyrosine: $p < 0.001$; HVA: $p < 0.001$; tryptophan: $p < 0.001$). Regarding 5-HT and norepinephrine levels, despite a trend towards a decrease and increase, respectively, the results were not significant (5-HT: $F_{(2,8)} = 1.24$, $p = 0.339$; norepinephrine: $F_{(2,11)} = 1.70$, $p = 0.227$).

4. Discussion

Exposure of the parental generation to contaminants can significantly compromise biological responses (e.g., behavior, growth, reproduction, hormones levels, enzymes, and gene expression) and the offspring viability (Schwindt, 2015). As fluoxetine is a drug with bioaccumulation potential (Pan et al., 2018; Yan et al., 2020), this SSRI could be transferred to the oocytes. Alterations in the storage and transfer of maternal factors (e.g., proteins, lipids and transcripts) to the offspring (Miccoli et al., 2017), which are essential for embryonic development, can directly affect their fitness. The results of this study confirm that exposure of adults to environmental concentrations of fluoxetine can transmit long-lasting effects to the next generation, suggesting the potential intergenerational inheritance and ecological relevance of the DoHAD paradigm in fish.

4.1. Parental exposure to fluoxetine leads to adverse effects on offspring morphogenesis

In zebrafish, the swim bladder plays a role in maintaining buoyancy, breathing, perceiving pressure fluctuations, reducing energy

	8 dpf		3-month-old	
	100 ng/L	1000 ng/L	100 ng/L	1000 ng/L
sert (slc6a4a)	↑	↑ *	↑	↑
mao	↑	↑ *	↑ **	↑ *
tph1a	↑	↑ **	↑	↑
dat (scl6a3)	↑	↑ *	↑ *	↑ *
vmat2 (scl18a2)	↓	↑	↑	↑ *
th2	↑	↑ *	↑	↑ *

Fig. 6. Changes in the expression of six selected monoaminergic genes in the offspring at 8 dpf and 3-month-old, after parental exposure to fluoxetine.

	8 dpf		3-month-old	
	100 ng/L	1000 ng/L	100 ng/L	1000 ng/L
Serotonin (5-HT)	62.26 *	91.70	77.66	54.11
Tryptophan	141.21 **	104.40	79.49	39.16 ***
Dopamine	167.05 *	189.05 **	91.93	29.84 **
Homovanillic acid (HVA)	84.10	71.48 *	90.77	36.57 ***
Tyrosine	153.93 *	100.15	98.72	61.10 ***
Norepinephrine	136.86	164.51 *	89.87	132.30

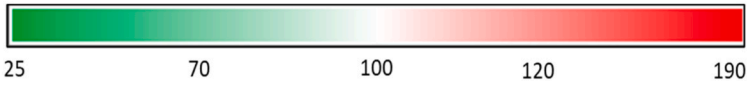

Fig. 7. Heat map diagram showing effects of parental exposure to fluoxetine on monoaminergic neurotransmitters levels of offspring at 8 dpf and 3-month-old. The colors in the heat map represent the deviation from the control offspring, with a gradient of green or red for values below or above the controls, respectively. The number within each cell corresponds to the mean of the results for each parameter normalized as percentage of their respective controls. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$; [8 dpf: One-way ANOVA with Dunnett's multiple comparison test (5-HT, tryptophan, dopamine, HVA and norepinephrine) or Kruskal Wallis test with Dunn's multiple comparison test (tyrosine); 3-month-old: One-way ANOVA with Dunnett's multiple comparison test (5-HT, tryptophan, dopamine, HVA, tyrosine and norepinephrine)]. Data from 3 independent assays.

expenditure in deeper environments (Sado et al., 2020), appearing inflated at 96 hpf. The offspring of organisms exposed to fluoxetine exhibited early hatching and delayed swim bladder inflation. Developmental effects similar to those observed in the offspring in this study were previously reported by other authors in zebrafish embryos directly exposed to a concentration range of 0.64 ng/L – 276.6 μg/L fluoxetine, between 24 h and 8 days (Airhart et al., 2007; Correia et al., 2023a; Cunha et al., 2016; Kalichak et al., 2016; Orozco-Hernández et al., 2022; Rodrigues et al., 2020). One possible hypothesis for the developmental effects observed in this study could be related to low metabolic activity (low yolk sac absorption) and, indirectly, lower respiration rates (Folmes and Terzic, 2014; Lima et al., 2020). The low metabolic activity can also be linked to the low heart rate observed in these organisms, smaller total body length and high incidence of malformations (Folmes and Terzic, 2014; Glazier, 2008; Illsinger and Das, 2010; Prasad et al., 2009). The ability to use oxygen for oxidative respiration during embryonic development is essential for normal development (Fathollahipour et al., 2018; Marsico et al., 2023), so further studies should investigate the potential of fluoxetine to induce metabolic changes in the early stages of fish. Impaired swim bladder inflation has also been attributed to a decrease in thyroid hormones (THs) levels (Godfrey et al., 2017; Stinckens et al., 2016) and the inhibition of deiodinases (enzymes involved in the activation or inactivation of thyroid hormones) in the early stages of fish development (Cavallin et al., 2017; Knapen et al., 2018; Van Dingenen et al., 2023). Future studies should investigate the involvement of TH levels on the observed effects of fluoxetine.

4.2. Behavioral responses in early life stages of fish are altered by parental exposure to fluoxetine

The offspring of 1000 ng/L fluoxetine exposed animals exhibited a decreased response to an acoustic/vibrational stimulus (or startle response). These results are in agreement with the data of studies with zebrafish larvae directly exposed (from 4 hpf to 8 dpf or 24-h exposure) to the same drug (Correia et al., 2023a; Faria et al., 2021b), and other drugs of the same class (Faria et al., 2022b). A decrease in BLA was also observed in offspring from animals exposed to 100 and 1000 ng/L fluoxetine, an effect that could be related with the significant decrease in the total body length observed in these larvae.

The test of habituation, a simple form of non-associative learning, is based in the reduction of the response to an irrelevant and repetitive stimulus (Best et al., 2008). The habituation of 1000 ng/L fluoxetine offspring was significantly slower than the control, which could be indicative of memory and learning problem. In a study performed with zebrafish larvae 8 days exposed to sertraline, an SSRI drug, a similar effect was observed, at concentrations lower than 1 μg/L (Faria et al., 2022b). Regarding the VMR, in the present study, the 100 ng/L offspring showed a decreased activity response. VMR demonstrates that fish can distinguish between light and dark and has been linked to predator avoidance (Burton et al., 2017). This result is in agreement with the results of Faria et al. (2021b), that reported that direct 24 h exposure to a higher concentration of fluoxetine (154.65 μg/L) resulted in a decrease in VMR in zebrafish larvae at 8 dpf. However, the increase in VMR observed in the 1000 ng/L offspring, exhibiting a decreased total body size, seems to be an effect activating specifically some of the neuronal circuits involved in this response (Pakan et al., 2018).

4.3. The effects of parental exposure of fluoxetine persist at a behavioral level in the offspring through the growth

In later life stages (juveniles and adults), although the delayed filling of the swim bladder is no longer evident, the metabolic alterations remain, manifested by high oxygen consumption by the offspring, behavioral alterations and altered expression of genes and levels of neurotransmitters of the monoaminergic system.

The increased oxygen consumption observed in 2- and 3-month-old fluoxetine offspring may indicate a possible alteration in the citric acid cycle (or Krebs cycle), suggesting a modulation of brain's energy metabolism due to parental exposure to fluoxetine (Scaini et al., 2010). Therefore, future studies should investigate the effects of fluoxetine and other SSRIs on the Krebs cycle.

In the juvenile stage (1 month old), an increase in locomotor activity in BLA and VMR was found in the offspring of 1000 ng/L fluoxetine treatment. In spite to this increase in motor activity, dopamine levels of these juveniles were significantly lower than those of the corresponding controls. Therefore, the most suitable explanation for these results is once again the observed differences in the total length, with the increased total body length of the offspring of 1000 ng/L fluoxetine treatments finally resulting in an increased motor performance. Studies about the direct effects of fluoxetine on juvenile fish are also scarce. Other authors also found effects of direct exposure to fluoxetine on the response to alternating changes in light conditions. Juvenile zebrafish exposed for 12 days to 1000 ng/L fluoxetine displayed increased swimming time during the dark period (Correia et al., 2022). The analysis of the results clearly shows that there is still a considerable lack of information on the potential effects of fluoxetine on juveniles, both in terms of direct exposure and the effects of parental exposure, supporting further studies on this subject.

In the behavior of the offspring at 2-month-old offspring, two different behaviors were initially performed: anxiety-like behavior (NTT) and social behavior (shoaling test). These behaviors are already present and well established at this age, being potentially sensitive to external disturbances such as stress and pharmacological agents (Kalueff et al., 2013; Kysil et al., 2017). Fluoxetine has been reported to have a clear anxiolytic effect in directly exposed fish (Egan et al., 2009; Kysil et al., 2017). At 2-month-old, fluoxetine offspring exhibited negative geotaxis, swimming longer distances and spending more time at the top of the tank, fewer transitions to the top and displayed a pronounced decrease in freezing behavior. All these factors indicate an anxiolytic effect of fluoxetine on zebrafish offspring from fish exposed to fluoxetine. This effect was maintained until adulthood (3 months), proving its persistence. These results are similar to those observed by other authors in direct exposures to fluoxetine (Stewart et al., 2014).

The tendency of adult fish to avoid bright-lit areas (or scototaxis) (Kysil et al., 2017; Maximino et al., 2010) is reversed by anxiolytic drugs, increasing their vulnerability to predators. In the present study, a negative scototaxis was observed in the offspring of fluoxetine-exposed fish, when they were 3 months old. This result supports the NTT data, suggesting an anxiolytic effect of fluoxetine. These results are in agreement with the data obtained in other studies with adults of zebrafish, medaka (*Oryzias latipes*) and mosquitofish (*Gambusia holbrooki*) directly exposed, between 10 and 14 days, to 0.01–100 μg/L fluoxetine (Ansai et al., 2016; Maximino et al., 2014; Meijide et al., 2018; Pittman and Ichikawa, 2013).

The NOT aimed to assess neophobic behavior, i.e., fear of an unknown object (in the case of our study, a LEGO figure). In zebrafish, the natural behavior is to stay away from this object (by staying close to the walls (thigmotaxis)), which is considered a potential predator (Johnson and Hamilton, 2017; Ou et al., 2015). In the present study, the offspring of both fluoxetine concentrations increased their permanence in the zone closest to the figure, a behavior consistent with a decrease in anxiety. The behavior of approaching the figure can also be seen as a measure of boldness (proactive behavior). Proactive or bold fish explore a new environment more quickly, and this behavior is associated with changes in serotonin levels and low basal cortisol levels, resulting in a possible modulation of the behavioral phenotype (Ferreira et al., 2023). On the other hand, the approach to the figure can also suggest an interference in the predator's escape behavior.

Social behavior in zebrafish persists throughout their lives (Hinz et al., 2017), allowing for recognition of conspecifics (same age and size) (Dreosti et al., 2015), early detection of predators, improved feeding and mating (Fontana et al., 2022). Social cohesion is strongly influenced by

anxiety state, with anxiogenic chemicals leading to decreased size of the shoal and anxiolytic chemicals leading to the opposite effect (Lachowicz et al., 2021). Therefore, the increased size of the shoal found in this study, in 2-month-old juveniles, from 100 ng/L fluoxetine-exposed parents. However, when this behavior was evaluated on 3-month-old organisms, the shoals of the 100 and 1000 ng/L fluoxetine offspring displayed greater cohesion. Interestingly, the effect on social cohesion was different from the observed effect on the SPT, more consistent with the anxiolytic effect found in the same adults from exposed parents. As shoaling test and SPT provide information on different elements of the social behavior, these assays often provide different outcomes. Results for SPT are similar to those reported by Giacomini et al. (2016) in zebrafish adults directly exposed for 15 min to 50 μg/L, and by Ansai et al. (2016), in *Oryzias latipes* adults directly exposed for 10 days exposed to 100 μg/L fluoxetine.

All these behavioral alterations could compromise their survival in their natural habitat by impairing their ability to evade predators, locate food, and reproduce.

4.4. Parental exposure to fluoxetine impairs the expression of genes and neurotransmitters levels involved in monoaminergic system in the offspring

To elucidate potential mechanisms related to the behavioral changes observed in the offspring, the monoaminergic gene expression levels were evaluated in the offspring. The monoaminergic system can be affected by direct exposure to fluoxetine (Lin et al., 2023), being one of the main mechanisms modulating various brain functions in fish species, like behavior (Backström and Winberg, 2017; Tayanloo-Beik et al., 2022) and physiological changes (Backström and Winberg, 2017; McDonald, 2017).

In 8 dpf offspring larvae (Group A), the parental exposure resulted in the upregulation of *slc6a4* (*sert*), *tph1a* and *mao* transcripts. The *slc6a4* encodes SERT, a transmembrane protein transporter responsible for the reuptake of 5-HT from the synaptic cleft to the presynaptic serotonergic neuron (McDonald, 2017) *tph1a* encodes the tryptophan hydroxylase, the rate-limiting enzyme in the synthesis of serotonin, and *mao* (monoamine oxidase) encodes the enzyme that catalyzes the oxidation of monoamines (Horzmann and Freeman, 2016). The observed upregulation of *tph1a* may have been triggered by reduced 5-HT levels, leading to a positive feedback loop on tryptophan hydroxylation, resulting in increased levels of serotonin (Ge et al., 2022). The increase in *sert* expression levels may also be related to the reduced levels of 5-HT observed, indicating that there is a large amount of this neurotransmitter being transported. These effects may be associated to the observed delay in the non-associative learning (habituation) in fluoxetine offspring. The neurons of the serotonergic system, associated with Mauthner cells (reticulospinal interneurons located in the hindbrain of fish) play an important role in regulating the non-associative learning process (Mu et al., 2012; Pantoja et al., 2016), and could have been affected by parental exposure to fluoxetine.

Catecholamines also play an important role in the regulation of movement and motor systems control (Howes et al., 2012; Schultz, 2013) and an impairment of the dopaminergic system leads to locomotion changes in teleosts (Jay et al., 2015; Yao et al., 2016). The levels of dopamine and norepinephrine, as well as the expression levels of *slc6a3* and *th2* in the fluoxetine offspring were significantly higher than in the control. Interestingly, levels of HVA, the final product of dopamine metabolism, were found to decrease. As *th2* encodes the tyrosine hydroxylase, the rate limiting enzyme in the catecholamines synthesis (Horzmann and Freeman, 2016), the observed increase in the expression of this transcript could be directly related to the observed increase in both dopamine and norepinephrine. The *slc6a3* gene encodes DAT (dopamine transporter), a transmembrane protein responsible for the transport and reuptake of dopamine from the synapse into the cytosol of dopaminergic neurons (Lamothe and Zhang, 2016). The increased levels of catecholamines observed in the fluoxetine offspring could also be related with the increase in *slc6a3* expression levels (Rudnick et al., 2014), leading to an increase in the transport and reabsorption of the neurotransmitter into dopaminergic neurons. Additionally, the reduced levels of HVA strongly suggest a potential impairment in MAO or catechol-*O*-methyltransferase (COMT), enzymes involved in the degradation of dopamine to HVA, and a concomitant increase in the dopamine β-hydroxylase to produce increased levels of norepinephrine (Irons et al., 2013). The decrease in BLA found in the fluoxetine offspring contrasts with the increased dopamine levels found in the same larvae, suggesting that it is the decrease in the total length of these larvae the main factor behind the decreased motor performance of these larvae (Irons et al., 2013). However, a specific effect of the parental exposure to fluoxetine on dopamine receptors in the offspring cannot be discarded.

When the offspring reached adulthood, the expression of monoaminergic genes remained high, and in some cases with marked increases in fold-changes. These data demonstrate that the synthesis pathways mentioned above remained affected.

Possibly, the persistence of behavioral effects observed until adulthood (negative geotaxis and scototaxis, decreased freezing behavior, changes in sociability and neophobic behavior), changes in the expression of genes and neurotransmitters levels involved in monoaminergic system, indicates the involvement of epigenetic alterations. In view of this possibility, more research is needed in this area in order to understand the epigenetic mechanisms that may be involved in the changes observed in offspring and how these may affect the survival and continuity of the species.

5. Conclusion

This study's results demonstrate that zebrafish exposure to environmentally relevant concentrations of fluoxetine has effects on its offspring, in terms of embryonic development and behavioral responses important for larvae survival. This exposure, even at low concentrations of fluoxetine, caused an increase in the expression levels of key genes of the monoaminergic system and neurochemical alterations. Given this evidence, the need to perform parental and multigenerational exposures to environmentally relevant concentrations of fluoxetine becomes evident. Further studies of this nature with different aquatic species could provide information on the potential environmental risks associated with the long-term effects across generations, reinforcing the importance of monitoring the presence of these psychotropics and other contaminants in the aquatic environment, requiring a reassessment of the ecological and evolutionary consequences of exposure to SSRIs in wild populations of aquatic organisms.

CRediT authorship contribution statement

Daniela Correia: Writing – review & editing, Writing – original draft, Methodology, Investigation, Funding acquisition, Formal analysis, Conceptualization. **Marina Bellot:** Methodology, Formal analysis. **Júlia Goyenechea:** Methodology, Formal analysis. **Eva Prats:** Resources, Investigation. **Hugo Moro:** Methodology, Investigation. **Cristian Gómez-Canela:** Methodology, Formal analysis. **Juliette Bedrossiantz:** Methodology, Investigation. **Niki Tagkalidou:** Investigation. **Carla S.S. Ferreira:** Investigation. **Demetrio Raldúa:** Writing – review & editing, Resources. **Inès Domingues:** Supervision, Methodology, Conceptualization. **Melissa Faria:** Writing – review & editing, Supervision, Resources, Methodology, Funding acquisition, Conceptualization. **Miguel Oliveira:** Writing – review & editing, Supervision, Methodology, Funding acquisition, Conceptualization.

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 byFoundation

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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.2024.141851.

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